



Modern trends in radiopharmaceuticals for diagnosis and therapy

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FOREWORD

Radiopharmaceuticals form the cornerstone of nuclear medicine. While the existing range of radiopharmaceuticals permits study of the structure and function of many important organs, there is a need for new radiopharmaceuticals that could be used to explore more subtle mechanisms of bodily functions. Significant progress has been achieved in this direction by the development of tracers labelled with cyclotron produced isotopes, including ^{11}C , ^{13}N , ^{15}O , ^{18}F and ^{123}I . Major efforts are also under way to translate this success into regular clinical practice by developing similar agents labelled with metallic radionuclides, particularly with the most widely used $^{99\text{m}}\text{Tc}$. The development of agents labelled with beta emitting isotopes for potential use in the targeted radiotherapy of various malignancies is also being widely pursued. In addition to laboratories in advanced countries, several developing countries are also interested in these areas and have been participating in research programmes organized by the IAEA. In keeping with its role to promote co-operation between Member States, the IAEA held an International Symposium on Modern Trends in Radiopharmaceuticals for Diagnosis and Therapy in Lisbon, Portugal, from 30 March to 3 April 1998. Two earlier symposia were organized on similar topics in Copenhagen, Denmark in 1973 and in Tokyo, Japan, in 1984. The proceedings of these symposia have been published and widely used as reference sources. To facilitate faster publication and more widespread availability, the IAEA has decided to publish the proceedings of this symposium as a cost-free TECDOC.

The symposium was organized into 14 sessions consisting of five on $^{99\text{m}}\text{Tc}$ radiopharmaceuticals, two each on therapeutic radiopharmaceuticals and radiohalogens/other isotopes and one each on bioevaluation, radiometric assay, medical isotope production, good radiopharmacy practice and technology transfer. In the proceedings the papers from multiple sessions on the same topic have been grouped together for the convenience of the reader.

The papers presented in the symposium reflect current and future developments in diagnostic and therapeutic agents. The largest number of papers presented dealt with $^{99\text{m}}\text{Tc}$, highlighting its continuing importance to nuclear medicine and the role of imaging as an important tool. The emerging interest in therapeutic radiopharmaceuticals based on beta emitting short lived isotopes such as ^{186}Re and ^{153}Sm was evident from the papers presented in two sessions devoted to this topic. Also of steady interest was the development of agents labelled with other established isotopes, radioiodine in particular and also ^{111}In and ^{67}Ga . Regulation, training and good manufacturing practices are important for ensuring safety in regular use of radiopharmaceuticals and were discussed in a separate session. The production of radiopharmaceuticals has become a regular activity in many developing countries, often facilitated by IAEA technical co-operation programmes and reports from representative countries were presented at the symposium.

The symposium was attended by 117 participants from 41 countries and one international organization. The IAEA gratefully acknowledges the assistance of the Government of Portugal and the Instituto Tecnológico e Nuclear, Sacavem, in providing technical, scientific and administrative support.

EDITORIAL NOTE

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INVITED LECTURE

RADIOPHARMACEUTICAL DEVELOPMENT AND CLINICAL NEEDS

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Abstract

The use of radionuclides for medical applications has continued to grow at a very rapid pace. The use of radiotracers for nuclear medicine imaging and for radiotherapy of cancer as well as certain benign disorders is firmly established as an important clinical modality.

Over the past ten years, nuclear medicine has experienced an evolution towards functional studies and novel therapeutic approaches. New radionuclides are required for these applications.

In the developmental stages, each new isotope has to go through a phase of careful scrutiny and evaluation, and practical concerns related to the cost of production and availability must be addressed.

The development of 18 F-labeled radiopharmaceuticals has opened a completely new area of investigation.

Research on bioconjugates (this term includes radiolabeled antibodies, peptides, receptor-specific and other bioactive molecules) has experienced rapid growth because of the promise of a number of these "bioactive molecules " to serve as selective carriers of radionuclides for tumor-associated and other specific antigens/receptors "in vivo".

The new concept of nuclear medicine, particularly when applied to the field of oncology is directed towards the physiological mechanisms and the study of molecular disfunctions.

The search for new radiopharmaceuticals thus aims at studying tumors at a tissue and molecular level. Examples of this new approach are scans utilizing the following substances:

- guanetidine and noradrenalin analogues such as meta-iodo-benzyl-guanidine labeled with Iodine-131 or Iodine-123 aimed at targeting neuroendocrine cells and their secretory granules.

- various monoclonal antibodies directed at different tumor types, both for diagnostic and therapeutic purposes. Radioimmunotherapy is considered particularly suited for treatment of tumors not easily amenable to surgery and for the treatment of small disseminated lesions.

- somatostatin analogs tagged with Indium - 111 or more recently with Yttrium-90, also for therapeutic purposes. Somatostatin receptor imaging often provides optimal information in terms of tumor localization, staging and prediction of the usefulness of medical therapy.

- several non-specific agents (Thallium-201 and Technetium-99m labeled agents MiBi and Tetrofosmin) the uptake mechanisms of which are not completely understood and which are used to measure cell membrane transport during cell division.

INTRODUCTION

The discovery of X-Rays in 1895 by William Konrad Roentgen was an enormous contribution to the advancement of the medical sciences and earned him a Nobel prize in 1901. Four months later, another physicist, by the name of Henri Becquerel, discovered natural radioactivity. In 1913, Georg de Hevesy introduced the concept of the radioactive marker, and was later awarded a Nobel prize in 1943. This concept, which constitutes the basis for the practice of Nuclear Medicine, is still current today.

The scope of Nuclear Medicine has grown tremendously in recent decades, especially in the eighties and nineties, and the specialty has acquired a greater autonomy in relation to other imaging modalities, since it permits, in a single step, the study of both morphology and function. The use of small amounts of drugs, devoid of pharmacological action, coupled with radionuclides which emit radiation detected by modern gamma-cameras, allows the study of organ and tissue function without interfering with that function. Computer science, as applied to Medicine, has also developed greatly, and specifically, in this specialty, is responsible for the formation of digitalized images and for the quantification of function. From the viewpoint of the patient, the techniques performed are simple and require only an intravenous injection or the oral administration of a radiopharmaceutical. Adverse reactions are extremely rare. The nuclear medicine physician then utilizes his/her techniques in order to try to answer the questions posed by the clinician, such as:

1. Is there any alteration present?
2. If so, where?
3. What is its extension?
4. Is it possible to determine the etiology of the alterations?
5. Is it possible to determine the degree of activity of the alterations?

The advances which have occurred in the biomedical sciences have made possible a different diagnostic approach. Whereas classically studies were aimed at the function of systems and organs, they can now be directed towards more elementary levels, namely the molecular and tissue levels. A disease process is not

or should not be looked upon as a strange entity which invades the body, but as a dissonance between one or more messenger systems [1]. These systems send messages to the cells indicating how and when they should divide, and how they should react to microorganisms or other foreign elements. In the realm of oncology, cancer is no longer treated as a disease of a specific organ, but as a disequilibrium between factors which promote and those which suppress growth.

Nuclear Medicine deals with the detection of photons by means of external detectors. The quality and quantity of an emission provides valuable information concerning the biochemical interactions between molecular structures. In this scenario, the patient is seen as a symphony of biochemical reactions, which when altered are responsible for a specific signal. This "signal" provides the diagnosis, the disease stage, the therapeutic response, or information regarding a potential secondary effect.

The new concept of Nuclear Medicine, particularly when applied to the field of Oncology, is directed towards the physiological mechanisms and the study of molecular disfunctions [2]. The search for new radiopharmaceutical agents thus aims at studying tumors at a tissue and molecular level. Examples of this are scans utilizing tagged antibodies (immunoscintigraphies), somatostatin analogues tagged with Indium-111, guanetidine, and noradrenalin analogues such as meta-iodo-benzylguanidine labeled with Iodine-131 or Iodine-123, Thallium-201, and isonitriles labelled with Technetium-99m.

1. SOMATOSTATIN RECEPTOR SCINTIGRAPHY

Somatostatin is a neuropeptide first discovered in the hypothalamus twenty years ago. It is produced in various tissues, such as the central nervous system, endocrine pancreas, and gastrointestinal system. It is a peptide consisting of 14 or 18 aminoacids, with a short half-life, which acts upon the central nervous system as a neurotransmitter. It inhibits growth hormone, insulin, glucagon, gastrin, serotonin,

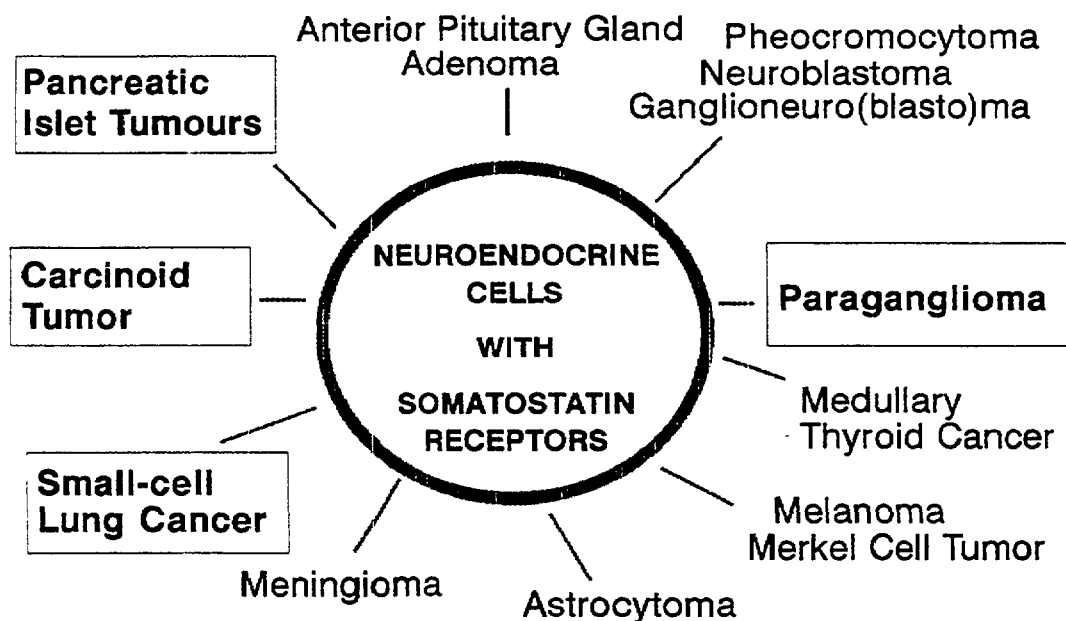


Fig. 1 - Tumors which express somatostatin receptors (6).

and calcitonin. Still other actions have been described, namely an anti-proliferative effect upon tumors and a modulating effect upon immunologic function [3]. Somatostatin receptors have been identified in cells of neuroendocrine origin, such as cells of the anterior hypophysis, pancreatic islet cells, and thyroid C cells. Other cells, such as activated lymphocytes, also have a large number of somatostatin receptors [4]. Fig.1 highlights the tumors which express a large number of somatostatin receptors. Due to the increase in somatostatin receptors observed in activated lymphocytes, Hodgkin's and non-Hodgkin's lymphomas, as well as other non-malignant diseases such as granulomatous and autoimmune diseases, also express large quantities of these receptors [5].

In 1982, octreotide, a somatostatin analogue with a long half-life, was first synthesised. In 1988, it was approved by the FDA for use in the treatment of carcinoid tumors and vipomas. This analogue was later tagged with Iodine-123 and used in 1989 for the *in vivo* detection of pancreatic tumors. The excretion of octreotide is preferentially hepatic, which is detrimental to the visualization of

abdominal tumors. A new derivative - pentatetreotide labeled with Indium-111 - is preferentially excreted by the kidneys and has been in use since 1992 for the study of malignant and non-malignant tumors, which express somatostatin receptors. The protocol for this examination consists of the intravenous administration of 200-250 MBq pentatetreotide-111In and the acquisition of planar whole body views at 4, 24, and 48 hours. Tomographic images are optional. In order to perform this examination, therapy with octreotide must be suspended one week ahead. Non-specific distribution of the radiopharmaceutical is seen in the hypophysis, thyroid gland, salivary glands, liver, spleen, and kidneys. Increased uptake of the product which persists in several images are indicative of pathologic localization in tissues rich in somatostatin receptors.

The sensitivity of this technique is very high for the diagnosis of neuroendocrine tumors: sensitivity greater than 80% for pheochromocytomas, neuroblastomas, paragangliomas, small cell tumors of the lung, carcinoid tumors, gastrinomas, vipomas, and other tumors originating in the endocrine cells of the pancreas. In insulinomas, the sensitivity is only 40%, due to the existence in these tumors of different types of receptors which do not take up the pentatetreotide. Furthermore, this technique has a very high sensitivity for the detection of non-endocrine tumors, such as meningiomas (100%), Hodgkin's and non-Hodgkin's lymphomas (85-95%), astrocytomas (60-70%) and breast cancer (75%) [6].

The good results obtained thus far with octreotide scintigraphy allow us to foresee the possibility of tagging other peptides and their derivatives for new applications in the field of Oncology.

2. IODINE-131/123-MIBG SCINTIGRAPHY

The study of neuroendocrine cells helped delve more profoundly into the workings of secretory cells, as well as into the clinical syndromes resulting from the secretion of their products. These cells are capable of producing and secreting

monoamines and peptides, with the possibility of both products coexisting, not only in the same cell, but also in the same secretory granule.

Neuroendocrine tumors derive embryologically from the neural crest (Table I). A striking characteristic of these tumors is the preservation of highly differentiated cellular function. They produce large quantities of active compounds, such as epinephrine, norepinephrine, dopamine, serotonin, calcitonin, and a variety of other hormones. The levels of these hormones and their metabolites can be determined in the blood and urine.

Table I.

NEUROENDOCRINE TUMORS
Pheocromocytoma
Neuroblastoma
Ganglioneuro(blasto)ma
Paragangliomas
Medullary Thyroid Cancer
Melanoma
Merkel Cell Tumor
Small-cell lung cancer
Carcinoid Tumor
Pancreatic Islet Tumors

In 1981, Wieland et al. [7] used the meta isomer of iodobenzylguanidine labelled with Iodine-123 and Iodine-131 to study the adrenal medulla in primates. MIBG (metaiodobenzylguanidine) has a structure similar to guanetidine (adrenergic blocker) and to norepinephrine (neurotransmitter). In the first trials performed using human subjects, in 1981, Sisson [8] was able to detect pheocromocytomas in 8 patients.

MIBG is taken up actively by the cells at the level of the cellular membrane [9]. Within the cell, MIBG is actively transported and stored in cytoplasmic vacuoles or granules. It is then set free and may undergo a reuptake mechanism.

The first studies were performed with ^{131}I -MIBG but, more recently, diagnostic examinations have been preferentially performed using the same molecule labelled with ^{123}I , due to the physical advantages and radioprotection aspects inherent to this radiopharmaceutical. Iodine-123 has the advantage of a shorter half-life (13.2 hours) and, above all, the absence of β radiation, as well as a lower gamma energy, which in turn is more suited to the instrumentation (gamma-cameras) used.

The protocol of this examination consists of the slow intravenous administration of 185 MBq of ^{123}I -MIBG. Whole body scintigraphy is performed 24 hours later. Tomographic examinations are performed as needed. The thyroid gland should be previously blocked through the administration, starting two days before the exam, of potassium perchlorate or Lugol solution.

The images obtained display an unspecific uptake of the product by the salivary gland complex, liver, spleen, bladder, and heart. There is a slight uptake by the adrenal glands in 60% of patients. Regions exhibiting greater uptake of the

Table II.

SUBSTANCES WHICH INTERFERE WITH UPTAKE
Cocaine
Reserpine
Tricyclic antidepressants
Phenylpropanolamine
Labetalol
Nifedipine

radiopharmaceutical are compatible with the presence of tumor tissue bearing the previously described properties. In the interpretation of these scans, it is particularly important to consider the interference in uptake and storage of MIBG by a large variety of substances (Table II), which should be withheld before the examination is performed.

For the diagnosis of pheochromocytoma and neuroblastoma, MIBG has been shown to have a sensitivity ranging from 90 to 95% and a specificity of almost 100% [9]. It has an important role not only in the diagnosis of the primary tumor, but also in the detection of metastatic lesions. It is thus a well established technique in the follow-up of patients diagnosed with neuroblastoma in order to evaluate therapeutic success, remission, or the recurrence of tumors.

For other neural crest tumors, the sensitivity values are inferior to those mentioned above, although specificity values remain high (Table III).

Table III.

NEUROENDOCRINE TUMORS	SENSITIVITY
Pancreatic tumors	60%
Paraganglioma	89%
Ganglioneuroma	100%
Lung cancer	15%
Schwannoma	75%
Carcinoid tumor	70%
Medullary thyroid cancer	35%

3. THALLIUM-201, 99mTc-MIBI, and 99mTcTETROPHOSMIN

In the field of Oncology, the main objective of scans using these radiopharmaceuticals was initially the possibility of distinguishing between residual tumor or recurrence from necrotic or scar tissue. This issue is often not settled by other imaging techniques, which favor anatomical information in detriment of function.

Thallium-201, a cyclotron-produced radiopharmaceutical which behaves in a similar fashion to potassium ion, is actively taken up by the cells. This uptake is dependent upon tissue vascularization, the viability of tumor cells, the integrity of the Na⁺/K⁺ pump mechanism, and the permeability of the cellular membrane.

More recently, radiopharmaceuticals such as MIBI (metoxiisobutyl-isonitrile) and tetrophosmin were produced. Both are lipophilic cations which diffuse across the cell membrane and concentrate in the mitochondria, their charge being dependent upon the energetic state of the cell. Tumor vascularization may be a limiting factor affecting uptake and retention will only occur as long as membrane potentials are intact. The great advantage of these radiopharmaceuticals is the possibility of using a 99m-technetium label. Technetium-99m, which results from the decay of Molybdenum in a generator, is the most commonly used radiopharmaceutical in diagnostic nuclear medicine techniques. These generators are present in all Nuclear Medicine Departments and are eluted daily for harvesting 99m-Tc. It is favorable both from radioprotection and from instrumentation standpoints, due to its short 6-hour half-life and its low 140 Kev gamma radiation.

The protocol for a specific scintigraphic technique, as concerns the dosage used, acquisition times and quantity of images obtained will vary depending upon the radiopharmaceutical used and upon the disease process at hand. The most noteworthy clinical indications are currently the following:

- (a) Differential diagnosis between recurrence and post-irradiation necrotic tissue in the follow-up of malignant brain tumors, namely astrocytomas and glioblastomas [10].
- (b) Calculation of the degree of necrosis, which may be considered a non-invasive indication of the pre-surgical response to chemotherapy [11] in osteosarcomas.
- (c) More recently, staging of lung tumors, indifferntiated thyroid carcinoma, and differentiation between benign and malignant breast nodules.

Although the mammogram retains its place as the primary examination for the detection of breast lesions, a considerable percentage of these exams are difficult to interpret, namely in women with breast rich in stroma or with fibrocystic disease. In these situations, surgical biopsies are commonly performed, which besides increasing diagnostic costs (use of the OR), significantly increases morbidity [12]. Breast scintigraphy, using radiopharmaceutical agents, has a high sensitivity (greater than 90%) for palpable nodules with diameter greater than 1 cm. Its specificity, already determined for studies performed with Thallium-201[13] and 99mTc-MIBI [14] ranges between 80 and 90% for the former and between 70 and 80% for the latter [14]. Sensitivities varying between 56 and 67% have been described for the detection of axillary nodes [15, 16].

4. IMMUNOSCINTIGRAPHY

The use of antibodies for the detection and treatment of cancer was initiated one hundred years ago. In 1885, Hericourt and Richet prepared an antiserum directed against osteogenic sarcoma and thus treated 50 patients with clinical results which were then considered positive [17]. Pressman, in 1953, was able to visualize an osteogenic sarcoma transplanted into an experimental animal using antibodies labeled with Iodine-131 [18]. After the identification of CEA (carcinoembryonic antigen) by Gold and Freeman in patients with colorectal cancer, Goldenberg, in 1972, using a technique he designated radioimmunodetection [19], employed anti-CEA antibodies labeled with Iodine-131 in heterotransplanted tumors, thus visualizing these tumors. In 1975, Kohler and Milstein introduced the hibridoma technique, by means of which monoclonal antibodies are produced through the fusion of immunized lymphocytes from an experimental animal and human myeloma cells [20]. This in turn paved the way for Mach et al. to perform the

first study in human patients with colorectal cancer using the monoclonal anti-CEA antibody labeled with Iodine-131 [21].

Nuclear medicine scans performed with labeled monoclonal antibodies (MABs) are designated immunoscintigraphies (ISGs) or radioimmunoscintigraphies (RISGs). They constitute a rare diagnostic modality, since they are based on a biological property of the tumor cell. They enable the detection of antigens expressed by the tumor cells by means of administered antibodies produced specifically against those antigens. The choice of antibody, the choice of radionuclide, the imaging technique and the experience of the observer are all decisive factors for achieving good results. The imaging protocol is variable depending upon the type of antibody used and the radionuclide used for labeling. The biodistribution of the radiopharmaceutical agent influences the time at which imaging is performed. Administration is by slow intravenous injection. In addition to whole body views, tomographic studies (SPECT) of anatomical areas suspected clinically or on morphological examinations may be performed.

This methodology has been extensively used in the follow-up of patients with colorectal cancer and malignant melanoma, among other tumors. In recent years, an extensive effort has been made for improving diagnosis and therapy of solid tumors. However, most solid tumors continue to constitute a serious medical problem, as evidenced by the short survival times of patients with this type of tumor [22, 23].

Colorectal cancer is the second most common tumor in the western world and still on the rise [23]. Immunoscintigraphy, with sensitivity values ranging between 89 and 95%, has revealed itself as a promising method in the post-surgical follow-up of patients with colorectal cancer when there is clinical and/or laboratory evidence suggesting tumor recurrence or metastases, especially when other imaging modalities, namely radiological techniques, are not able to confirm these situations [24, 25].

Malignant melanoma is a very aggressive tumor. A collaborative European study [26] which included 200 patients concluded that immunoscintigraphy is a sensitive method (82 to 87%) in the follow-up of these patients after resection of the primary tumor. It also increases detection of lesions previously undiagnosed by other imaging techniques, which warrants a modification of the initial stage and often also of the therapeutic approach [27].

Ovarian carcinoma is a tumor with a high mortality rate, usually diagnosed at a late stage in the disease process. Its diagnosis and staging usually require an exploratory laparotomy.

Surgery is the mainstay for evaluating therapeutic efficacy when patients are subjected to chemotherapy. Chatal et al. [28] have demonstrated positive predictive values of 78% and negative predictive values of 85% for immunoscintigraphies employing monoclonal antibody OC-125, directed against CA-125 antigen expressed by these tumors. This group of investigators has also shown that the association of immunoscintigraphy and computerized tomography increased the aforementioned to values to 86 and 90%, respectively.

The variety of monoclonal antibodies with diagnostic applications has increased continuously and many other types of tumors have been studied. The value of this method will eventually depend upon many factors, among which are the size of the tumor, its vascularization, the degree of necrosis, the quantity of antigen present on the surface of its cells, and the presence or absence of circulating antibody and its reactivity.

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^{99m}Tc RADIOPHARMACEUTICALS

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CURRENT DIRECTIONS IN RADIOPHARMACEUTICAL RESEARCH

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Abstract

Much of current radiopharmaceutical research is directed towards the development of receptor-binding tracers which are targeted towards biochemical processes. These may be extra or intracellular in nature and hold promise for an imaging approach to tissue characterisation *in-vivo*. Many of these products are based on proteins which range in size from large monoclonal antibodies to small neuropeptides and share a radiolabelling chemistry based on the use of bifunctional chelating agents. Although developed initially for use with indium-111, considerations of cost and isotope availability have continued to direct the efforts of many researchers towards the use of technetium-99m. While polypeptide-based radiopharmaceuticals may be useful for imaging peripheral cell-surface receptors, access to sites of interest within the cell, or in the brain, requires the development of small lipophilic molecules with retained ability to interact with intracellular targets. The design and synthesis of these compounds presents a particular challenge to the radiopharmaceutical chemist which is being met through either a pendant or integrated approach to the use of technetium coordination with particular emphasis on technetium (v) cores. Progress continues to be made in the application of targeted radionuclide therapy particularly in the development of radiopharmaceuticals for the treatment of malignant bone disease. Methods for labelling antibodies with a great variety of cytotoxic radionuclides have now been refined and their use for radioimmunotherapy in the treatment of haematological malignancies shows great promise. The major medical areas for application of these new radiopharmaceuticals will be in oncology, neurology and inflammation but the increasingly difficult regulatory climate in which drug development and health-care now operate will make it essential for researchers to direct their products toward specific clinical problems as well as biological targets.

INTRODUCTION

The 1990's has seen major changes in the management of health care in many western countries. From being a largely unrestricted resource with open-ended budgets and complete clinical freedom, health-care provision has become highly controlled with established protocols, tight budgets and internal markets. Drug development has also become more tightly regulated. Radiopharmaceuticals now have to comply with the full range of regulations which control conventional drugs and this has enormously increased their development costs. The number of major commercial radiopharmaceutical players in the field is also decreasing following mergers and take-overs in the Industry and this limits the possibilities for commercial exploitation of academic discoveries. There have also been major upheavals in the institutions undertaking radiopharmaceutical development.

Many of the national nuclear centres around the world either have been privatised or are under pressure to make financial profits. Funding for academic research in universities has also been cut successively in real terms for many years.

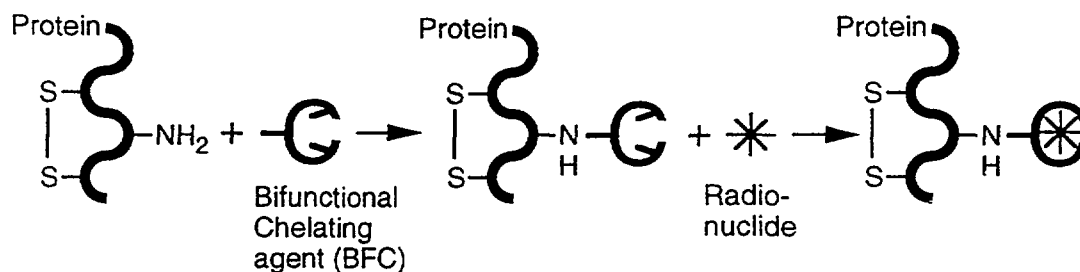
There are many reasons for wanting to undertake radiopharmaceutical research and for many years academic curiosity has been the main driving force for many scientists. However, these recent changes suggest that, for success in this field, development projects in the future will have to be much more directed and focused than was needed in the past. In particular, thought needs to be given to the ultimate clinical application of the product under development and the likelihood of it overcoming the regulatory and financial hurdles in its way.

To be successful, new radiopharmaceuticals must fulfil a clinical need. Imaging techniques can play a role either in primary diagnosis and staging of the disease or in monitoring or judging the success of a course of treatment. In the past, many academic radiopharmaceutical research programmes have not addressed this question. Often the programme is concerned with the labelling and application of a particular interesting compound with no real thought given to its future possible application. Even when a particular biological target is being pursued, the question of whether it addresses a real clinical need is often not considered. A good example is the use of radiolabelled monoclonal antibodies for tumour imaging. Over the last fifteen years, an enormous amount of work has gone into development programmes based on these materials and this has resulted in radiopharmaceuticals which can effectively image cancer with sensitivities and specificities comparable or superior to other imaging modalities. But, however successful the particular imaging technique developed, unless it influences the management of a particular group of patients, it will not find routine application in clinical practice, and so far at least, this seems to be the situation for these new radiopharmaceuticals.

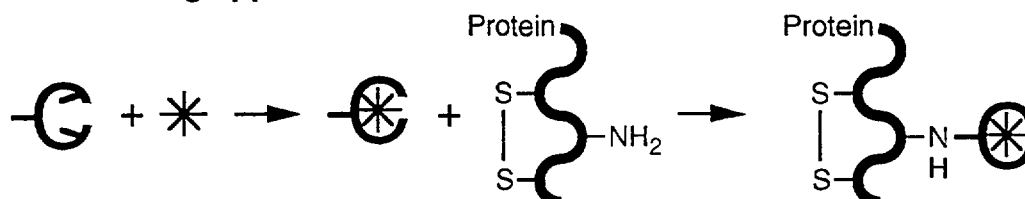
The most direct influence of Nuclear Medicine on patient care can be made using therapeutic radiopharmaceuticals which will play a more important role in the future. In managed care programmes, restrictions are increasingly being placed on the introduction of expensive new therapies into clinical practice. This represents both a challenge and an opportunity for new radiopharmaceuticals. In such a restrictive environment any new product must provide not only a record of safety and efficacy but also an improvement over existing established treatments and this will make it harder for new therapeutic radiopharmaceuticals to find their place in routine clinical practice. However, an alternative route to acceptance of expensive new modalities is to define a subset of patients who are most likely to benefit from this particular treatment. A unique strength of Nuclear Medicine is its ability to combine the use of diagnostic and therapeutic radionuclides for the same target and it seems likely that matched pairs of diagnostic and therapeutic radiopharmaceuticals will be used increasingly in the future to fulfil this role.

At a scientific level, in recent years radiopharmaceutical research has taken a new direction. In the 1970's most research was concerned with labelling a wide range of compounds with potential for

A : Indirect radiolabelling of pre-formed conjugate



B : Pre-labelling approach



C : Direct radiolabelling

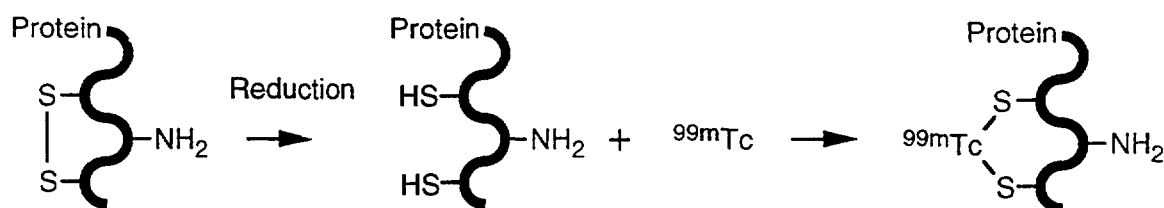


Fig 1. Radiolabelling approaches for proteins and peptides.

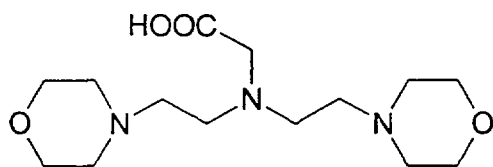
uptake in organs of tissues of interest. Some of the agents which arose from this work still form the basis of useful clinical Nuclear Medicine investigations most notably technetium-99m labelled methylene diphosphonate (MDP) for bone scanning. In the 1980's emphasis was placed on the design of new technetium cores, the primary incentive being to develop perfusion tracers for major organs of interest. This work resulted in the development of HMPAO, MIBI and MAG-3. In the 1990's while some exploration of new technetium cores continues, most research has been directed towards new applications for established cores. In recognition of the strengths of emerging competing imaging modalities such as MRI, increasing emphasis has been directed towards radiopharmaceuticals which can interact with physiological processes such as cell-surface receptors, enzymes and other metabolic systems.

This review describes, in general terms, some aspects of current radiopharmaceutical research which have appeared in the recent published literature or have been the subject of conference presentations. Several of these topics are also the subject of more detailed reviews in this publication. However some specialised areas of work are deliberately excluded from this chapter, in particular, no attempt is made to cover developments in the field of Positron Emitting Tomography.

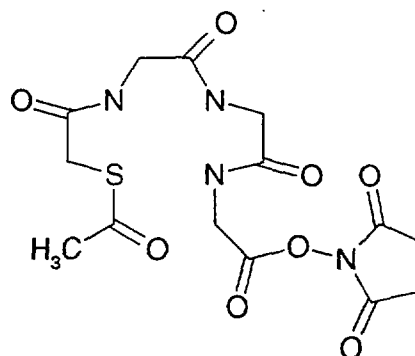
Radiolabelled proteins and peptides.

This field represents the biggest growth area in radiopharmaceutical research over the last decade. It stems originally from the development of monoclonal antibodies and an appreciation of their potential for targeting tumour associated and other restricted antigens in Nuclear Medicine. While the earliest work in this area took place with radionuclides of iodine, an appreciation of the limitations of iodine-131 in particular and the possibilities of a range of metallic radionuclides led to the development of a number of bifunctional chelating agents which were able to act as acceptor sites for radiometals on the peptide backbone of the antibodies as shown in Fig. 1A. The first bifunctional chelates were based upon the well established chelator diethylenetriaminepentaacetic acid and, by conjugation to lysine amino-acid side chains in the proteins were used to label antibodies with indium-111 with considerable success. A common characteristic of all these reagents was that for ease of synthesis one of the carboxylic acid side chains in DTPA was used as the point of attachment to the protein[1]. While stability and crystallographic studies later suggested that this pragmatic approach was perfectly valid when used for the hepta-coordinate complexation of indium, both theoretical considerations, and later experimental data suggested that it was inappropriate for labelling with other metals such as yttrium-90 which prefers octa-coordination for optimum stability. Accordingly, several series of second-generation bifunctional chelates were designed which could satisfy the coordination requirements of these radiometals. Some of these were also open-chain chelators based on DTPA albeit using more complex synthetic pathways which allowed sites of protein conjugation to be engineered into the backbone of the molecule thus leaving all five carboxylates together with five nitrogen atoms with the potential to participate in metal complexation as shown in Fig. 2[2]. In an attempt to provide still greater complex stability a number of closed-cage or macrocyclic chelators were also developed[3]. These reagents have the advantage that they are less liable to attack by competing cations which are present in the biological environment, however they have the potential disadvantage that while complex dissociation is slow, radionuclide association is also slow and radiolabelling of these tracers can take several hours at ambient temperatures[4].

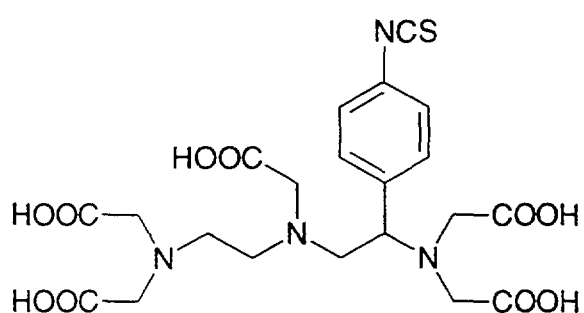
A logical extension of the use of bifunctional chelates for radiolabelling with the trivalent metals was their use for technetium-99m and a number of chelates were consequently designed for use with this radionuclide. A complication arose however when the simple labelling procedures developed for indium-111 were applied to technetium-99m. It became clear that while technetium could certainly bind to appropriately designed chelators conjugated to proteins, the radionuclide could also bind adventitiously to other potential coordination sites offered by the peptide structure of the protein itself[5]. The drawback of this eventuality was the likelihood that the weakly-bound radioisotope would subsequently dissociate from the antibody in a biological environment. An alternative strategy was therefore developed in which the technetium was first labelled to the chelator prior to conjugation to the antibody as illustrated in Fig. 1B[6]. This pre-labelling approach has the advantage that it prevents 'non-specific' binding of the radionuclide to the protein. The technetium is



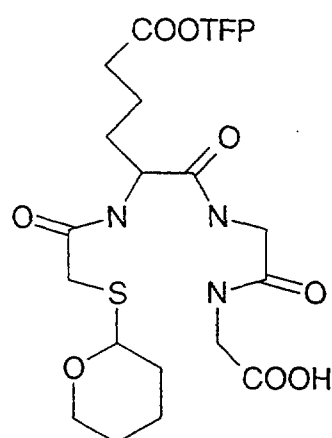
DTPA dianhydride



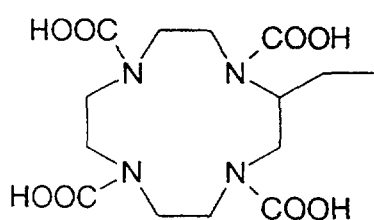
S-acetyl N-hydroxysuccinimide ester of MAG3



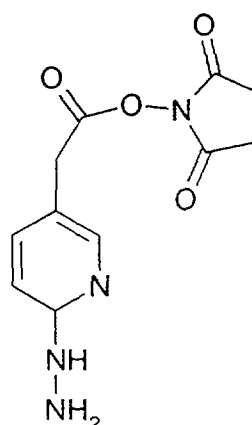
Isothiocyanatobenzyl DTPA



Tetrafluorophenyl ester of an adipic acid - MAG3 analogue



Bifunctional DOTA



N-hydroxysuccinimide ester of HYNIC

Fig 2. Some bifunctional chelating agents for radiolabelling proteins and peptides.

only complexed in the high affinity site offered by the bifunctional chelating agent. The disadvantage of this method is its relative complexity. It is more time consuming than the pre-conjugation approach and the labelling efficiencies achieved are generally less. Nevertheless, this approach provides the most control over the labelling chemistry of any of the antibody labelling methods and a new radiopharmaceutical based on this technology recently received marketing approval. The realisation that technetium will bind, albeit often with low affinity, to sites on the antibody molecule led to a further exploration of direct labelling. It was discovered that there were in fact both high and low affinity binding sites present in the molecule with the former being subsequently identified as thiol-based[7]. Labelling methods were then developed in which the number of these high affinity binding sites was augmented by reduction of the disulphide bridges present in the molecule as shown in Fig. 1C[8]. These methods have been proven to be simple, rapid and efficient for labelling antibodies with technetium-99m and products based on this technology have also found their way through to market approval.

Although these various labelling strategies were developed originally for monoclonal antibodies, it was soon recognised that they were also applicable to many other proteins and polypeptides of potential interest. In recent years, however, the greatest interest has been directed towards their use for labelling very small peptides. The discovery that the radioiodinated somatostatin analogue ^{123}I -Tyr³-octreotide could be used for imaging somatostatin receptors on tumours [9] led to the development initially of a DTPA -conjugated octreotide for imaging with indium-111 and subsequently to a whole host of peptide conjugates which have been labelled with a variety of metallic radionuclides for both imaging and therapy. Owing to the rapid pharmacokinetics of these small peptides, the short-lived technetium-99m is widely regarded as their radiolabel of choice and much effort is currently being expended to refine the methods used for antibody labelling in order to render them suitable for use with these peptides. A major difference is found between the use of different bifunctional chelates for labelling antibodies and their use for peptides in that, while different chelates undoubtedly influence the stability and metabolism of labelled antibodies, their effect upon the overall pattern of biodistribution is relatively small. The overriding influence on antibody biodistribution is that of the large antibody molecule itself. This is not the case with peptides. Small changes in the structure of a peptide conjugate can radically change its biodistribution. This is apparent from a comparison of the distribution of the first two peptide radiopharmaceuticals used in clinical practice. Iodinated Tyr³-octreotide is cleared mainly through the liver and the hepatobiliary tract while indium labelled DTPA octreotide is cleared almost entirely through the kidneys. Technetium-labelled somatostatin analogues have so far shown a pattern of distribution somewhere between the two[10, 11]. Since accumulation of activity in the gastrointestinal tract can impede the detection of tumour deposits in the abdomen, it would be advantageous to alter this pattern of excretion accordingly and work is currently in hand to identify the most critical factors responsible for these patterns of biodistribution.

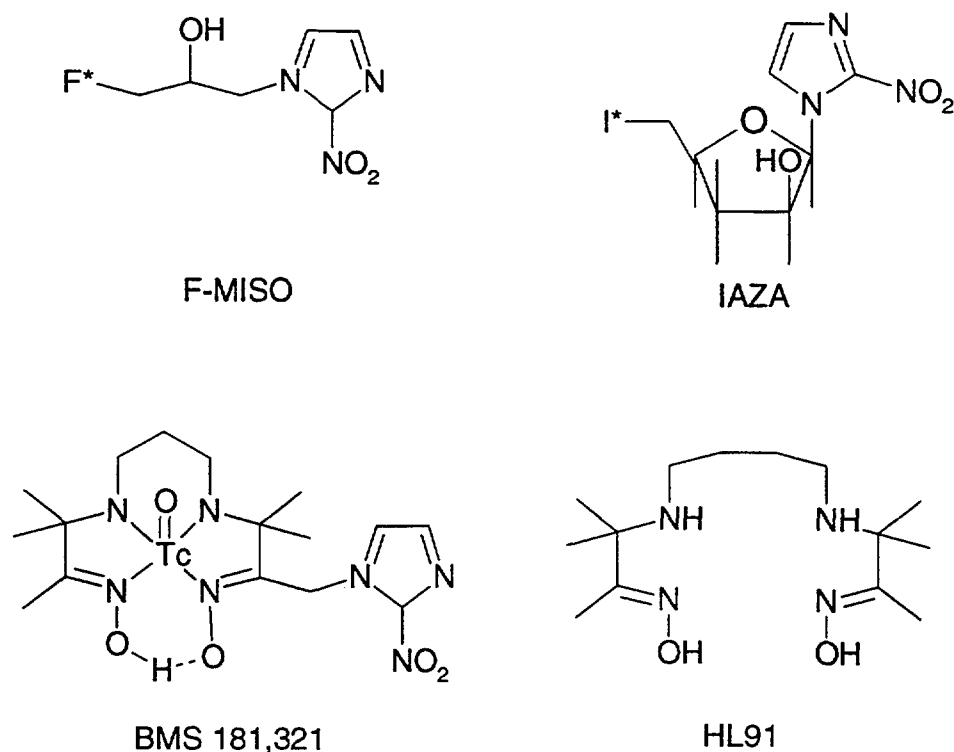


Fig 3. Radiopharmaceuticals for imaging hypoxia.

Radiopharmaceutical research in cancer is not limited to the use of antibodies and peptides. While these compounds are directed towards sites on the outside of cells, other tracers are being explored which interact with intracellular targets. Radiolabelled nucleosides and analogues thereof have been developed which are able to image cell proliferation[12] and also have application in strategies for monitoring gene therapy[13] and radiolabelled oligonucleotides are under scrutiny for imaging mRNA transcription[14]. A number of research groups have also made progress in the design of tracers for imaging cell hypoxia. These agents have potential for predicting the response of tumours to external beam radiotherapy which is strongly influenced by the oxygen potential of the cell. They may also be useful in cardiology for distinguishing areas of necrotic from hypoxic cardiac tissue. Based on the trapping of 2-nitroimidazoles following intracellular reduction, several analogues have been produced labelled originally with iodine-123 [15] or fluorine-18[16] and subsequently with technetium-99m[17] as shown in Fig. 3. However a serendipitous discovery during a research programme at Amersham International resulted in a non-nitroimidazole technetium-labelled molecule which is also trapped by reduction in hypoxic cells [18] and this compound is now in the late stages of the drug registration process.

Targeted Radiotherapy

Following the successful use of antibodies in tumour imaging, consideration was given to their use for targeting of therapeutic radionuclides. Numerous therapy studies have since been performed using either radioiodinated antibodies or antibody-chelate conjugates labelled with beta-emitting

TABLE I. Selected radionuclides with potential for targeted radiotherapy.

Radionuclide	Half-life	decay mode	energy of disintegration
Astatine-211	7.2h	EC, α	3.2-87 KeV electrons 5.8 MeV alpha
Bismuth-212	61m	α, β, γ	2.7-40keV electrons 6 MeV alpha 727 KeV gamma
Bismuth-213	45m	α, β, γ	
Copper-67	2.6d	β, γ	141 KeV beta* 185 KeV gamma
Iodine-125	60.1d	EC	0.7-30 KeV electrons 35.5 KeV gamma
Iodine-131	8d	β, γ	181 keV beta* 364 KeV gamma
Phosphorus-32	14.3d	β	695 keV beta*
Rhenium-186	3.7d	β, γ	329 keV beta* 137 KeV gamma
Rhenium-188	0.7d	β, γ	764 keV beta* 155 KeV gamma
Samarium-153	1.9d	β, γ	225 keV beta* 103 KeV gamma
Strontium-89	50.5d	β	583 keV beta*
Tin-117m	13.6d	IT	0.6-158 KeV electrons 159 KeV gamma
Yttrium-90	2.7d	β	935 keV beta*

*average

radiometals, in particular Yttrium-90[19]. While there have been anecdotal reports of success, examples of sustained responses of solid tumours to such treatments have been very few. In contrast however, many haematological malignancies have shown a profound response to radioimmunotherapy[20, 21] due in part to the improved access of the antibody to malignant cells in these semi-liquid tumours and in part to their greater radiosensitivity. In the field of targeted radiotherapy, much deliberation has been given to the relative merits and drawbacks of the large number of potential therapeutic radionuclides some of which are listed in Table I. At a radiobiological level, the most important factors are generally considered to be particle energy (and consequent path length) and half-life which influences both the time over which the target will be irradiated and also

the dose rate. However at an economic and logistical level the most important factors are cost and availability. In the absence of clear scientific evidence that a shorter or longer path-length or a higher or lower dose-rate is superior, then the radionuclide which is most widely and reliably available for the most acceptable cost will be the choice of many researchers and for this reason iodine-131 and yttrium-90 remain the most widely explored radionuclides for radioimmunotherapy. The treatment of common carcinomas remains the holy grail in radiopharmaceutical research, but, as indicated above, the direct use of labelled antibodies has shown only limited efficacy so far. A promising development, however, is the use of pre-targeted radioimmunotherapy in which small rapidly diffusible therapeutic radiopharmaceuticals are targeted towards antibody conjugates pre-localised in the tumour. This approach appears to improve both the efficacy and therapeutic index of radioimmunotherapy and shows real promise for the future[22]. In the treatment of haematological malignancy, in particular leukaemia, the problem is not one of efficacy but toxicity. Sufficiently high doses can be delivered to malignant cells to potentially cure a high proportion of patients. However, the pattern of infiltration of this disease into the bone marrow produces islands of tumours within a sea of normal haematopoietic stem cells. The long path length of high energy beta-emitters which are targeted to the tumour cells also irradiate and kill these normal cells resulting in a potentially fatal leucocytopenia. For this reason increased interest has been directed towards the use of shorter path-length radionuclides in particular alpha-emitters. For some years a number of groups have shown that alpha-emitting radioimmunoconjugates of bismuth[23], lead,[24] and astatine[25] have efficacy in animal models of cancer. However, recently Phase I clinical studies of bismuth-213 labelled antibodies have begun in patients with leukaemia [26]stimulating great interest that these highly cytotoxic radionuclides will find their place in targeted radiotherapy.

The success of radiolabelled peptides in imaging somatostatin receptors has also generated ideas for targeting cytotoxic radionuclides to these receptors. Initial attempts centred on the use of internal conversion electrons from therapy with high doses of indium-labelled octreotide, however more recently clinical studies have been initiated with a number of different yttrium-90 labelled somatostatin analogues showing promising early signs of efficacy.

Developments in radionuclide therapy have not been confined to proteins and peptides. Among the most fruitful areas of research in recent years has been the development of radiopharmaceuticals for the palliative treatment of malignant bone pain. Four front-runners have emerged which have now received either marketing approval or are in late stage clinical trial; Strontium (^{89}Sr) chloride, Samarium (^{153}Sm) EDTMP, Rhenium (^{186}Re) EHDP and Tin ($^{117\text{m}}\text{Sn}$) DTPA[27, 28]. All show a similar degree of efficacy despite a broad range of physical decay characteristics. The success achieved in treating bone pain together with anecdotal reports of resolution of metastases following Strontium therapy has raised interest in the possible wider therapeutic application of these products for treatment of metastatic bone cancer. Clinical trials to explore this possibility are now in progress but as doses are increased in an attempt to achieve efficacy, problems are likely to be experienced with haematological toxicity since high-energy beta particles targeted to the bone will also irradiate

the normal bone marrow. An advantage here might be demonstrated for the use of Tin-117m which emits only low energy electrons and may spare the normal bone marrow[29].

INFLAMMATION AND INFECTION

This field has provided a fruitful area for radiopharmaceutical research in recent years. In fact the arena has become rather crowded with a number of interesting products competing with each other for acceptance within a limited market. Many of the new products in this area are also based on polypeptides - antibodies, cytokines and peptides - and use radiopharmaceutical chemistry identical to that employed in the field of oncology. Lack of space prevents a detailed description of the broad range of compounds under development and the interested reader is referred to a recent review[30].

NEUROLOGY/PSYCHIATRY

The third major area of endeavour in radiopharmaceutical research has been in the development of agents for imaging neuroreceptors in the brain. This period of time has also brought major advances in molecular biology and has resulted in the unequivocal identification of a number of new sub-types of receptors for the common neurotransmitters. This in turn has stimulated a search by large pharmaceutical companies for drugs which bind selectively to one or more of these receptor sub-types. For many years this field has been dominated by PET chemistry. Owing to the existence of suitable radionuclides of carbon, oxygen and nitrogen, radioactive forms of almost any new drug with properties identical to the parent compound can be synthesised. This, of course, is not the case with single-photon emitting radioisotopes. Almost any radiolabelling procedure requires the insertion

Table II. SPECT radiopharmaceuticals for neuroreceptor imaging

Neuroreceptor system	Radioligand
Muscarinic	123-I QNB
	123-I Iododexetimide
5HT reuptake transporter	123-I- β -CIT
5HT 2a	123-R-91150
Dopamine reuptake transporter	123-b-CIT, FP-CIT
	Technepine
	TRODAT-1
Dopamine D2	123-I IBZM
	123-I IBF
	123-I epidepride
GABA/benzodiazepine	123-I Iomazenil
	123-I NNC 13-8241

of a foreign element into the drug molecule with unavoidable effects upon its physicochemical and biological properties. In order to keep the size of this foreign atom as small as possible iodine has always been the radiolabel of choice in this field and a number of radioiodinated tracers which bind to neuroreceptors have recently been developed. The aim in developing agents which bind to peripheral receptors such as those expressed on tumour cells is to produce a biologically stable molecule which binds selectively with high affinity to the receptor while showing an advantageous pattern of biodistribution and excretion as described above. In neurotransmitter research there is the additional requirement that the tracer must be able to cross the blood-brain barrier in order to gain access to the receptor of interest. PET radiopharmaceuticals now exist for all of the most important neurotransmitter systems - cholinergic, serotonergic, dopaminergic, GABA/benzodiazepine and opioid. In many cases selectivity for certain receptor subtypes can be demonstrated. Radioiodinated SPECT ligands have so far become established for only some of these as listed in Table II. A number of these radioligands show low receptor selectivity. β -CIT, for example, binds to both 5HT and dopamine receptors. Further research in this area however will continue to refine the selectivity of these agents and produce further tracers for those neuroreceptors not yet addressed.

Although neuroreceptor imaging agents labelled with iodine-123 can produce high quality images, the poor availability of this radionuclide places severe restrictions upon their widespread application. While it is hoped that the existence of new clinically-useful tracers will stimulate more producers to make iodine-123, it seems likely that universal application of brain receptor imaging agents will depend upon the development of technetium-99m labelled analogues. Because of the need to build into the radioligand not only the large technetium atom but also the chelate structure necessary to complex it, the design of such molecules makes great demands upon the ingenuity of the radiopharmaceutical chemist. As described above, a neuroreceptor-imaging radiopharmaceutical must not only be able to bind selectively with high affinity to the receptor, it must also be able to reach its target by passing through the blood-brain barrier and this places severe restrictions on the nature of the molecule. It must be relatively small, ideally less than 600 Da and it must be lipophilic with a log P of about 2[31]. The traditional approach, taken in the design of ligands for peripheral receptors is to attach a suitable technetium coordination site to the receptor-binding molecule, probably via a small spacer to reduce the effect of steric hindrance on receptor interaction - the so called pendant approach. However, the net result of such a design would almost certainly be a molecule too large to pass efficiently through cell membranes. The alternative is to try and build a technetium coordination site into the receptor-ligand itself in such a way that the technetium and its core resemble a part of the recognition site for the receptor. This is known as the integrated approach and is illustrated in Fig. 4.

Examples of the successful application of the pendant approach can be seen in two radiopharmaceuticals for imaging the dopamine re-uptake system. Both are tropane derivatives similar in structure to cocaine and the CIT series of compounds as shown in Fig. 5. Technepine[32] uses an amide/amine/dithiol core conjugated via a three carbon spacer to the molecule via the tropane

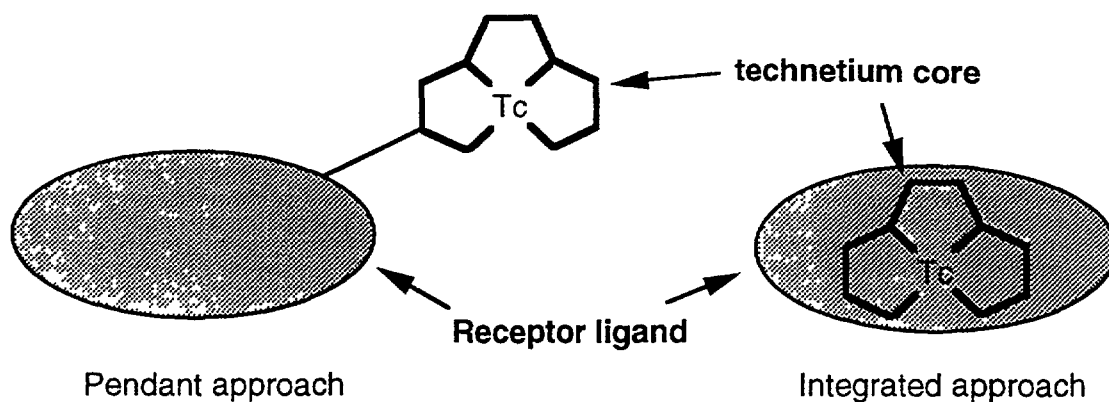


Fig 4. Pendant and integrated approaches to radioligand design.

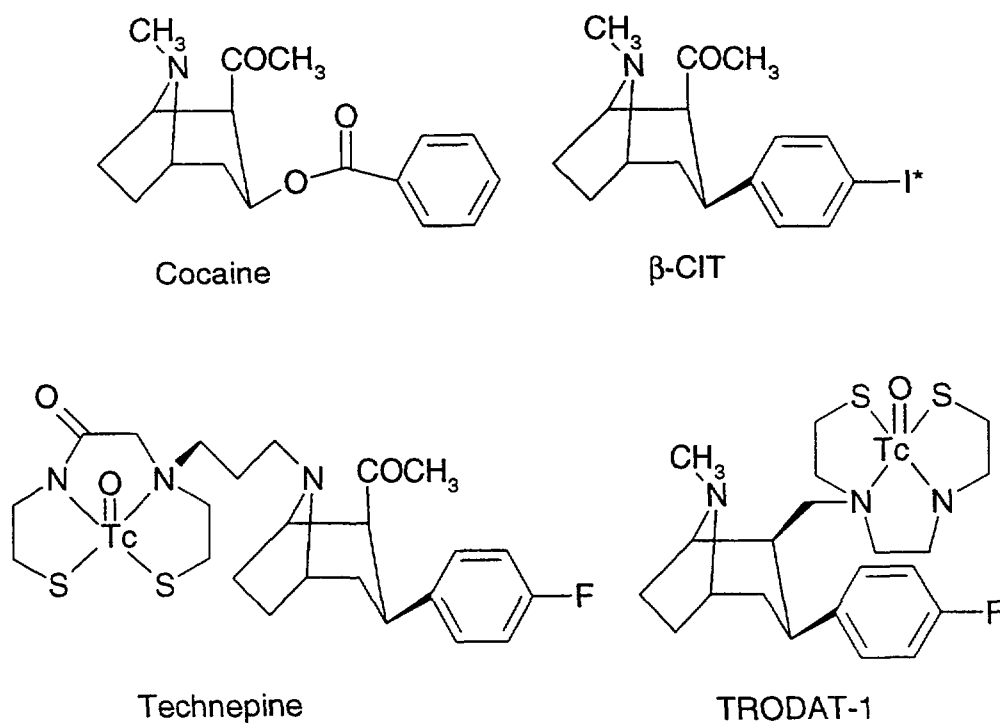


Fig 5. Radioligands binding to the dopamine reuptake receptor.

nitrogen while TRODAT-1[33] uses a diamine dithiol system attached via the 2β position of the tropane ring. Both molecules show good localisation in dopamine reuptake receptors in the living brain.

Because of the greater demands imposed by the integrated approach to radiopharmaceutical design, results in this field are not so far advanced, however a number of publications have pointed the way to progress along this road. Among these is the work of Johannsen and co-workers who have integrated technetium cores into different portions of the molecule ketanserin while still retaining a significant degree of receptor-binding affinity and brain uptake[34]. An example is shown in Fig. 6.

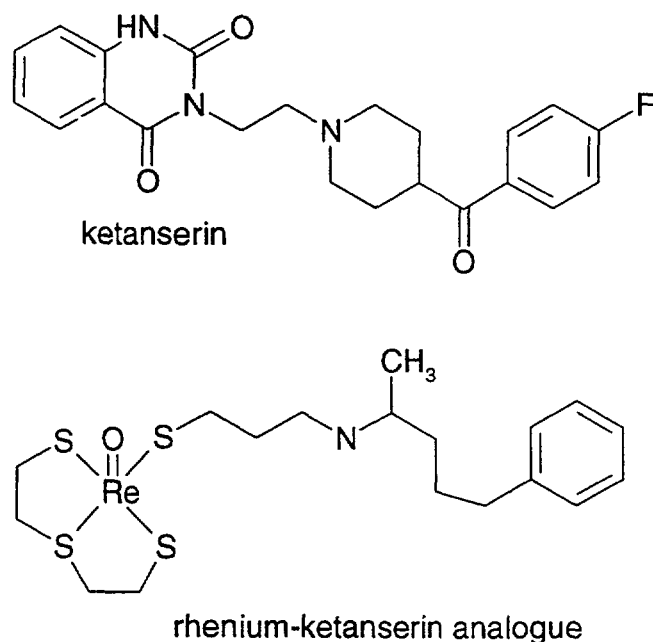


Fig 6. Ketanserin and an integrated rhenium conjugated analogue.

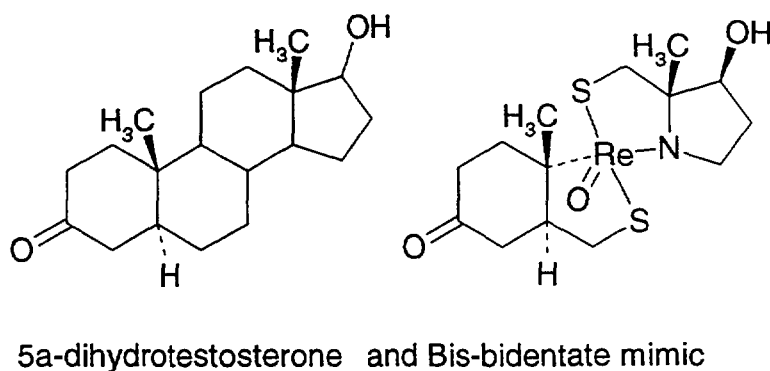


Fig 7. Design of an integrated rhenium labelled steroid analogue.

Perhaps the most extensive study in technetium core integration has been undertaken not in the field of neuroreceptor imaging but in that of oncology. Steroid hormones, like the neuroreceptors, have very restricted requirements for receptor binding. Steroid receptors, although peripheral, are largely intracellular and any radiotracer must therefore also be able to cross cell membranes. Although radioligands designed using a pendant approach have shown some success[35], the large multi-ring structure of the steroid hormone has provided an attractive target for an integrated approach [36] and a number of model compounds have been synthesised as shown in Fig. 7.

CONCLUSION

In recent years major progress has been made in the design of new radiopharmaceuticals based on both small synthetic ligands and larger naturally occurring molecules such as proteins and polypeptides. All of these new tracers share the characteristic that they interact with the biological processes underway in their cellular targets and therefore potentially provide unique information unavailable through any other imaging modalities. Progress has also been made in the design and clinical application of therapeutic radiopharmaceuticals for use in the treatment of haematological malignancies and bone pain. These products will form the basis of the next generation of diagnostic and therapeutic radiopharmaceuticals and indicate a healthy future for Nuclear Medicine in the first decades of the next millennium.

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EVALUATION OF ^{99m}Tc -ior t3 MoAb AS A RADIOTRACER IN RENAL GRAFT ACUTE REJECTION

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Abstract

Diagnosis of renal graft acute rejection is made mainly by clinical signs, humoral and functional changes, and is confirmed by histopathological studies. Many efforts have been made to find a radiopharmaceutical for early diagnosis of renal graft rejection.

^{99m}Tc -ior t3 monoclonal antibody was evaluated as a radiotracer in renal graft acute rejection. Schwarz's method was used with different molar relations 2ME:IgG and different tin-chelates were explored. The selected formulation was studied by chromatography and challenge studies. Once proved the quality of the radiopharmaceutical a freeze-dried Kit was performed. Its radiochemical purity and stability was studied for several months. FPLC and immunoreactivity studies were included. The sterility and apirogenicity was certified by the Center of Research and Development of Drugs. A clinical trial was started with patients who have received a renal graft eleven days before. An amount of 1.1 GBq of ^{99m}Tc -ior t3 MoAb was administered and planar scintigraphic images were recorded.

A molar relation of 2000:1 (2ME:IgG) with 30 min produced the best reduction of the MoAb. From the different tin-chelates, the tin-pyrophosphate produced the highest radiochemical purities. The MoAb freeze dried Kit contains 1.0 mg of protein and 14 μg of Sn^{+2} , this quantities guaranteed a shelf life of 6 months. The quality controls and challenge studies showed purity higher than 95.0 % and a per cent of dissociation close to 20 % was seen with DTPA, 80 % with HSA and 48 % with Cys.

Our preliminary immunoscintigraphic results showed a good correlation among scintigraphy, a high rate of counts Renal Graft/Opposite side and the cause of graft loss. The small number of cases does not permit to make a definite conclusion. However, in some clinical situations the use of ^{99m}Tc -ior t3 MoAb could help as an early and specific non-invasive diagnosis of renal graft rejection.

1. Introduction.

Diagnosis of renal graft acute rejection is made mainly by clinical signs, humoral and functional changes, and is confirmed by histopathological studies (1).

Several radiopharmaceuticals have been evaluated as " radiotracers " of renal graft acute rejection, among them the ^{99m}Tc -Sulphur Colloid (2), radiolabeled Fibrinogen (3), ^{111}In -Platelets (4) and ^{67}Ga -Citrate (5). These agents had poor or none specificity(6). Baum (7) in 1990 made the first specific diagnosis of acute rejection using the Monoclonal Antibody (MoAb) t11 (Anti-CD2) labeled with ^{99m}Tc in patients with renal and cardiac grafts.

The aim of this report is to show the elaboration of an ior t3 MoAb freeze-dried Kit, its behaviour, stability and potential utility as a radiotracer in renal graft acute rejection in a group of patients with renal grafts.

2. Material and methods.

2.1. Radiolabeling and evaluation of tin-chelates.

The ior t3 MoAb (Anti-CD3), isotype IgG2a was provided by the Center of Molecular Immunology (CIM) at a concentration of 5.0 mg/ml. Schwarz 's method (8) was used with molar relations of 2ME: IgG of 1000: 1, 2000: 1 and 5000: 1 during 30 min. Finally a relation 2000: 1 was chosen for the elaboration of freeze-dried Kits.

Once the reduction process was completed, the reduced MoAb was applied on a column (syringe of 10 ml of volume) filled with Sephadex G-50 (Pharmacia) previously equilibrated with Buffer Phosphate 0.1 M, pH-7.4 (degassed with gaseous nitrogen). Fractions of 3.0 ml were collected in sterile flasks, aliquots of 50 ul were taken of the different fractions and the optical density at 280 nm was determined. The IgG concentration was estimated by the molar extinction coefficient (E-1.4). A pool was made with the fractions with highest values of reduced IgG concentration. An aliquot was taken and kept frozen until the estimation of free thiols groups was made.

A comparison was performed with different tin-chelates, Sn-MDP, Sn-Pyrophosphate and Sn-Gluconate. These Kits were made by the Institute of Energetics and Nuclear Research (Sao Paulo, Brasil). A flask of the above mentioned Kits received a volume of steril saline solution (SSS) and was mixed. An aliquot was taken and added to a flask which contain 250 ug of reduced ior t3 MoAb. The mixture was shaken and a volume of pertechnetate was added. Thirty min later an aliquot was taken and a quality control was made.

2.1.1. Preparation of freeze-dried Kits.

Initially was elaborated a first batch which contained 250 ug of reduced ior t3 MoAb and 2.8 ug of Sn+2 per flask. This Sn+2 belongs to the Sn-Pyrophosphate Kit made by brazilian colleagues. This mixture was sterilized by filtration through 0.22 u sterile membranes and deposited in sterile flasks. These were frozen and dried by 24 hours.

Three new batches nominated 951018, 960306 and 971202 were elaborated with a modification, in this case the amount of reduced MoAb was increased to 1.0 mg and the Sn+2 content was increased proportionally too. An amount of Sodium Gentisate was included. This Sn-Pyrophosphate-Gentisate solution was elaborated every time the Kit was made in our center. The rest of the procedure was performed as was described above for the first batch. Once finished the lyophilization process the flasks were kept at 4° c until they will be used.

From these new batches a group of flasks were sent to certify their sterility and apirogenicity.

2.1.2. Radiochemical purity controls.

Two flasks of the freeze-dried Kits were taken and labeled with 1.1 GBq of pertechnetate obtained from a 99Mo-99mTc (Elumatic II) provided by AMERSHAM Int. Thirty min later a sample was taken and applied on strips (0.6x7cm) of Whatman 3 MM. Ascending paper chromatography (APC) was developed. As solvents Metil Ethyl Ketone (MEK) and steril saline solution (SSS) were used. The strips were dried at 80° c and they were cut in the middle portion. Each one was counted in an automatic gamma counter and the per cent of purity was considered the fraction of radioactivity, which remained at the origin. A sample of the radiopharmaceutical was applied on a column (1x30 cm) filled with Sephadex G-25 M, previously calibrated and equilibrated with Buffer Phosphate 0.1 M, pH-7.4 (degassed with nitrogen). The per cent of recovery was estimated against a sample of the volume applied on the column. The controls have been made monthly along the first eight months after the Kit elaboration.

2.1.3. FPLC study.

Some flasks which contain 1.0 mg of ior t3 MoAb freeze-dried Kit were sent to the Center of Molecular Immunology (CIM) for analysis. Briefly, a volume of SS was added to a flask and the IgG concentration was adjusted to 0.5-1.0 mg/ml of SS. An aliquot was applied on a column of Superose 12. The elution was performed with Buffer Phosphate 0.05 M, pH-7.4 and the flow was regulated to 0.5 ml/min. Several fractions were collected and the optical density (at 280 nm) was recorded automatically.

2.1.4. Challenge studies.

Challenge studies with DTPA (2000 fold molar), Human Serum Albumin (100 fold) and Cysteine (3000 fold) were performed at different times. Briefly, the labeled MoAb was incubated with the ligand at room temperature. Samples were removed and applied on strips of Whatman 3 MM. These strips were developed with SS. The rest of the procedure was made as described in 2.1.2.

2.1.5. Immunoreactivity determination.

This was performed in the CMI, three flasks at least of each batch were evaluated. The content of a flask was dissolved with SSS and the concentration was adjusted to the required value. Selected volumes of the MoAb Kit and fresh blood were mixed in the cell of the FACS equipment. This made the separation of T Lymphocytes itself and recorded the percentage of MoAb bound to T Lymphocytes. The procedure was repeated with native ior t3 MoAb and the results were compared and expressed in per cent of immunoreactivity related to the native ior t3 MoAb.

2.1.6. Thiol assay.

A volume of 50 ul of reduced MoAb was added to 50 ul of Ellmans ' reagent (9) and the mixture was diluted with 3.0 ml of Buffer Phosphate 0.1 M, pH-8.0 and incubated at room temperature for 15 min. Optical Density was measured in an U V spectrophotometer at 412 nm. The number of free thiols was calculated by comparison with a standard curve obtained by the assay of a series of Cysteine standards ranging from 0.01 to 1.0 mM.

2.1.7. Clinical trial.

Once certified the sterility, apirogenicity and immunoreactivity of the freeze-dried Kits (951018, 960306 and 971202) a clinical trial was started with renal graft patients who received the kidney between 7-21 days before. The patients expressed their signed approbation to be included in the trial. All national and international regulations for human application of murine immunoglobulins were strictly observed. A subcutaneous test was made with 10 ug of ior t3 a day before the immunoscintigraphy will be done.

The patients received 1.0 mg of labeled ior t3 MoAb with a radioactivity between 1.1-1.3 GBq . Planar scintigraphic images were recorded between 10 min and 24 hours with a Gamma Camara Sophy-20P Sopha Medical. The rate of counts between renal graft (RG) and its opposite side (OS) was determined at different times. Once concluded the scintigraphy a biopsy was made and the correspondent histopathological study was performed.

3. Results.

3.1. Radiolabeling.

Table I shows the concentration of free thiols according to the molar relations 2ME: MoAb used. The ior t3 MoAb expressed a 4.7 % of free thiols without 2ME.

After a reduction with 1000: 1 (2ME :MoAb) the proportion of thiols groups was duplicated. An increase of the molar relation to 2000 :1 and even more to 5000 : 1 only produced a small increase in the proportion of free thiol groups.

The influence of ligands is presented in Table II. It was evident that Pyrophosphate (PPi) resulted the best ligand, because of independently of the amount added the per cent of purity obtained was highest even at 24.0 hours. For MDP, the increase of Sn+2 concentration, which implies an increase of MDP concentration, produced a progressive reduction of the radiochemical purity, especially at 24 hours. The Gluconate (GLU) apparently produced a high per cent of purity, but this result was accompanied of a strong opalescence in the solution and a posterior sedimentation of a white precipitate.

Table I

Estimation of free thiols by Ellmans ' reagent

2ME	MoAb	uM -SH	Per cent -SH
	0	11 3±1 3	4 7±1 1
1000	1	14 0±1 0	10 7±1 4
2000	1	16 1±1 4	12 3±0 9
5000	1	20 5±1 2	14 9±0 8

n = 3

Table II

Influence of the ligands on the radiochemical purity

ug Sn+2	Per cent of purity on Saline		
	MDP	PPi	GLU*
1 04	96 0±1 0	99 8±0 1	99 0±0 2 a
	95 0±0 7	99 7±0 2	99 2±0 5 b
2 08	96 0±0 8	99 8±0 2	95 0±1 1
	99 0±1 0	99 4±0 5	98 0±0 9
5 20	88 0±1 3	99 7±0 2	95 0±0 7
	78 0±0 8	99 6±0 2	98 0±0 9
10 40	76 0±1 4	95 0±0 8	88 0±1 3
	67 0±0 9	99 4±0 2	99 0±0 6

n=3

MoAb-250 ug
a-1.0 hr b-24 hr *-Opalescence

3.1.1. Freeze-dried Kits and purity controls.

The four batches answered positively to sterility and apirogenicity tests. This assured the quality of the freeze-dried Kit as intravenous pharmaceutical formulation before the administration to the patients.

Figure 1 presents the quality control made to the different batches with SSS. The first batch (without nomination) corresponds to the lowest mass of MoAb and Sn+2. Its shelf life was shorter than 90 days. Batches nominated 951018 and 960306 keep their high radiochemical purities even 180 days later. Column chromatography showed a 95.0 % of radioactivity associated with the protein peak. The recovery was always higher than 96.0 %. This results means a low proportion of tin radiocolloids in the labeled MoAb.

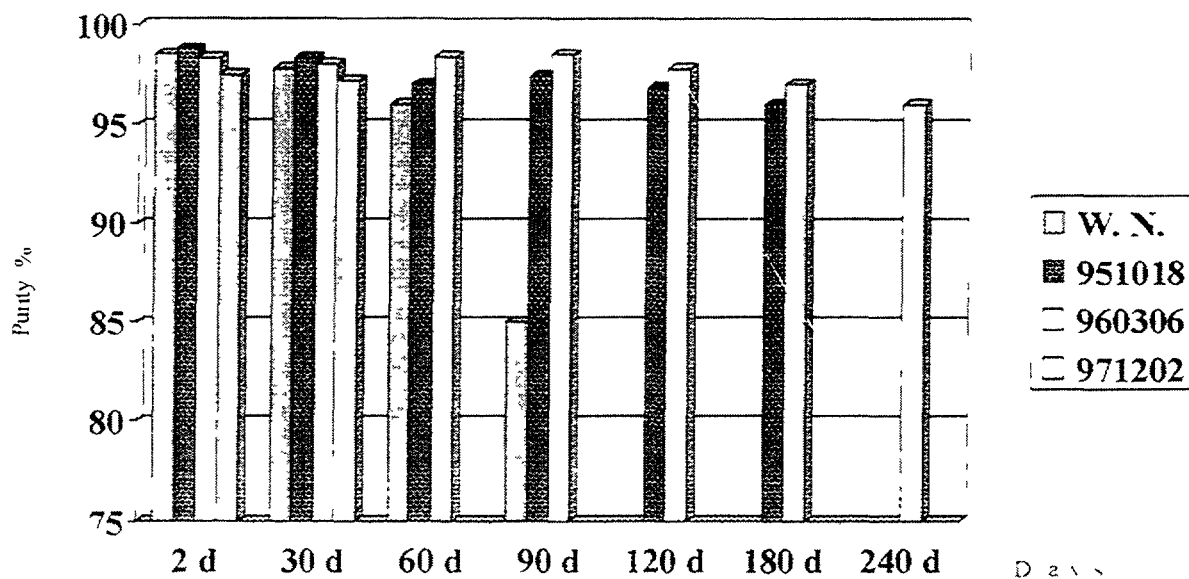


FIG 1. Stability of the radiochemical purity for the different batches.

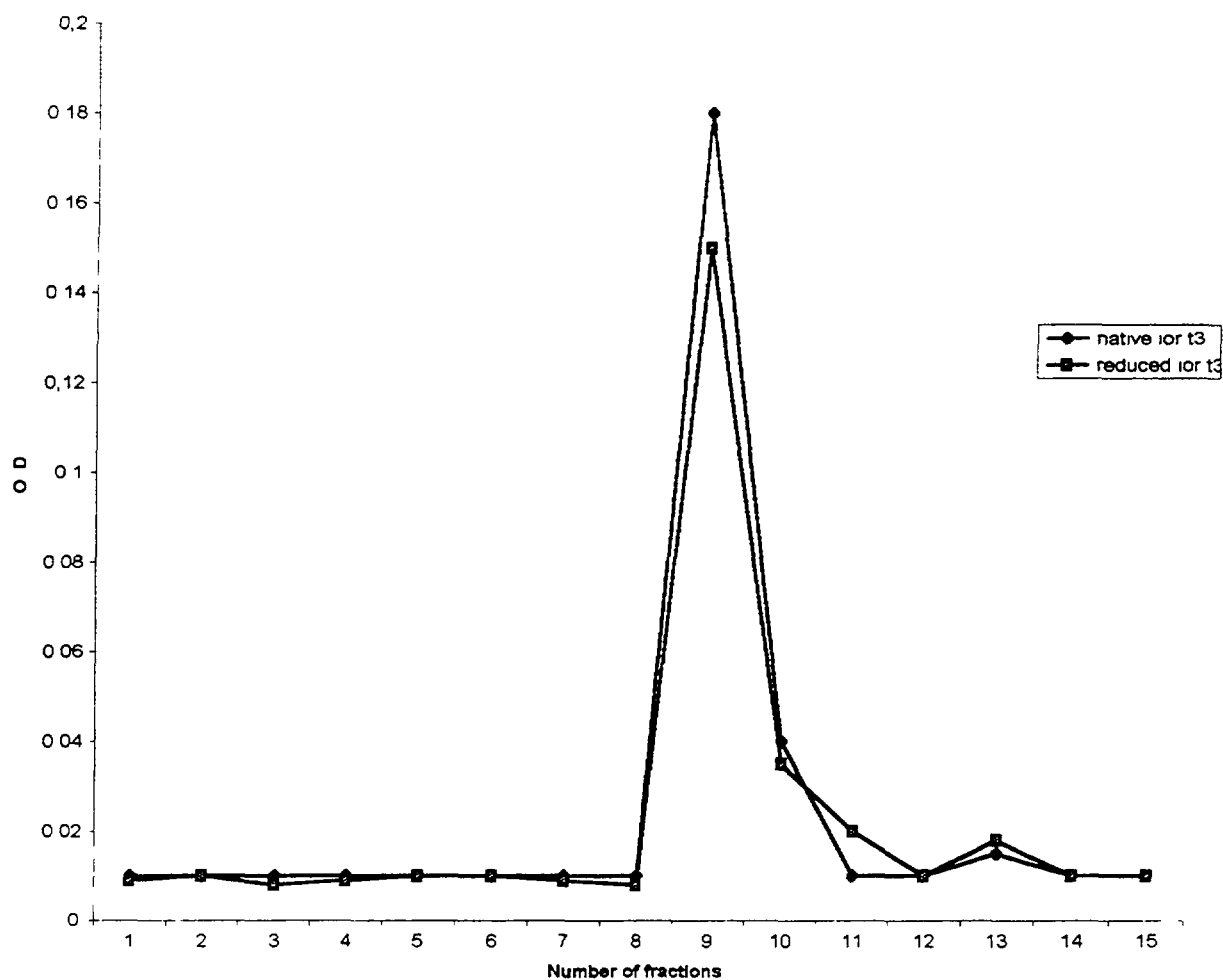


FIG. 2. FPLC of ior t3 on Superose 12.

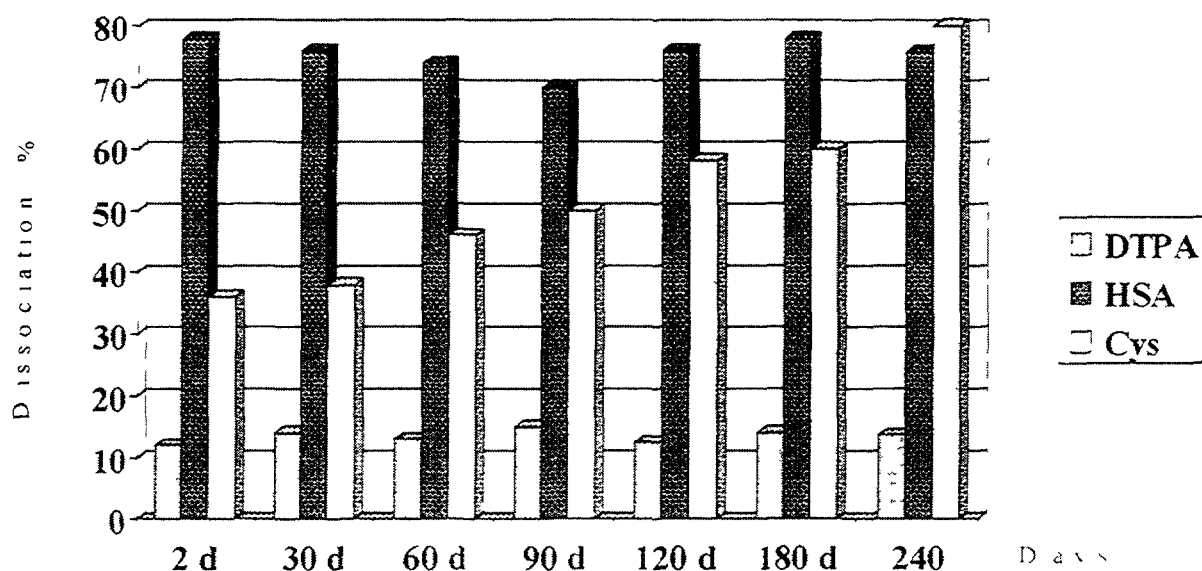


FIG. 3. Challenge studies of the 960306 batch.

3.1.2. FPLC study.

Figure 2 shows a characteristic chromatogram seen for the freeze-dried Kits on Superose 12. This presents mainly a peak, which corresponds, to native MoAb and the area represented a 98.4 %. No aggregates were detected and the percentage of fragments was lower than 1.5 %. These results were similar for the different batches and remained approximately constant during the firsts 180 days.

3.1.3. Challenge studies.

Figure 3 shows the challenge studies made against DTPA (2000/1), HSA (100/1) and Cys (3000/1) for 960306 batch at 4.0 hours. A per cent of dissociation close to 14.0 was seen for DTPA, which remained practically constant during 180 days. However, HSA produced a high dissociation level close to 80.0 %, which remained all the time. At 24 hours, a light decrease of the dissociation to 70.0 % was seen. Cys had an increased dissociation level; its highest value was reached at 240 days.

3.1.4. Immunoreactivity determination.

The immunoreactivity study showed that reduced and lyophilized ior t3 MoAb recognized between 73.0-83.0 % of T Lymphocytes in comparison to native MoAb. This result was the same for batches nominated 951018 and 960306.

Table III

Immunoscintigraphy, rate of counts and histopathology			
Patient number	IS	RG/OS	Histopathology
1	+	20	A R
2	-	13	A T N
3	+ -	16	A T N
4	-	13	B C
5	-	12	B C
6	+ -	14	A T N

A.R.-Acute Rejection A.T.N.-Acute Tubular Necrosis B.C.-Borderline Changes

3.1.5. Thiol assay.

The determination of free thiol groups demonstrated a generation of 6-7 thiol groups when a molar relation between 1000 and 2000: 1 was used. An ulterior increase to 5000 : 1 did not increase significantly the fraction of thiol groups.

3.1.6. Clinical trial.

Table III summarizes the studied patients, the criterion of positive (+), negative (-) or doubtful (+ -) immunoscintigraphy (IS), counts relation RG/OS (Renal Graft/Opposite Side) and histopathological diagnosis. There was in this small series of patients a good correlation among IS, counts relation and the cause of graft loss.

4. Discussion.

The most popular method for radiolabeling immunoglobulins corresponds to Schwarz (8). This use as a reducing agent 2-Mercaptoethanol (2ME) which originates a fraction of free thiols. However, the molar relation 2ME:IgG depends of the immunoglobulin to be reduced, because of murine immunoglobulins seem to be more resistance to reduction (10) or they can reorganize faster the broken disulfide bridges.

The ior t3 MoAb presents a low proportion of free thiols in its structure. The treatment with 2ME showed a saturation curve, that is a considerable increase of free thiols with the lowest molar relation and lower increases for the highest molar relations.

Our findings with different tin-chelates showed that a MoAb might answer in a very particular way, according to the tin-chelates, its components and elaboration. The Pyrophosphate was the weaker ligand in this series and permitted a high radiochemical purity and stability of the labeled MoAb. It showed the lowest affinity for 99mTc against the reduced MoAb. The Gluconate anion produces a Sn+2 precipitation as a hydroxide. This was explained because of for this particular tin-chelate the tin was added as a complex of tinasc (tin ascorbate) and this chemical form was decomposed when it came in contact with the protein solution.

The mass of MoAb and tin, the addition of tin preservatives and the procedure of lyophilization (11) determine the shelf life of a freeze-dried Kit. This fact was made evident in the first batch (without nomination); the low mass of tin and the absence of sodium gentisate drove us to reach a shelf life shorter than 90 days. The ulterior increase to 1.0 mg of MoAb, the correspondent increase of tin and the incorporation of gentisate as a tin preservative, make us to increase its shelf life to 180 days.

As ascending paper chromatography is not able to discriminate between labeled MoAb and tin radiocolloids, a Sephadex G-25 M column chromatography was used (12). This demonstrated that a 95.0 % of radioactivity was associated to the protein peak and more than 96.0 % of the applied radioactivity was recovered in the eluate. This fact guarantees a tin radiocolloids proportion lower than 4.0 %. The ulterior quality controls showed that this radiocolloid level was kept approximately constant.

Molecular modifications introduced on the structure of the MoAb by the Kit formulation can be detected only by means of size-exclusion chromatography (13) or SDS-PAGE (14). The chromatography on Superose demonstrated that a reduction with molar relation 2000:1 does not originate the formation of molecular aggregates and the fragmentation was within acceptable limits (15).

Challenge studies made against DTPA and Cysteine evidence the 99mTc binding on high affinity sites on the MoAb structure (16), because of the percentages of dissociation were similar to those found by other authors (17,18). For HSA, nevertheless, a high dissociation percentage was found as reported Alauddin (19). This can be attributed to the high molar relation used. Another factor, which could influence

is that our HSA contains several preservatives agents, which may modify the affinity of ^{99m}Tc on the MoAb structure.

Immunoreactivity study demonstrated a good biologic activity of the ior t3 MoAb after the Kit formulation. This means that the procedure did not imply molecular modifications, which affect the recognition of CD3 membrane complex on the T Lymphocytes.

The clinical trial have been made in a small group of patients and results resembles to Baum 's report (7) and confirmed the potentiality of labeled ior t3 MoAb as a radiotracer of renal graft rejection, because of a good correlation was observed between results and the cause of graft loss. Nevertheless, the small number of cases does not permit to make conclusions yet.

5. Conclusions.

The elaboration of ior t3 MoAb freeze-dried Kit was made. It has a shelf life of 180 days and the radiopharmaceutical could help to an early and specific non-invasive diagnosis of renal graft acute rejection.

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SODIUM METABISULFITE: A NEW REDUCER AGENT FOR DIRECT LABELLING OF IMMUNOGLOBULINS WITH ^{99m}Tc

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Abstract

A method for direct labeling of antibodies with ^{99m}Tc is described. Sodium Metabisulfite (SMB) was evaluated as a new reducing agent of disulfide bridges of the IgG molecule. Under selected experimental conditions, radiochemical purities of the label higher than 90.0 % were achieved in most of the experiments. On the basis of the obtained yields an empiric equation which describes the system was computed as : $Y = 93.6 + 2.1X5 - 1.36X6 + 0.9X7$. The absence of $X4$ indicated that the purification on Sephadex of the reduced IgG was not relevant to the labeling process. The most important variables were tin ion concentration ($X5$), the volume of Tin-Tartrate solution ($X6$) and the incubation time between reduced IgG and Tin-Tartrate solution ($X7$). A low aggregation and fragmentation of the IgG molecule was obtained when a SMB/IgG molar relation of 750/1 was used. The stability of the ^{99m}Tc -IgG was assessed by DTPA, HSA and L-Cysteine challenge studies. An acceptable level of dissociation was observed in presence of DTPA and L-Cysteine, when the Tartrate anion was used. However the transchelation level for HSA was about 80.0 per cent. These preliminary results showed that SMB could be a useful reducing agent for direct labeling of IgG. Further studies are needed to correctly evaluate it.

1. Introduction.

Nowadays the polyclonal and monoclonal antibodies are widely used for immunoscintigraphic diagnosis of different diseases. Initially these molecules were labeled with iodine 131 or 123 and indium 111. Although ^{99m}Tc is often considered to be the superior label for radioimmunodiagnosis for reasons of cost, availability and imaging properties, in spite of its relatively short physical half-life. The existing labeling methods may be classified into two types : indirect and direct approaches (1). Direct methods employ a reducing agent in order to transform a number of disulfide bridges in the protein to free thiols, which are able to bind technetium very efficiently due to their strong chelating capacity. These approaches are appealing because the antibody conjugation is avoided, the reduction is easily controlled, a higher specific activity is obtained, the procedures are simple to perform and offer easy product formulation. Several groups have employed different reducing agents such as stannous ion (2), 2-Mercaptoethanol (3,4), dithiothreitol (5) and others.

Each of these procedures for direct labeling have their advantages and drawbacks. There is no consensus about which of the methods is superior (6). Therefore the development of faster and easier methods in the labeling of monoclonal antibodies keeps on (7,8).

The aim of the present work is to evaluate Sodium Metabisulfite (SMB) as a new reducing agent for the direct labeling of antibodies with technetium- 99m because is non-toxic (9).

2. Materials and methods.

Lyophilized human polyclonal non-specific IgG was supplied by the Center for Hemoderivatives (Havana, Cuba).

A non-saturated (10) factorial design $2^{(7-4)}$ was performed to study the influence of seven independent variables (Xi) on the labelling efficiency (Y). It was used for Xi a low level (-) and a high level (+) for the initial 16 experiments, which were done at least twice. The Xi include : X1- mg IgG/ml (2 and 5), X2- SMB/IgG (500/1 and 1000/1), X3- reduction time (30 and 60 minutes), X4- Sephadex chromatography (No and Yes), X5- Sn+2 concentration (0.14 and 0.56 $\mu\text{g}/\mu\text{l}$ HCl-0.02 N), X6- μl of Sn-Tartrate solution (100 and 200) and X7- incubation time of reduced IgG and Sn+2 (0 and 15 minutes). The Sn+2 (X6) has 50 μmoles of Tartrate/ml of solution and the selected concentration of Sn+2 (X5).

2.1. Radiolabeling.

The procedure was started taking an amount of dried SMB and put it in a flask. The required volume of IgG dissolved in 0.1 M Phosphate Buffer , pH-7.4 or Saline (NaCl-0.9 %) was added and mixed .Solution was previously degassed by boiling in a gaseous nitrogen. The flask was closed and incubated a selected time. The mixture was not applied on a small column of Sephadex G-50 (previously calibrated and equilibrated with degassed Phosphate Buffer) or was applied. In the last case it was taken the fractions with reduced IgG. An amount of Sn-Tartrate was added and immediately or 15 minutes later 370 MBq of $^{99\text{m}}\text{Tc}$ was added. Purity was determined by Ascending Paper Chromatography (APC) with Metil Etil Ketone and Saline on strips of Whatman 3MM paper. Strips were cut in the middle point, radioactivity was measured and expressed as per cent of radioactivity at the origin (R_f -0.0). Finally five more experiments (17 to 21) were done with Xi in the middle level (0), excepts X4 which was kept at low level (No Sephadex purification).

2.1.1. FPLC and SDS-PAGE studies.

The reduced IgG obtained in this last condition (750/1) was analyzed by means of FPLC (11) and SDS-PAGE (12) . Briefly, a sample of native IgG was applied on a Superose 12 column previously calibrated and equilibrated with Phosphate Buffer. Flow was adjusted at 0.53 ml/minute and several fractions were collected and their absorbance at 280 nm was determined. The column was washed extensively with buffer and the correspondent reduced IgG was applied, several fractions were collected and their optical density was measured . A SDS-PAGE study was performed under nonreducing conditions on the native IgG and reduced IgG (750/1) . Briefly, samples for assay were prepared at a SDS concentration of 0.5 % and were not immersed in a boiling water bath prior to gel loading. Molecular weight standards were prepared in the same manner. Samples were resolved in a 3.5 % acrylamide stacking gel and a 8.0 % running gel . Gels were run at 7.5 mA/gel until the tracking dye reached the bottom. Each gel was fixed with 10 % trichloroacetic acid, stained with Coomassie Brilliant Blue G-250 and destained in 25.0 % methanol . Gels were read by means of Molecular Analyst SoftWare provided by Bio-Rad firm.

2.1.2. Sephadex G-25 M study.

The $^{99\text{m}}\text{Tc}$ -IgG was applied on Sephadex G-25 M to determine the fraction of radiocolloids (13) . Briefly, a sample was applied in a closed (1x7 cm) column and it was measured in a dose calibrator. The elution was performed with 10 void volumes of Saline solution. The column was closed and the radioactivity was measured again. The remained radioactivity was considered the percent of radiocolloids.

2.1.3. Assay of different ligands for the tin solution.

The influence to exchange the Tartrate anion for Gluconate (BDH), MDP and Citrate (BDH) was studied. The ligand concentration was maintained at 50 umoles/ml of solution, excepts for MDP, because in this case we used the Sn-MDP Kit provided by AMERSHAM Int., and was not possible to adjust the Sn+2 and MDP concentrations at the same time. We preferred to adjust Sn+2 concentration to the middle level (0.35 ug/ul HCl-0.02 N) and MDP concentration was not adjusted as we did to the others ligands.

The labeling was performed as described previously in the middle level for all the independent variables, excepts X4, which was kept at low level (No Sephadex).

2.1.4. Challenge studies in presence of DTPA, HSA and L-Cysteine.

Challenge studies in presence of DTPA (2000 fold molar excess), HSA (100 fold) and Cys (3000 fold) were performed in order to investigate the stability of the label. 99mTc-IgG was incubated in the transchelating solution at room temperature, samples were removed at different times and applied on Whatman 3MM using as eluant Saline following the procedure previously described. In this system the labeled IgG remain at the origin while DTPA, HSA and Cys migrate. The IgG was labeled according to the conditions described for the middle level (excepts X4-No Sephadex) of the experimental design.

2.1.5. Thiol assay.

Fifty ul of reduced and purified IgG sample was mixed with 50 ul of 5,5 dithio bis-(2-nitrobenzoic acid) 3.96 mg/ml (Ellmans reagent) and diluted to 3.0 ml with 0.1 M Phosphate Buffer pH-8.0. The mixture was incubated at room temperature for 15 min and absorbance measured in an UV spectrophotometer at 412 nm. The number of thiols was obtained by comparison with a standard curve obtained by the assay of a series of cysteine standards ranging from 0.01 to 1.0 mM.

2.1.6. Statistical analysis.

All data were processed by using SPSS for Windows. Its mean and standard deviation were calculated. The statistical tests were performed with a significant level $\alpha = 0.05$.

3. Results.

3.1. Influence of the studied variables.

Table I shows the experimental design and mean values of purity (Y) for the initial 16 experiments. On the basis of the obtained yields the empiric equation which describes the system behaviour was computed as : $Y = 93.6 + 2.1X5 - 1.36X6 + 0.9X7$

The absence of X4 means that the presence or not of SMB in the labeling process was irrelevant, however the concentration of stannous ion (X5) and the incubation time (X7) affect positively the yield improving the labeling efficiency. On the other hand the volume of the tartrate solution (X6) affects negatively the yield. The rest of the studied variables have no significant influence on the labeling of IgG under the selected experimental conditions ($p > 0.05$).

The radiochemical purity of the 99mTc-IgG was higher than 90.0 %, except for the experiment number 14 (85.3 %). It demonstrates a reasonable binding of Tc99m into the IgG molecule under selected conditions and the efficiency of SMB as a reducing agent. The percents of free 99mTc-pertecnetate and 99mTc-Tartrate were lower than 1.0 % and 5.0 % respectively for all the experimental points.

Table I
Experimental design and results of yields (Y) expressed in per cent

Number	X1	X2	X3	X4	X5	X6	X7	Y(%)
1	+	+	+	+	+	+	+	98.3
2	-	+	+	-	-	+	-	90.3
3	+	-	+	-	+	-	-	95.2
4	-	-	+	+	-	-	-	95.5
5	+	+	-	+	-	-	-	89.9
6	-	+	-	-	+	-	+	96.6
7	+	-	-	-	-	+	+	90.9
8	-	-	-	+	+	+	-	93.6
9	+	+	+	-	-	-	-	95.0
10	-	+	+	+	+	-	+	94.0
11	+	-	+	+	-	+	+	90.0
12	-	-	+	-	+	+	-	95.4
13	+	+	-	-	+	+	+	95.0
14	-	+	-	+	-	+	-	85.3
15	+	-	-	+	+	-	-	97.3
16	-	-	-	-	-	-	-	95.6
17	0	0	0	-	0	0	0	91.6
18	0	0	0	-	0	0	0	91.7
19	0	0	0	-	0	0	0	92.0
20	0	0	0	-	0	0	0	92.6
21	0	0	0	-	0	0	0	93.2

Table II
Molecular species obtained for Native (Na) and Reduced (Re) IgG by SDS-PAGE

Fraction	mm	Rf	Molecular mass (kDa)	Percentage	
				Na	Re
1	11,3	0,12	121,9	88,3	78,6
2	19,9	0,21	104,4	2,6	12,3
3	35,7	0,37	77,4	1,3	
4	44,2	0,46	65,1	6,3	3,9
5	51,3	0,53	55,7	0,5	
6	80,9	0,84	25,6	0,5	5,2

3.1.1. FPLC and SDS-PAGE studies.

Figure 1 shows chromatograms obtained by FPLC on Superose 12 of native and reduced IgG. There are two main peaks, the first corresponds to protein aggregates present in the native IgG and the second properly to the IgG. The reduced IgG has a similar profile to the native IgG. There is no increase in the formation of aggregates and no fragmentation was detected by U V measurements. Table II presents the fractions, their molecular mass and respective per cents for native and reduced IgG obtained by SDS-PAGE. A molecular fragmentation of the native IgG is seen. This corresponds to a decrease of 9.7 % in fraction number 1 and a similar increase in fraction number 2 in the reduced IgG.

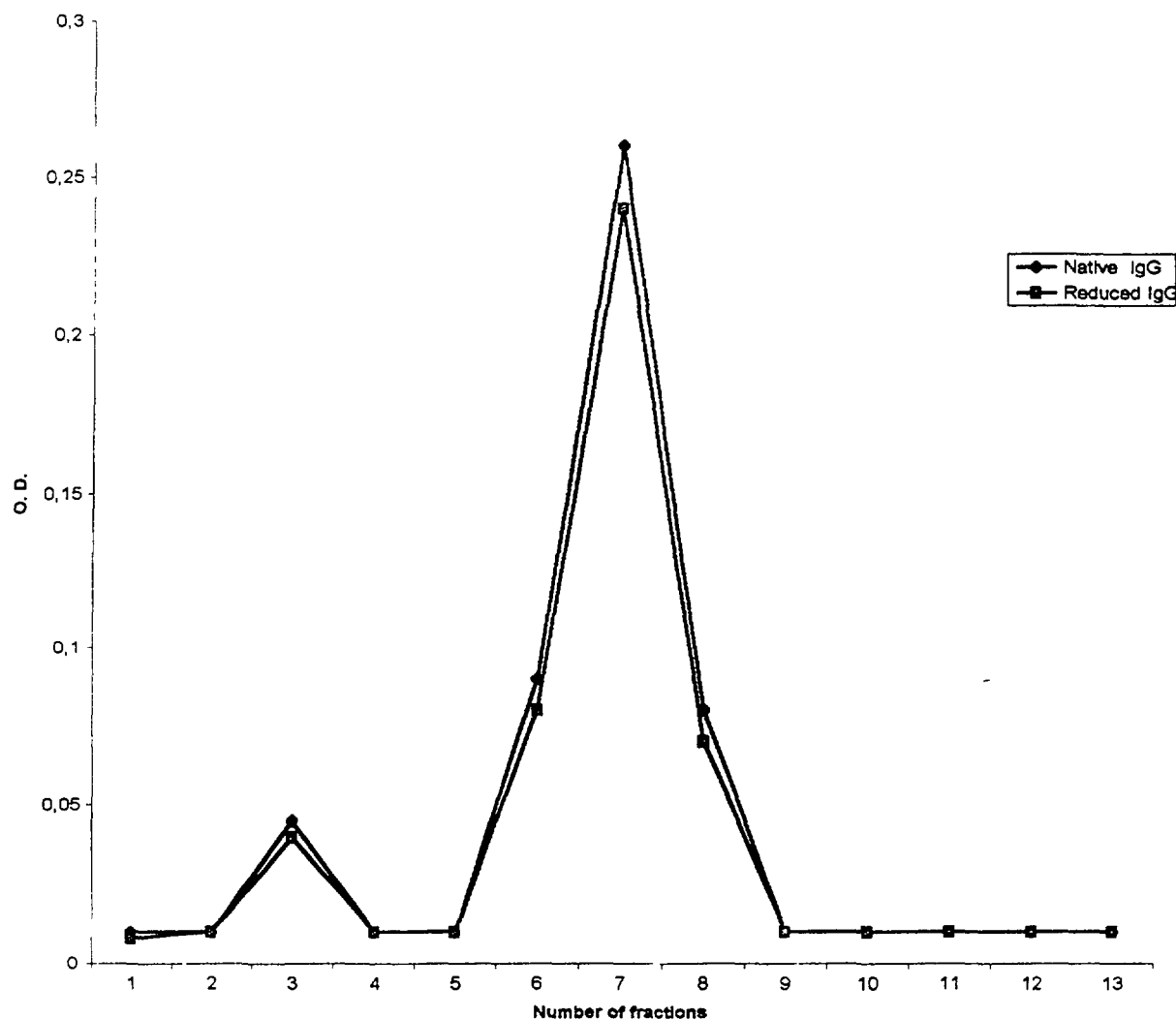


Fig. 1 FPLC chromatograms of Native and Reduced IgG

3.1.2. Sephadex G-25 M study.

The amount of radiocolloid detected by Sephadex G-25 M was lower than 3.0 % for the most of the sixteen experiments. In some cases the dose calibrator was unable to detect residual radioactivity. These results confirmed the hypothesis that almost all the radioactivity at the origin in Saline was associated to IgG and not to radiocolloid.

3.1.3 Assay of different ligands for the tin solution.

Figure 2 shows the influence to use gluconate (GLU), MDP and citrate (CIT) in the tin solution on the labeling process. As a rule in MEK radiochemical purities between 91.0 and 99.5 per cent were achieved. In Saline, high differences were obtained according to the anion used. The highest value corresponded to citrate and the lowest to MDP. Tartrate (TAR) anion reached a 92.0-93.0 per cent of purity. This value was close to Gluconate and approximately 4.0 % lower than the value achieved by citrate (CIT) anion.

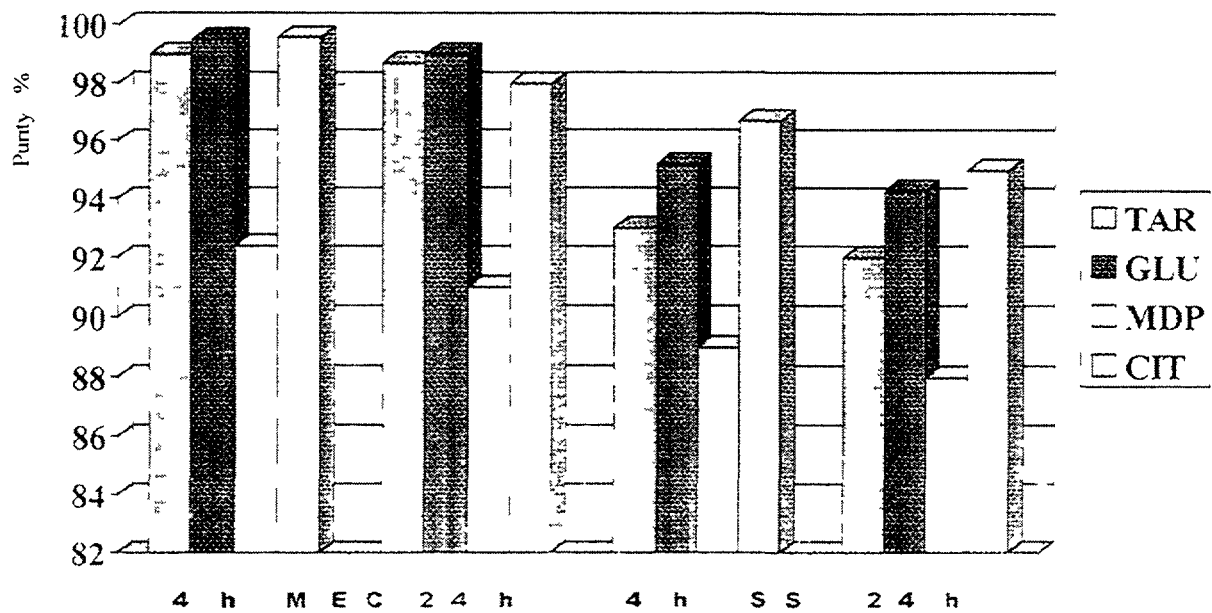


Fig. 2 Radiochemical purities of ^{99m}Tc -IgG obtained by different anions

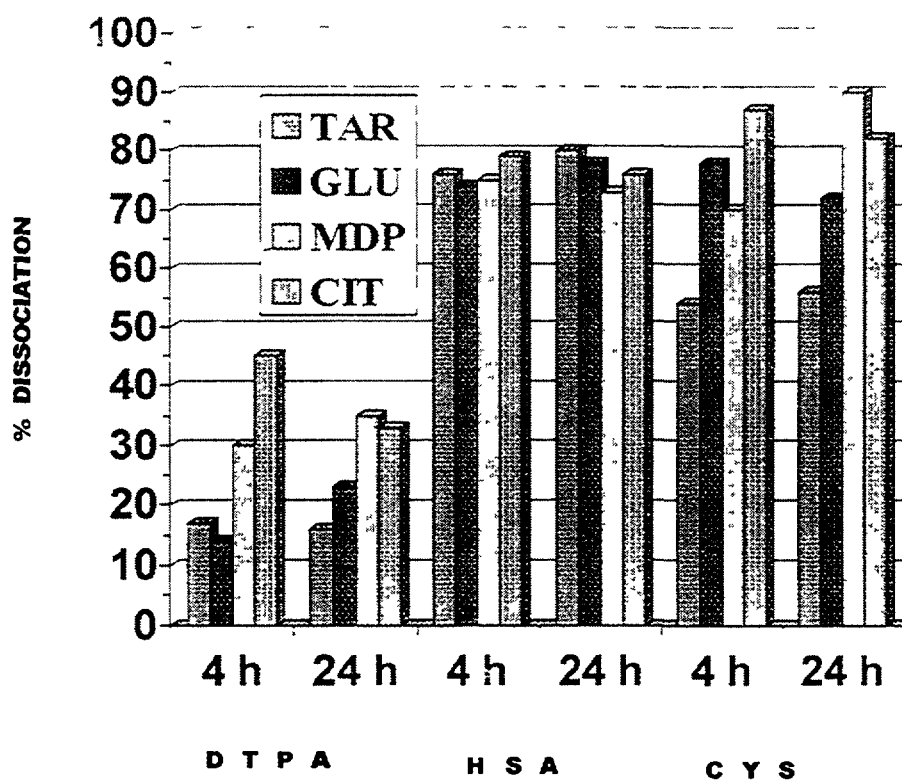


Fig. 3 Challenge studies of ^{99m}Tc -IgG

3.1.4. Challenge studies in presence of DTPA, HSA and Cysteine

The challenge studies were performed with the IgG labeled with Tc-99m under conditions of the middle level of the experimental design. In the Figure 3 were shown the results of the DTPA, HSA and Cys challenges at two different times. DTPA outcomes showed a low level of dissociation for tartrate and gluconate anions, the MDP and citrate were higher in comparison to these two previously mentioned. In the case of HSA a high level of dissociation was seen, which was time dependent for tartrate and gluconate anions. Cysteine showed a dissociation closed to 60.0 % for tartrate anion, the others assayed anions produced higher levels which varied between 70.0 and 90.0 %.

3.1.5. Thiol assay

The native IgG presented only 2-3 free -SH groups per mol. Thiol assays demonstrated an amount of ten -SH groups per mol when the human IgG was treated with a molar relation of 750/1 (SMB/IgG) during 45 min. This represented an increase of 7-8 groups after the reduction step.

4. Discussion.

Sodium metabisulfite (SMB) is a very common reducing agent in radioiodination (15), but it has not been reported before to reduce the disulfide bridges in the IgG molecule.

The final empiric equation obtained through the experimental design revealed that the presence of SMB was irrelevant on the labeling process. This fact and its toxicity of 10 g as lethal dosis (9) permits to eliminate the purification step and makes the process shorter and easier.

The empiric equation revealed that tin (II) ion concentration, the volume of Sn-Tartrate solution and the incubation time were the most important studied variables. From the equation it is observed that increasing the tin concentration and the incubation time of the reduced IgG with the Sn-Tartrate solution a rise of the 99mTc-IgG is achieved. Rhodes (16) reported the function of tin (II) in the prevention of the reactive sulfide groups created by the reduction of disulfide moieties, according to the reaction $R-SH + SnX \longrightarrow R-S-Sn-X$. The determined high positive influence of these two parameters (+ 2.1X5 and + 0.9X7) for stannous ion concentration and incubation time respectively could be related to the fact described above. The negative influence of the volume of the tartrate solution (- 1.36X6) might be in relation with the rise of the tartrate anion which may compete with the reduced IgG for 99mTc.

Interesting was the fact that the variation of the SMB/IgG in the range of 500/1 to 1000/1 practically did not affect the labeling yield. No other molar ratios were studied. Further studies should be performed to investigate better all these facts.

Results of FPLC and SDS-PAGE have demonstrated that molar relations SMB/IgG of 750/1 do not produce a high aggregation or fragmentation of the IgG molecule. Unreported results showed us that when using a molar relation of 7500/1, this produced an IgG fragmentation near to 50.0 %.

Challenge studies demonstrated that the 99mTc was bound to high affinity sites of the protein molecule when the Tartrate anion was used. The dissociation levels in presence of DTPA and Cysteine were in accordance with data reported by other authors (17,18). In case of HSA the stability of the label was low. It could be attributed to the high molar ratio employed. In the literature there is no consensus related to the HSA challenge (19,20) and the authors use different molar relations of this chelator. Another factor which could explain the high dissociation levels is the fact that we have used our own human serum albumin and some of the preservative agents added, may affect the 99mTc affinity in the

IgG molecule. Surprising was the high dissociation seen with Cys for gluconate, citrate and MDP, especially for the two formers, because of they are weak ligands which should not compete against reduced IgG for ^{99m}Tc . This fact is not easy to explain adequately on the light of our present knowledge. For MDP, however, this result may be influenced for the MDP concentration which was not possible to adjust as was made for the other ligands, and an increased concentration of MDP could decrease the labeling of reduced IgG.

Achieved preliminary results suggest that SMB is able to reduce disulfide bridges in the structure of IgG, allowing labeling efficiencies higher than 90.0 %. Since only human IgG was evaluated further studies are necessary before affirming its use like a new reducing agent for the direct labeling of antibodies with ^{99m}Tc .

5. Conclusion.

Sodium metabisulfite could be considered a promising reducing agent for the direct labeling of immunoglobulins with ^{99m}Tc .

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RADIOLABELLING OF RC-160: PRELIMINARY RESULTS



XA9847975

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Abstract

Vapreotide (RC-160) was labelled with ^{125}I using Chloramine-T and Iodogen methods and with $^{99\text{m}}\text{Tc}$ by a direct method with sodium ditionite as reducing agent in the presence of ascorbic acid.

Several methods of purification and quality control were evaluated. Yields of the reactions and of purification steps were calculated. The results obtained for the radioiodination reactions showed higher yields when limiting Chloramine-T method was used. Labelling of RC-160 with $^{99\text{m}}\text{Tc}$ indicated better yields when high radioactivity concentration of the radionuclide was used.

Stability of the products obtained was assessed at different post-labelling times by selected quality control methods: Sep-Pak cartridge as purification method and chromatography by RP-HPLC and ITLC-SG using saline solution as solvent. It was demonstrated that ^{125}I -RC-160 and $^{99\text{m}}\text{Tc}$ -RC-160 were stable during five weeks (at $-20\text{ }^{\circ}\text{C}$) and 6 hours (at room temperature) respectively.

Preliminary biodistribution of $^{99\text{m}}\text{Tc}$ -RC-160 in normal rats and mice were done showing different biological behaviour compared with control animals injected with pertechnetate.

In conclusion, RC-160 was successfully labelled with both radionuclides, with radiochemical purity higher than 95%. These results encourage further research work in animal models as well as to investigate the biochemical behaviour of radiolabelled peptide.

1. INTRODUCTION

Radiolabelled receptor specific bio-molecules promise to play a major role in diagnostic as well as therapeutic applications of malignant tumours. There are several peptides which may form the basis of useful radiopharmaceuticals for imaging a wide range of major diseases, especially those related with alterations at receptor levels.

Vapreotide, a somatostatin analogue, tyrosine-3 octapeptide (RC-160), is a potential agent for diagnostic purposes [1].

In this study, techniques and methodology for labelling RC-160 with I-125 and with Tc-99m were investigated.

2. MATERIALS AND METHODOLOGY

RC-160, provided by I.A.E.A., was labelled with radioiodine using Iodogen (Pierce) and Chloramine-T (1,8 μ g Cl-T, without reducing agents) (Sigma Chemical Co.). Several methods of purification and/or quality control of the radiolabelled compound were evaluated: ionic exchange resin (Dowex 1x8); Sep-Pak C-18 cartridges (Waters Associates) [2]; gel filtration (Sephadex G-25 Pharmacia, PD-10 columns); electrophoresis SDS-PAGE (gel 15%, 6x8 cm, V=100 volts, time 30, 45, 60 and 90 min); ITLC-SG (Gelman) chromatography (MeOH 85%, TCA 20% or NaCl 0.9% as solvents); RP-HPLC with UV detection at 280 nm as well as radioactive detection (0.4x30 cm, MCH-10 C18 column, Varian). HPLC analysis was performed using methanol 100% as solvent A and 0.9% NaCl as solvent B (flow rate 1ml/min) [3]. Gradient was programmed to start from 40% A reaching 80% A in 20 minutes; initial composition was regenerated at 25 minutes. In line radioactivity determination was not always possible, so in those cases, samples were collected and measured in a solid scintillation counter.

RC-160 was labelled with ^{99m}Tc by direct method using sodium dithionite as reducing agent in the presence of ascorbic acid [4]. The final $\text{Na}_2\text{S}_2\text{O}_4$ concentration was $2\text{ }\mu\text{g}/\mu\text{l}$ reaction mixture. Freshly eluted pertechnetate with 12 mCi/ml - 85 mCi/ml concentration range was used. Sep-Pak purifications (using C18 cartridge) were performed. Chromatographic separation methods were the same as previously indicated for labelling with iodine. RP-HPLC control assays were performed for RC-160, ascorbic acid, sodium dithionite, ethanol, free pertechnetate and iodide. Biological distribution was assessed in normal rodents, 1 hour after i.v. administration of technetium peptide. After the sacrifice, organs and blood, muscle and skin samples were dissected, weighed and activity measured.

In order to determine the "in vitro" radiochemical stability of the RC-160 labelled with ^{125}I and with ^{99m}Tc , controls in ITLC and RP-HPLC at analitic conditions mentionned before were done.

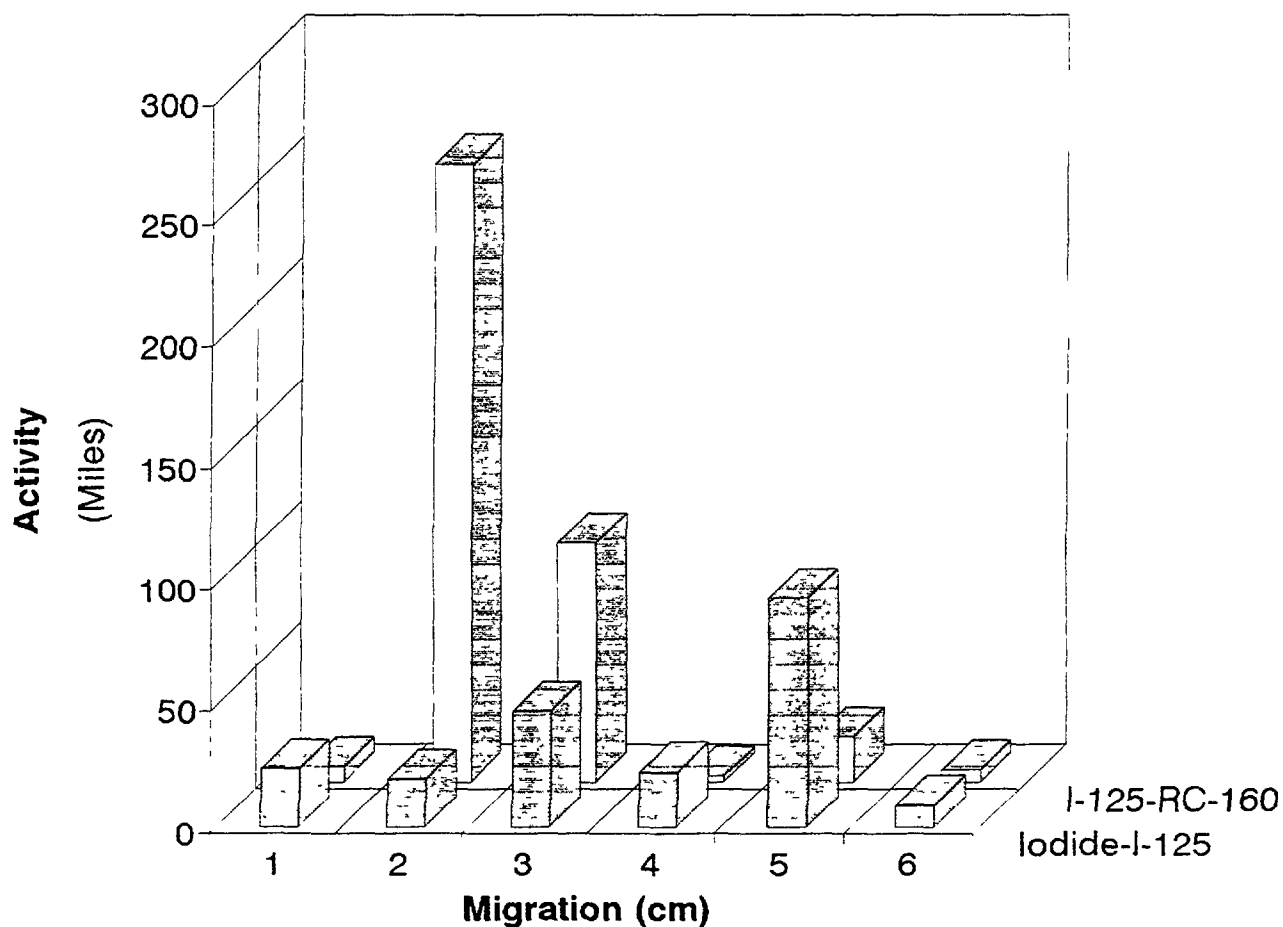


Fig. 1: SDS-PAGE Electrophoresis

Run time: 45 minutes

3. RESULTS

Sep-Pak cartridge was selected as purification method because of its capability to separate labelled molecules from free radionuclides in a simple and effective way.

Comparable results were found for the different quality control methods except for the system ITLC-SG chromatography with methanol 85%. SDS-PAGE was useful to resolve labelled radioiodinated compound from iodide with a 45 minutes run time (Fig.1). Considering the agreement among the other quality control methods, RP-HPLC and ITLC-SG/NaCl 0.9% were selected.

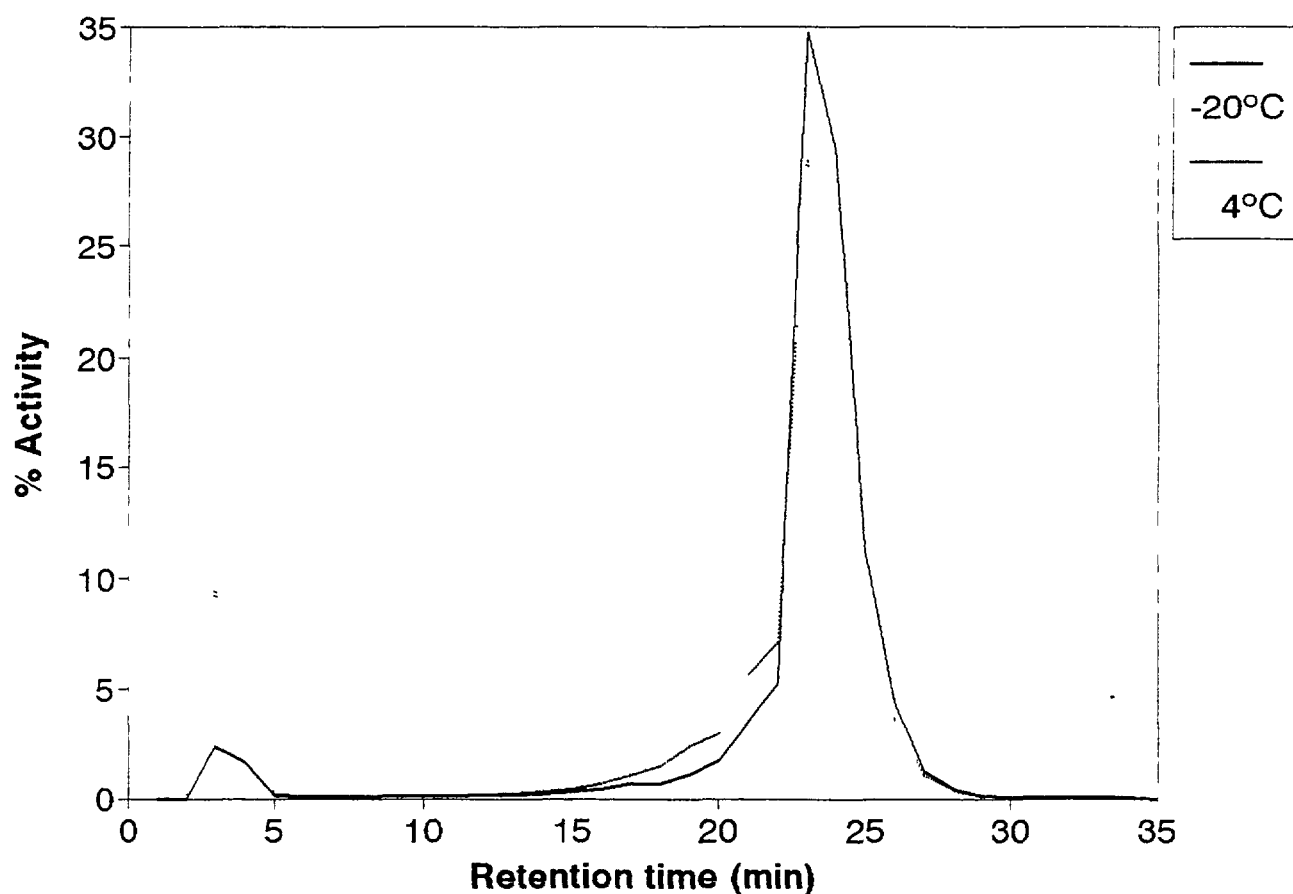


Fig. 2: ^{125}I -RC-160

HPLC analysis 37 days after labelling

The radioiodination reactions showed higher yields when Chloramine-T was used: 60% or more versus less than 50% for iodogen method. Specific activities were $240\mu\text{Ci}/\mu\text{g}$ - $274\mu\text{Ci}/\mu\text{g}$ and the radiochemical purity was 98% (calculated by HPLC). Loss in the radiolabelling steps, including adsorption was up to 20%.

Total yield for radiolabelling of RC-160 with $^{99\text{m}}\text{Tc}$ was better when high concentration radioactivity solutions were used. More than 90% of ethanolic fraction activity from Sep-Pak elution were recovered in the second and third half milliliters and less than 2% remain in the cartridge. The hydrophilic radiochemical impurities by HPLC were less than 4% and was consistent with the results of ITLC-SG chromatographic system.

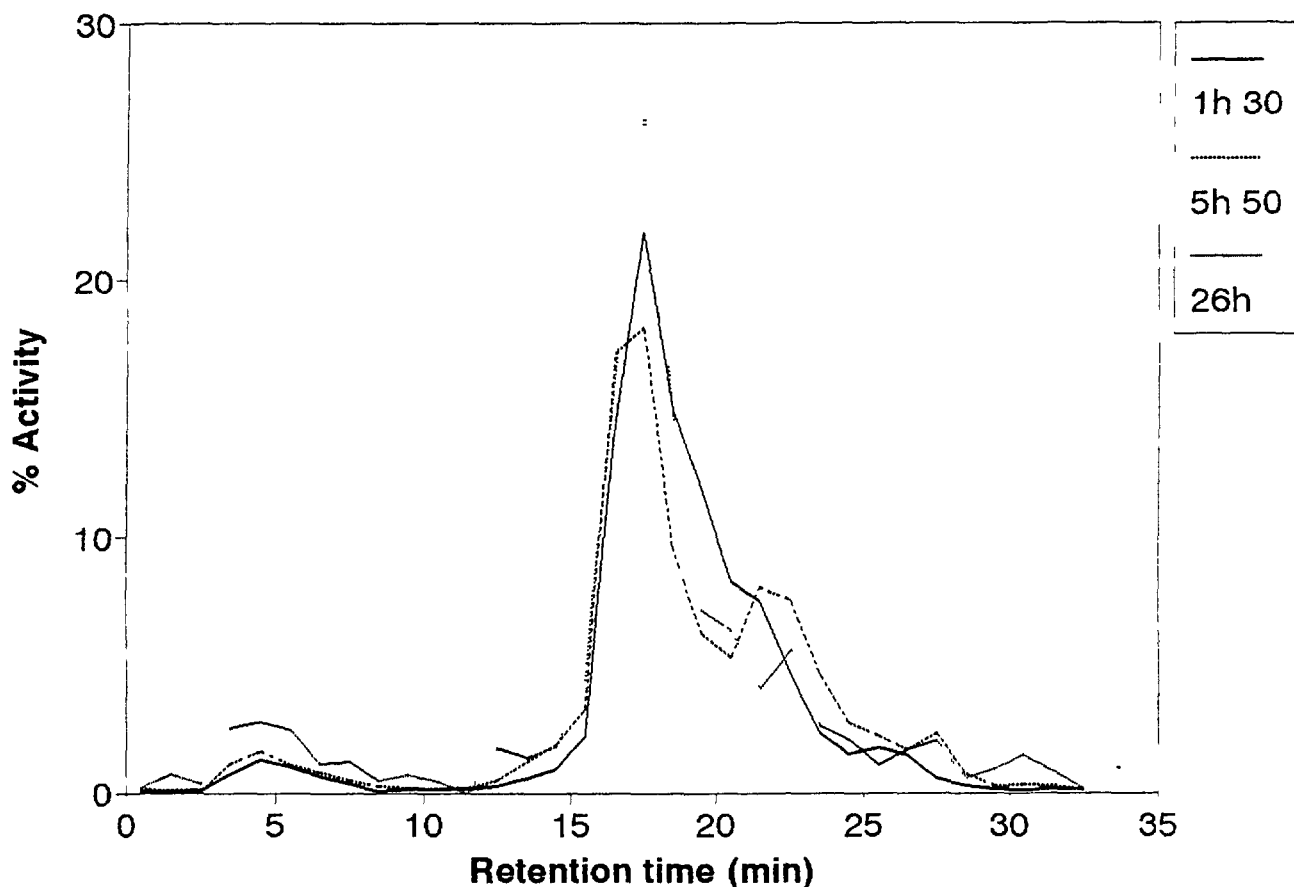


Fig.3: $^{99\text{m}}\text{Tc}$ -RC-160

HPLC at different times post labelling.

Biodistribution results indicated high blood clearance (less than 0.10% ID/g at 1 hour) and high uptake in intestines (more than 80% of injected activity) which is in agreement with the lipophilic characteristics of the compound. Very small or negligible activity was found at heart, lung, thyroid, muscle, brain and stomach. Uptake in adrenals and pancreas was not significantly different from the results obtained for pertechnetate so longer biodistribution times are going to be tried.

Figures 2 and 3 showed the typical HPLC profiles for the labelled molecules at different times post-labelling and storage conditions. It was verified that at -20°C, the radioiodinated molecules do not have a significant increase of hydrophilic impurities ($4\% \pm 2\%$) during 5 weeks, but at 4°C, an increase of up to 13% for the same period of time was observed. For the ^{99m}Tc labelled molecule, no significant difference was observed in radiochemical purity during 6 hours. At longer times, the hydrophilic species increase from less than 3% to more than 10% (at $t=24$ hours stored at room temperature).

4. CONCLUSIONS

RC-160 was successfully labelled with ^{125}I and with ^{99m}Tc , leading to high radiochemical purity compounds with standardized physicochemical quality control methods and well recognized "in vitro" stability. Therefore, these results and the local experience achieved through these activities will encourage further basic research activities mainly in the field of biological and biochemical behaviour of radiolabelled peptides for peripheral membrane receptors.

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INVITED LECTURE

RADIOLABELED PEPTIDES: EXPERIMENTAL AND CLINICAL APPLICATIONS

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Abstract

Radiolabeled receptor specific biomolecules hold unlimited potential in nuclear medicine. During the past few years much attention has been drawn to the development radiolabeled peptides for a variety of diagnostic applications, as well as for therapy of malignant tumors. Although only one peptide, In-111-DTPA-(D)-Phe¹-octreotide, is available commercially for oncologic imaging, many more have been examined in humans with hematological disorders, and the early results appear to be promising. Impetus generated by these results have prompted investigators to label peptides with such radionuclides as Tc-99m, I-123, F-18, Cu-64, and Y-90. This review is intended to highlight the qualities of peptides, summarize the clinical results, and address some important issues associated with radiolabeling of highly potent peptides. While doing so, various methods of radiolabeling have been described, and their strengths and weaknesses have also been discussed.

Introduction

During the past few years much attention has been given to the diagnostic applications of radiolabeled peptides and significant progress has been made. While one peptide is available commercially for routine oncologic imaging, several others are either in clinical trials or being developed for different indications. It is generally considered that the database with radiolabeled peptides is not yet sufficient enough that it warrants their use for therapeutic applications. However, a few groups have taken the lead and initiated studies, the early results of which appear to be promising.

Receptor specific peptides plays an important role not only in the diagnostic and therapeutic applications of neoplastic diseases, but also in the pathogenesis of other diseases. The receptor specificity of peptides has therefore generated excitement in the field of nuclear medicine and hopes are high that investigations will lead to the development of many clinically useful radiopharmaceuticals. It must be

kept in mind, however, that not only are novel peptides discovered at a rapid rate, but also new interactions of previously known peptides are being discovered. This wealth of information has made it difficult to keep the knowledge updated and to make a choice of peptide for use in a chosen application.

This article is intended to describe characteristics of peptides, their advantages and disadvantages for clinical applications, and the challenges met in their development as radiopharmaceuticals.

Peptides

By definition any compound consisting of 100 or fewer amino acids is considered as a peptide. However, "Peptides" one of the eleven journals with the word peptides in the title tends to prefer manuscripts about peptides containing fewer than 50 amino acids (1). Consensus is less on the definition of "polypeptides." By an arbitrary definition a polypeptide consists of 100-200 amino acids. For the purpose of the journal "Peptides" compounds with chains of 200 or more amino acids are generally referred to as "proteins" (11).

There are more than 6000 known peptides. A peptide is customarily named by the function by which it was originally discovered. Some of them have drawn a lot more attention than others. One review article published in 1994 stated that 298,105 papers were published on 42 peptides since 1962, of which 6840 were on insulin alone (2). A few of these peptides are listed in Table 1. As to the peptides in radiopharmaceutical chemistry, N-For-met-Leu-Phe, also known as FMLP, was labeled with In-111 in my laboratory in 1981 (3). However, it was not until 1994 that a peptide-based radiopharmaceutical, In-111-DTPA-(D)-Phe¹-octreotide, was approved by the FDA for imaging tumors. Although a few more have been radiolabeled and injected into patients, the interaction of the nuclear medicine community with radiolabeled peptides is relatively new.

Most of the peptides are non-polar, hydrophobic, or polar and hydrophilic. Only three (Arg, His, Lys) amino acids are cationic and two (Asp, Gly) are anionic. The hydrophobic and hydrophilic forces are especially important for peptide-protein interactions, making some peptides more toxic than others (4).

Advantages and disadvantages of peptide-based radiopharmaceuticals:

Like monoclonal antibodies, peptides are also receptor specific. As radiopharmaceuticals they have several advantages over murine monoclonal antibodies. Most peptides are endogenous and

bioactive analogs or key amino acid sequences of large protein molecules. These are less likely to produce any antigenic reaction. Peptides are much smaller in size and generally have rapid blood clearance than monoclonal antibodies. Yet they can often obtain high concentration in target tissues, leading to high target tissue/blood radioactivity ratios. Peptides can also withstand harsher chemical conditions of pH and temperature, making radiolabeling parameters more flexible and less damaging to the biological activity of a peptide. Peptides are relatively less expensive and can be easily synthesized in a short period of time using a conventional peptide synthesizer.

Peptides can, however, induce pharmacologic effects in much smaller quantities, and can also be more susceptible to *in vivo* proteolysis by endogenous proteases than monoclonal antibodies. Most useful peptides are therefore modified or molecularly engineered so that their enzymatic destruction could either be slow or inhibited. Common approaches toward this goal have been to cyclize peptides constructing one or more disulfide bridges and to substitute D-amino acids for L-amino acids, or both. However, when labeled with radionuclides, often peptides can and do fail to follow the biodistribution of their C-14 incorporated counterparts.

Radionuclides for peptides:

The peptide, octreotide, that sparked the interest in radiolabeled peptides in nuclear medicine is labeled with In-111. This radionuclide is a cyclotron produced, has a half-life of 67 hours and decays with the emission of two gamma rays (173 KeV -89% and 247 KeV -94%) and by electron capture. Although used widely in nuclear medicine In-111 is expensive, contributes to the cost of the radiopharmaceutical and adds to the inconvenience as it must be ordered well ahead of time of its use.

The faster blood clearance of peptides justifies the use of shorter lived radionuclides such as Tc-99m. With a six hour half-life, 140 KeV (90%) gamma ray emission and universal availability on a generator, Tc-99m is the most commonly used radionuclide in diagnostic imaging. Efforts are therefore plentiful in developing Tc-99m labeled peptides for diagnostic imaging. In addition to this, peptides have been labeled with positron emitting radionuclides, as well as with radionuclides of therapeutic importance. These are given in Table 2 and some of the radiolabeled peptides for their proposed applications in Table 3.

Table 1

Some biologically active peptides with their primary function and receptor specificity

Peptide	Primary Function
ACTH (adrenocorticotrop (h) in)	Hormone of the pituitary gland (SH mutates glucocorticoid production)
Bombesin	Active in CNS GI tract. Suppresses feeding in rats. BN and GRP receptor specificity.
BNP (brain natriuretic peptides)	BNP receptors exist in human cardiac tissue
Calcitonin	CRF receptors
CCK (cholecystokinin)	CCK receptors, homology to VIP and PACAP receptors. CCK receptors expressed on SCL and ovarian cancer.
GRP (gastrin releasing peptide)	GRP/neuromedin B (NMB) receptors
Glucagon	Specific for receptors expressed by endocrine pancreatic B cells.
α -MSH (α -melanocyte stimulating hormone)	Regulation of skin pigment, α -MSH receptors expressed on human melanoma cells.
Oxytocin	Uterus contracting and lactation stimulating hormone. Crosses BBB.
PACAP (pituitary adenylate cyclase activating peptide)	Includes secretin, glucagon, calcitonin, parathyroid hormone. PACAP I & II/VIP receptors are expressed on a variety of human tumors.
Somatostatin analogs	Somatostatin receptors I-V. Growth hormone release inhibiting factor. Receptors are expressed on a variety of tumors.
Vasopressin	Antidiuretic hormone. Hepatic V _{1a} receptor specific.
VIP (Vasoactive Intestinal Peptide)	Is involved in a wide range of biological activities. VIP I and VIP II receptors are expressed in high density on various types of tumors.
Neurotensin	Receptors are expressed on SCL carcinoma, H colon carcinoma and H-meningioma.

Table 2

Radionuclides labeled to peptides for SPECT, PET, and Therapeutic Applications

Planar or SPECT Imaging	Reference
In-111 $t_{1/2}$ -67 hrs, γ -173 KeV (89%), 247 KeV (94%)	5-7
Tc-99m $t_{1/2}$ -6 hrs, γ -140 KeV (90%)	8-12
I-123 $t_{1/2}$ 13.3 hrs, γ -159 KeV (83%)	13-15
Ga-67 $t_{1/2}$ 78 hrs, γ -93 KeV (40%), 184 KeV (24%), 296 KeV (22%)	16
PET Imaging	
F-18 $t_{1/2}$ 110 mins, β^+ -(97%)	16-17
Cu-64 $t_{1/2}$ 12.8 hrs, β^+ -(19%)	18
Y-86, $t_{1/2}$ -14.6 hr, β^+ -(26%)	16
Therapeutic Applications	
Y-90 $t_{1/2}$ -2.66 d, β^- , 2.27 MeV (100%)	19
Re-188 $t_{1/2}$ -16.7 hrs, β^- -2.12 MeV (100%), -155 KeV (10%)	20,21
In-111 $t_{1/2}$ -67 hrs, 173 KeV (89%), 247 KeV (94%)	22
I-131 $t_{1/2}$ -8.3 d, β^- -806 KeV (max), γ -364 KeV (82%)	23

Radiolabeling Peptides:

Peptides have been labeled with radionuclides by using four different methods which are briefly discussed below. In general, peptides can be modified for radiolabeling without affecting their biological characteristics. However, precautions should be taken that the sequence of certain groups of functional amino acids involved in receptor specificity is neither altered, nor should the chelating agents added exert steric hindrance that would alter the biological activity of the peptide. Most peptides have been modified at the N terminus to facilitate chelation of radionuclide, but in certain peptides, such as VIP, modifications at N-terminal H¹ can significantly alter its biological activity (1,30,31). Since conjugation of most bifunctional chelating agents is facilitated by formation of an amide bond, ϵ amino groups where such conjugation is undesirable are protected during the synthetic procedure and then deprotected. Steric hindrance created by an added bulky group can also effect the biological activity of a peptide.

Table 3

Radiolabeled Peptides Evaluated for Various Applications

Tumor Imaging	Reference	Tumor Therapy	Reference
In-111-DTPA-(D)-Phe ¹ -Octreotide**	5	In-111-DTPA-(D)-Phe ¹ -Octreotide*	22
I-123-(3)-Tyr-Octreotide*	14	Y-90-DOTA-(D)-Phe-Octreotide*	19,22
I-123-VIP*	13	Re-188-RC-160	20,21
Tc-99m-P-829*	10		
Tc-99m-RC-160	11		
F-18-Octreotide	17		
Cu-64-Octreotide	18		
<u>Imaging Vascular Thrombosis</u>		<u>Imaging Infection</u>	
Tc-99m-P280*	24	In-N-for-MLF (FMLP)	3
Tc-99m-Bitistatin	25	Tc-99m-Nor-for-MLFK	28
Tc-99m-DMP-444*	26	Tc-99m-Tuftsia*	29
Tc-99m-CSVTG	27		

** Approved by FDA in 1994

* Evaluated in humans

Subsequent to such a modification and radiolabeling net charge on peptide can also alter. In general, lipophilic peptides are sequestered by the liver and can clear by hepatobiliary excretion whereas hydrophilic peptides are usually removed rapidly from plasma by glomerular filtration in the kidneys. Indium-111-DTPA-Phe¹-Octreotide clears primarily through the kidneys whereas I-123-Tyr³-Octreotide has predominant hepatobiliary excretion (14). Furthermore, Breeman et al (32) have reported that the uptake of radioactivity in somatostatin receptor positive tissues is a bell-shaped function of the injected mass of the peptide. Tissue distribution of a given peptide may vary depending upon the nature of a radionuclide used for radiolabeling and upon the animal species used. While approaching for radiolabeling and biological evaluation of a peptide, all these parameters must be carefully considered.

Radioiodination of peptides:

Radioiodination of a peptide is exclusively carried out at the aromatic amino acid Tyr. Peptides such as VIP (Tyr¹⁰ and Tyr²²) and RC-160 (Tyr³) which contain Tyr have been radioiodinated without any structural modifications of the native peptide. In order to facilitate radioiodination in other peptides such as Sandostatin, tyrosine has been added in place of Phe³ which is considered to be the amino acid not important for its biological activity. For radioiodination most investigators use iodogen as oxidizing agent (13) although chloramine-T has also been successfully used (11). In order to prevent oxidation of other amino acids, long incubation times of the reaction mixtures containing oxidizing agents have been avoided and mono and diiodo species formed have been separated using reverse phase HPLC.

Direct labeling:

This method is limited to labeling peptides only with Tc-99m or Rhenium radionuclides and is applicable to radiolabeling of only those peptides which are cyclic (11,12,20,21). In this method, the disulfide bond is reduced either with sodium ascorbate or stannous chloride. The sulfhydryls resulted by the reduction serve as strong chelating groups for reduced Tc-99m, or rhenium radionuclides. The method is simple, generally efficient, does not require conjugation of bifunctional chelating agents, blocking or deblocking of functional groups and the subsequent purification and characterization of the required product. Biological activity of the peptides labeled by this method is not altered (11,12) and the spatial topography of the side chains essential for somatostatin receptor binding is maintained (33). This method, however, cannot be used for peptides that are not cyclic.

Bifunctional chelating agent (BFCA) method:

This is the most common method applied for radiolabeling of peptides. It has been used to label peptides with all radionuclides given in Table 2, except F-18 and Iodine-123. A large number of chelating agents have been synthesized and covalently linked to the N terminus of a chosen peptide. The conjugation reaction can be performed in liquid or in solid phase using resin on which the peptide is synthesized. In solution phase the length of the process is compounded by protecting side chain groups either by Boc (t-butyloxycarbonyl) or Fmoc (9-fluorenylmethyloxycarbonyl). Conjugation of the BFCA on resin on which a given peptide synthesized is facilitated by pre-protected side chain groups. Furthermore, by using this procedure, deblocking of the peptide side chains and removal of the peptide

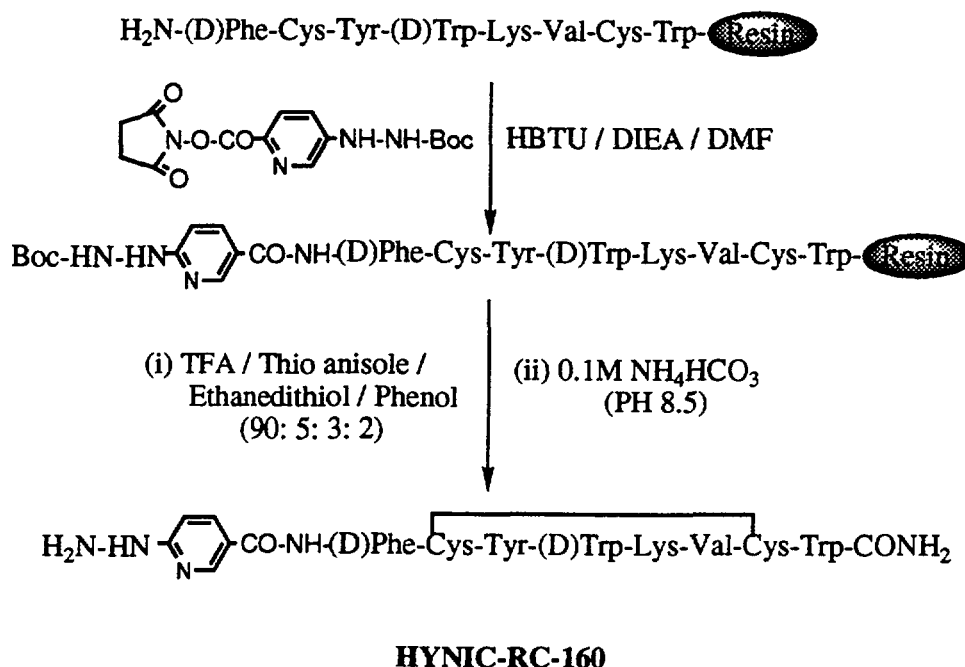


Fig 1 Schematic presentation for the preparation of HYNIC-RC-160 (11,12)

from the resin is achieved simultaneously. In solid phase synthesis removal of solvents and impurities also becomes less cumbersome. The process on resin is generally considered to be more efficient than that in the liquid phase. A typical procedure in which RC-160, a somatostatin analog (D-Phe-Cys-Trp-D-Trp-Lys-Val-Cys-Trp) also known as vapreotide, was conjugated with Hynic (hydrozino nicotinamide) is briefly described below and schematically presented in Fig 1.

RC-160 was synthesized commercially and obtained as its carboxamide on 5-[4-(9-fluorenylmethoxy carbonyl) (amino methyl)-3,4 dimethoxy phenyl] valeric acid (PAL) resin (Lysine residues blocked). A five fold molar excess BFCA, BOCHYNIC (6-Boc- hydrazinopyridine-3-carboxylate) was activated with 2 (1H-benzotriazol-1-yl) 1,1,3,3-tetramethyl uronium hexafluorophosphate (HBTU) for 20 minutes, added to the resin in dimethylformamide (DMF) and allowed to react at 22°C for three hours. Using a sintered funnel, the resin was washed with DMF, followed by dichloromethane and vacuum dried. The peptide was cleaved from the resin and deblocked with 95% TFA, diethylether was added and the mixture was cooled at -20°C for 4-5 hrs. The resultant precipitate was dissolved in 0.1M ammonium bicarbonate solution (pH-8.5) and stored overnight for cyclization. The reaction mixture was then separated on a preparative HPLC using Rainin's Dynamax

(2.14 x 41.4 cm) column and a gradient solvent consisting of 0.1% TFA in water (Solvent A) and 0.1% TFA in acetonitrile (Solvent B). The flow rate was 6 ml/min and Solvent B increased from 50% at 1 minute to 90% at 45 min. Fractions were collected, lyophilized and analyzed using matrix assisted laser desorption ionization (MALDI) mass spectrometer. The fraction with the expected molecular weight was then chosen for radiolabeling. Thus, even in solid phase this method is long, laborious, and can be result in poor yield.

Hybrid peptide method:

Hybrid peptide procedure, developed in our laboratory for chelation of Tc-99m and possibly for other radionuclides such as Re-186, Re-188, and Y-90 is a simple and yet efficient one step method. In this method, the chelating moiety consists of a group of peptides itself which is synthesized along with the synthesis of the primary peptide. This eliminates the number of secondary synthetic steps required for synthesis and purification of a BFCA and its conjugation to the peptide. For validation of this concept we have synthesized several peptides ranging from 4 to 28 amino acids using Gly-(D)-Ala-Gly-Gly as a chelating moiety which provides N₄ configuration for chelation of radionuclides. The primary peptide can be extended with the required chelating moiety either at N or C terminus. Although we have not yet studied the stability constants of these metal chelates, our experimental data indicate that the complex is stable when challenged with such strong metal chelating agents as human serum albumin, cysteine, and DTPA (34). In order to avoid steric hindrance due to the bulky metal chelate, we have inserted a spacer molecule, α -amino butyric acid, between the primary peptide and the chelating moiety. Our data, *in vitro* and in experimental animals, have indicated that the biological activity of hybrid peptides resembles that of the primary peptide.

The advantage of this method is that it eliminates the multiple steps for synthesizing chelating agents and their conjugation to the peptide which, as described previously, is time consuming and inefficient. With the hybrid method, a usable final product is achieved without additional efforts, cost and compromising the yield. This method also allows one to choose a group of amino acids as a chelating agent as may be required for a chelation of a given radionuclide. This also allows one to add peptides of choice that may alter net charges on the peptide, which may change their pharmacokinetics, as shown by Lister-James et al (10). Additionally, this method is applicable to label straight chain or cyclic peptides.

In our laboratory the hybrid peptides have been formulated into a simple kit for instant labeling with Tc-99m. A small (μg) quantity of a peptide is lyophilized with a required quantity of SnCl_2 in phosphate buffer solution at pH-12 and stored at -20°C . At the time of preparation sodium pertechnetate (Tc-99m-O_4) solution is added to the vial and after incubation at 22°C or 100°C as the case may be, pH of the solution is adjusted to pH-6.5 with the addition of precalculated quantity of phosphate buffer, pH-4. The radiolabeling yield is quantitative and radiochemical purity of the preparation is confirmed by analytical HPLC.

A proposed structure of one such hybrid peptide, Tc-99m-TP1201, is given in Fig. 2. This peptide is being developed in our laboratory for imaging vascular thrombosis.

Experimental and Diagnostic Applications:

Routine applications of radiolabeled peptides are centered around oncologic imaging with In-111-DTPA-(D)-Phe¹-octreotide (5). Given in Table 4 are some early results of the efficacy of In-111-DTPA-(D)-Phe¹ scintigraphy as compared to the somatostatin receptor expression determined by autoradiography (36). The high sensitivity of detection and the excellent correlation with the *in vitro* data for most tumors is impressive. However, not so well detected with In-111-DTPA-(D)-Phe¹-octreotide scintigraphy appear to be the tumors of the breast, pituitary glands, thyroid, and pancreas and astrocytoma. In Table 5 are compared the scintigraphic results of I-123-MIBG and In-111-DTPA-(D)-Phe¹-octreotide (36), which depict the excellent results of In-111-(D)-Phe¹-octreotide. In addition to In-111-DTPA-(D)-Phe¹-octreotide, a large number of other radiolabeled peptides have also been evaluated.

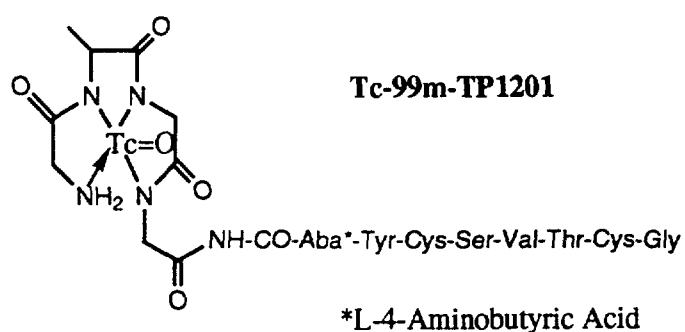


Fig. 2 Proposed structure of Tc-99m labeled Gly-(D)-Ala-Gly-Gly-Aba-Tyr-Cys-Ser-Val-Thr-Cs-Gly, (TP-1201) (27)

Table 4
Results of In-111-DTPA-(D)-Phe¹-Octreotide scintigraphy in patients with malignant tumors compared to the expression of somatostatin receptors as determined by autoradiography (36)

	In vivo	Scintigraphy	In vitro	Receptor status
GH-producing pituitary tumor	7/10	70%	45/46	98%
TSH-producing pituitary tumor	2/2	100%	—	—
Non-functioning pituitary tumor	12/16	75%	12/22	55%
Gastrinoma	12/12	100%	6/6	100%
Insulinoma	14/23	61%	8/11	72%
Glucagonoma	3/3	100%	2/2	100%
Unclassified APUDoma	16/18	89%	4/4	100%
Paraganglioma	33/33	100%	11/12	92%
Medullary thyroid carcinoma	20/28	71%	10/26	38%
Neuroblastoma	8/9	89%	15/23	65%
Phaeochromocytoma	12/14	86%	38/52	73%
Carcinoid	69/72	96%	54/62	88%
Small cell lung cancer	34/34	100%	4/7	57%
Non-small cell lung cancer	36/36	100%	0/17	9%
Meningiomas	14/14	100%	54/55	98%
Breast cancer	37/50	74%	33/72	46%
Exocrine pancreatic tumors	0/24	9%	0/12	0%
Astrocytoma	4/6	67%	14/17	82%
Non-Hodgkin's lymphoma	59/74	80%	26/30	87%
Hodgkin's disease	23/24	96%	2/2	100%
Sarcoidosis	23/23	100%	3/3	100%
Wegener's granulomatosis	4/4	100%	—	—
Tuberculosis	6/6	100%	2/2	100%
Grave's disease thyroid	9	a	1	—
Grave's ophthalmopathy	25	b	—	—

Table 5

Comparison of results with In-111-DTPA-(D)-Phe¹-Octreotide and I-123-MIBG scintigraphy in patients with comparable tumors (36)

Tumor type	Percentage of positive scans (number)	
	In-111-Octreotide	I-123-MIBG
Phaeochromocytoma	86% (14)	88% (> 1000)
Neuroblastoma	89% (9)	91% (841)
Paraganglioma	100% (33)	52% (25)
Medullary thyroid carcinoma	71% (28)	35% (178)
Carcinoid	96% (72)	70% (237)
Endocrine pancreatic tumor	80% (56)	25% (4)

in humans. These include I-123-VIP for imaging VIP receptor specific tumors (13), Tc-99m-DMP-444 and Tc-99m-P-280 for imaging DVT (24,26), Tc-99m-P-748, and I-123(Tyr³)-octreotide for imaging tumors (14,35) and Y-90-DTPA-octreotide for treating tumors (19,22). Generally the absolute uptake of radiolabeled peptides in a given lesion is small, but the rapid blood clearance of agents renders target tissue/blood ratio high, leading to a high degree of success in clinical results. Impetus generated by these observations has led many investigators to label a variety of peptides with Tc-99m and evaluate them in experimental animals. Many of these are labeled with Tc-99m and also include agents for imaging infection and vascular thrombosis as shown in Table 3.

Therapeutic Applications:

In-111-DTPA-(D)-Phe¹-octreotide and Y-90-DOTA-octreotide, are in the early stages of their use in tumor therapy. Although In-111 is a gamma emitting radionuclide (Table 2), it decays with the emission of conversion electrons and x-rays that form the basis for its application in therapy. It has been estimated that the radiation dose to a cell with 10 µm diameter with a decay of a single In-111 atom is 0.135 rad (37). Up to 500 mCi of In-111-DTPA-Phe¹-Octreotide has therefore been used as a single therapeutic dose. Results of any systematic clinical trials are unknown at the time of this writing but general comments on the outcome of results are encouraging.

Lately Otte et al and Krenning et al (19,22) have used the β^- emitting Y-90 (Table 3) as a tracer for similar applications. Octreotide is also labeled with Y-90 using DOTA (1,4,7,10-tetraazacyclododecane-N,N', N', N'', N'''-tetra acetic acid) as a chelating agent conjugated at D-Phe¹ and the early results in the first few patients are encouraging (38).

In summary, in a relatively short period of time, the progress made with radiolabeled peptides is impressive. It ranges from the development of a wide variety of techniques to radiolabel peptides to evaluate them for their potential in significant clinical and experimental applications. The glimpses of the subject provided here may neither do justice to the capacious volume of literature existing on the subject, nor may it provide a vision to its reader. We wish to remind the readers, however, that with the space limitation, this article was intended only to serve as a brief introductory review and we hope that it serves the purpose.

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REACTION WITH GLUTATHIONE. A POSSIBLE MECHANISM INVOLVED IN RODENT BRAIN RETENTION OF A ^{99m}Tc SNS/S COMPLEX CONTAINING A PENDANT ESTER FUNCTIONALITY

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Abstract

The synthesis, characterization of $\text{M}^{\text{VO}}\{[\text{CH}_3\text{CH}_2\text{N}(\text{CH}_2\text{CH}_2\text{S})_2](p\text{-S-Ph-COOCH}_2\text{-CH}_3)\}$ (M: ^{99m}Tc : **I**, Re: **II**) is presented in this work, where a pendant ester function is attached to the monothiolate ligand. Chemical structure of **I** is established after chromatographic comparison with **II**, synthesized in macroscopic amounts. Complex **II** is fully characterized by classical methods of analysis showing that the compound adopts a distorted trigonal bipyramidal configuration around the metal. The two sulfur atoms of the tridentate ligand and the oxo group form the basal plane, while the remaining nitrogen atom of the tridentate ligand and the sulfur atom of the monothiolate ligand occupy the apices of the bipyramid. In vitro challenge experiments with glutathione (GSH) in neutral aqueous medium demonstrate, that **I** suffers nucleophilic attack by GSH and thereby transformation to a more hydrophilic daughter metal compound. Formation of the latter depends on time and GSH concentration. Tissue distribution in mice shows minor retention in brain. As rodent brain presents no esterases to hydrolyze the ester group of **I**, while the intracerebral content in GSH amounts to 2 mM, the above described mechanism is suspected for the observed brain retention. However, in primate brain cells retention of **I** may additionally involve the hydrolysis of the ester function to the corresponding acid, as already revealed by preliminary in vitro experiments using esterase incubates.

1. Introduction

Brain imaging with SPECT is currently possible by means of two major ^{99m}Tc radiopharmaceuticals, [^{99m}Tc]HM-PAO and [^{99m}Tc]ECD. Both of these radioagents have been developed and introduced in the clinic rather recently. Chemically they are small-size, neutral and lipophilic ^{99m}Tc complexes that cross the intact blood brain barrier (BBB) by passive diffusion, but are then retained in brain tissue by different mechanisms. While [^{99m}Tc]HM-PAO suffers intracellular nucleophilic attack by glutathione (GSH) and transformation to hydrophilic species, [^{99m}Tc]ECD is metabolized to the corresponding free acid form after hydrolysis of at least one of its pendant ester functionalities by brain esterases [1-4].

We report herein the synthesis, stability and biodistribution in mice of $^{99m}\text{TcO}\{[\text{CH}_3\text{CH}_2\text{N}(\text{CH}_2\text{CH}_2\text{S})_2](p\text{-SPh-COOCH}_2\text{CH}_3)\}$, **I**. Compound **I** carries a pendant ethyl ester group on the monothiolate coligand, that — like in [^{99m}Tc]ECD — may undergo hydrolysis to the acid form by brain esterases in primate brain cells. The structure of **I** is established by comparison with $\text{ReO}\{[\text{CH}_3\text{CH}_2\text{N}(\text{CH}_2\text{CH}_2\text{S})_2](p\text{-SPh-COOCH}_2\text{CH}_3)\}$, **II**. The latter is synthesized in macroscopic amounts and fully characterized by classical methods of analysis. Stability of **I** versus glutathione is monitored by chromatographic methods (HPLC). Eventually, stability data is correlated to the biodistribution profile of **I** in mice with the aim to investigate if and to what extent GSH is involved in the retention mechanism of this compound in mice brain.

2. Experimental Section

2.1. Synthesis of ligands

The ligands L^1H_2 [$\text{CH}_3\text{CH}_2\text{N}(\text{CH}_2\text{CH}_2\text{SH})_2$] and L^2H ($p\text{-HS-Ph-COOCH}_2\text{CH}_3$) are prepared according to modified reported protocols [5-7].

2.2. Synthesis of the [$^{99m}\text{TcO}^{\text{V}}$]-(L^1L^2) complex (tracer level), *I*

The ^{99m}Tc complex *I* {(ethyl *p*-thiolatobenzoate) [N-ethyl-N,N-bis(ethylene-2-thiolato)amino]}oxotechnetium-99m is prepared as follows: A glucoheptonate (GH) kit vial (Gluco/Demoscan) is reconstituted with physiological saline (10 mL). An aliquot thereof (1 mL) is mixed with [^{99m}Tc]NaTcO₄ solution (0.5 - 1.0 mL, 5 - 10 mCi) and the forming [^{99m}Tc]Tc^V-GH solution is added to a centrifuge tube containing equimolar amounts (< 0.02 mmol) of L¹H₂ and L²H ligands. The mixture is agitated vigorously by a vortex mixer and left to react at room temperature for 10 min. The lipophilic complexes are then extracted in CH₂Cl₂ (3 x 1.5 mL) and the combined organic extracts dried over MgSO₄ and filtered. The radioactivity content in both the aqueous and organic phases is measured separately and the yield calculated (Yield: > 85%).

2.3. Synthesis of the [ReO^{V}]-(L^1L^2) complex (carrier level), *II*

To a 0.5 M AcONa solution in MeOH (4 mL) Re^VOCl₃(PPh₃)₂ (166.6 mg, 0.2 mmol) is suspended. Then N-ethyl-N,N-bis(ethylene-2-thiol)amine (33 mg, 0.2 mmol) and ethyl *p*-mercaptobenzoate (36 mg, 0.2 mmol) in MeOH (1.0 mL) is added dropwise under stirring. The mixture is refluxed until a clear solution is obtained and then H₂O (10 mL) is added. The product is extracted in CH₂Cl₂ and the organic extracts dried over MgSO₄ and concentrated by vacuum evaporation. After addition of MeOH and slow evaporation green crystals separate. The crystals are soluble in CH₂Cl₂ and CHCl₃, slightly soluble in MeOH and EtOH and insoluble in Et₂O, pentane and H₂O.

Yield: (70 mg) 71%; IR (KBr, $\nu_{\text{max}}/\text{cm}^{-1}$): 947 (Re=O str.); UV/Vis ($\lambda_{\text{max}}/\text{nm}$): 406; ¹H-NMR δ_{H} (250 MHz, CDCl₃, Me₄Si): 1.33 (3H, t, CH₃CH₂N), 1.4 (3H, t, CH₃CH₂COOC), 2.69 (2H, m, *exo*, NCH₂CH₂S), 2.85 (2H, m, *exo*, NCH₂CH₂S), 3.4-3.5 (4H, m, *endo*, NCH₂CH₂S), 3.9 (2H, q, CH₃CH₂N), 4.35 (2H, q, CH₃CH₂COO), 7.69 (2H, dd, *o*-Ph-H),

8.1 (2H, dd, *m*-Ph-*H*); *elemental analysis*. % found: C 33.01, H 4.38, N 2.61, S 17.01; % calculated for $C_{15}H_{22}NO_3S_3Re$: C 32.95, H 4.06, N 2.56, S 17.59.

2.4. High performance liquid chromatographic analysis

The purification of **I** from the radiolabeling reaction mixture, as well as corroboration of structure with **II** is achieved by HPLC. Thus, the CH_2Cl_2 reaction solution containing **I** is reduced to a small volume by a mild nitrogen stream and loaded on a NP column. The column is isocratically eluted by $CH_2Cl_2/MeOH$ 99/1 at a 1 mL/min flow rate and the major radioactive peak collected.

Co-injection of aliquots thereof and a solution of **II** in CH_2Cl_2 on the HPLC column is conducted by application of a twin detector system: i) a photometric diode array detector for monitoring the Re complex and ii) a gamma detector for detecting the ^{99m}Tc species, under the following HPLC conditions: A RP Techsil CN column (10 μ , 4.6 mm x 250 mm) is eluted at a 1 mL/min flow rate by a TEAP/EtOH (TEAP: triethylammonium phosphate buffer pH 7.1) gradient system: 0 to 70% EtOH from 3 to 20 min. Thus, corroboration of structure between complexes **I** and **II** is investigated. For the analysis of incubates of **I** with GSH the same HPLC conditions are applied as above.

2.5. In vitro stability versus glutathione

The CH_2Cl_2 is expelled from the collected HPLC fraction containing purified **I** by a mild nitrogen stream and **I** is redissolved in EtOH. A part of the ethanolic solution is incubated at 37°C with a 1 mM and a 0.1 mM GSH solution in phosphate buffer pH 7.4. Aliquots of the above are subjected to HPLC analysis at 1, 15, 30, 60 and 120 min time intervals.

2.6. Animal distribution studies

The organic solvent of the HPLC purified **I** is expelled under a mild nitrogen stream and EtOH is added, so that a 30% EtOH final solution is obtained. Aliquots (0.1 mL, 2-3 μ Ci) are then injected each through the tail vein in 3 groups of five male Swiss Albino mice (30 ± 2 g). The animals are sacrificed by cardiac puncture at 5, 30 and 60 min time intervals. Tissues of interest are excised, washed and weighed. The radioactivity content of each sample is measured on an automated gamma counter and the percentage of injected dose per organ (%ID/organ) and per gram (%ID/g) are calculated by comparison to standard solutions. The brain to blood ratio (Br/Bi) is calculated by dividing the respective %ID/g values.

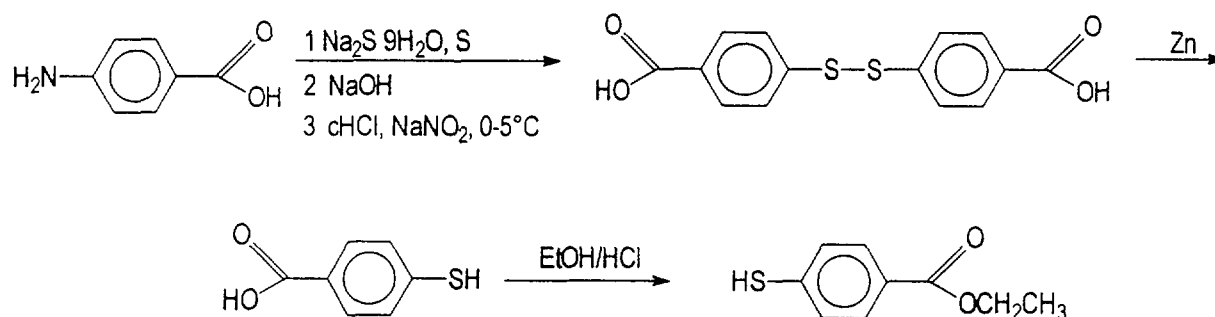
3. Results and Discussion

We report herein on the synthesis and characterization of $M^VO\{[CH_3CH_2N-(CH_2CH_2S)_2](p-S-Ph-COOCH_2CH_3)\}$, (M: ^{99m}Tc : **I**, Re: **II**), where a pendant ester group is introduced on the monothiolate ligand. Biodistribution in mice of complex **I** forming at tracer level is performed. This compound is a potential brain imaging agent, whose brain retention mechanism may mimic either that of $[^{99m}Tc]ECD$, or of $[^{99m}Tc]HM-PAO$, the two main commercially available brain radiopharmaceuticals today. Thus, it may either be hydrolyzed by brain esterases to the acid derivative (like $[^{99m}Tc]ECD$), or suffer nucleophilic attack and transformation to a more hydrophilic product (like $[^{99m}Tc]HM-PAO$). Both mechanisms are intracellular and lead to the trapping of radioactivity in brain cells for time sufficient to conduct SPECT [1-4]. In this work, we expect to reach some initial conclusion as to which one of the above mechanisms is involved in the brain retention of **I**. For this purpose, we investigate the susceptibility of **I** to GSH attack in vitro and its biodistribution in mice.

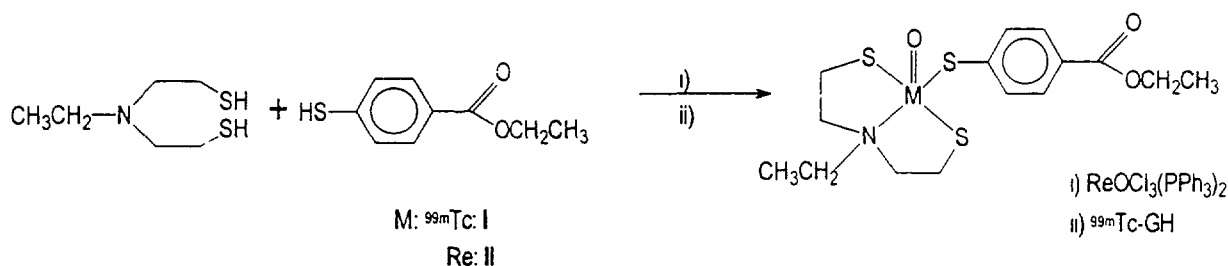
Synthesis of the ester modified monothiolate ligand proceeds according to a published protocol, as described in Scheme 1 [7]. Complexes **I** and **II** are obtained by reacting

equimolar quantities of the aminedithiolate (L^1H_2) and the monothiolate ligand (L^2H) on the $Re^VOCl_3(PPh_3)_2$ and $[^{99m}Tc]Tc^V$ -GH precursor respectively, as presented in Scheme 2. As the thiolate groups are deprotonated upon binding to the MO^{3+} core, neutral complexes are finally produced [8-11].

Compound **II** is purified by crystallization and characterized by classical analytical methods (Experimental Section). Briefly, the IR spectrum demonstrates a characteristic band at 947 cm^{-1} assigned to the $Re=O$ stretching vibration and consistent with reported values [10]. Similarly, the UV/Vis spectrum shows a maximum at 406 nm characteristic of Re^VO -S charge-transfer-band(s). The 1H -NMR spectrum is consistent with a d^2 electronic configuration in a distorted trigonal bipyramidal field [10]. The parent ligand peaks with the exception of the three thiolate protons are clearly seen in the spectrum. Comparison with reported values for similar oxometal species clearly suggests the formation of only the *syn* isomer of **II** [10,11]. The latter conclusion is further supported by preliminary X-ray analysis



Scheme 1: Synthesis of the ethyl *p*-mercaptobenzoate ligand



Scheme 2: Preparation of $^{99m}Tc^V$ O- and Re^VO - L^1L^2 complexes **I** and **II**

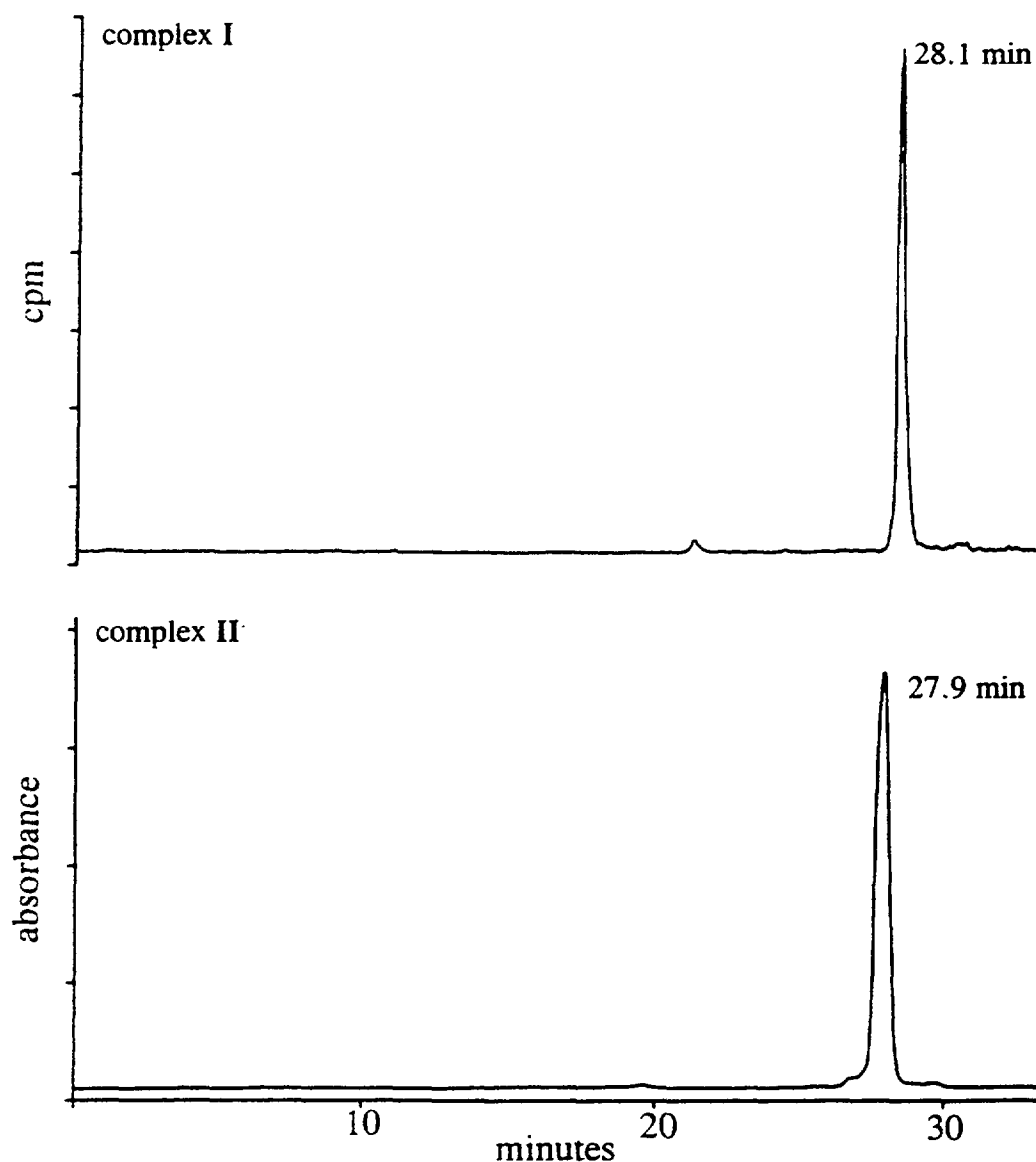


Figure 1: Chromatographic comparison of complexes I and II by HPLC; complex I: gamma detection, complex II: UV detection (for HPLC conditions see text)

of II. According to this data, the basal plane is defined by the two sulfur atoms of the SNS ligand and the oxo group, whereas the two apices of the distorted trigonal bipyramid are occupied each by the nitrogen atom of the SNS ligand and the sulfur atom of the monothiol.

Corroboration of structure between complexes I and II is achieved by HPLC adopting both photometric (Re) and radiometric (^{99m}Tc) detection modes and a comparative chromatogram is presented in Fig. 1. The identical t_R values of 28 min exhibited by the two analog complexes, suggest formation of isostructural metal species.

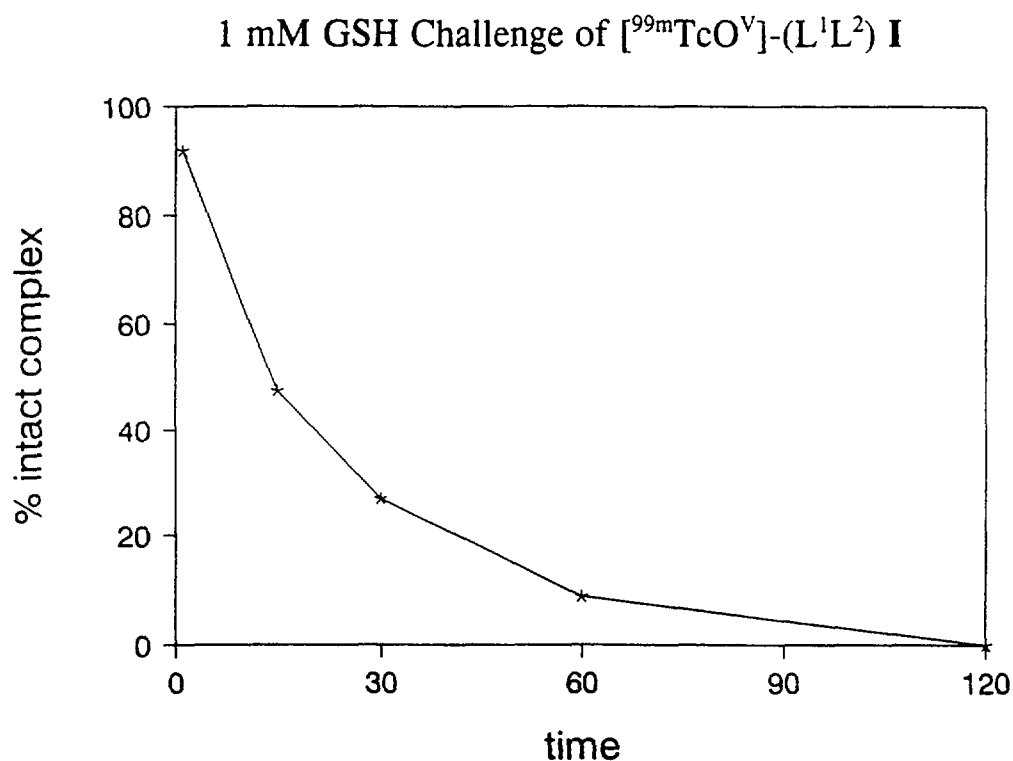


Figure 2: Nucleophilic attack of GSH on **I** with time; 1 mM GSH concentration, pH 7.4 and 37°C incubation temperature

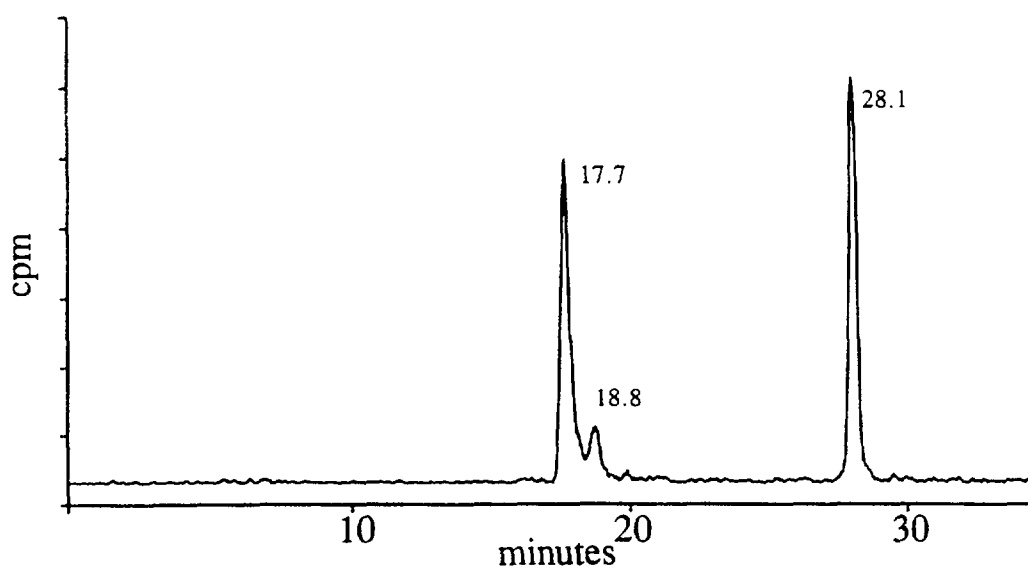


Figure 3: Representative radiochromatogram of a 15 min incubate of **I** with GSH; 1 mM GSH concentration, pH 7.4 and 37°C incubation temperature (for HPLC conditions see text)

Table 1: Comparative tissue distribution data for **I** as % ID/organ in mice at 5, 30 and 60 min time intervals; each value is the average calculated from 3 animals

organ/ time	I		
	5 min	30 min	60 min
Blood	12.12±1.41	9.38±0.80	8.26±1.39
Liver	32.07±1.49	40.09±2.86	33.96±8.53
Kidneys	6.92±0.55	3.42±0.37	2.38±0.36
Intestines	5.25±0.43	8.93±1.26	12.04±0.94
Lungs	5.15±0.45	2.34±0.34	1.75±0.12
Brain	1.05±0.07	0.26±0.05	0.2±0.01
Br/Blood ^a	0.41	0.15	0.13

^aBrain/Blood : % ID/g in the brain divided by % ID/g in the blood.

Incubation of **I** with GSH at pH 7.4 and a concentration of either 0.1 or 1 mM leads to formation of a more hydrophilic daughter compound, as demonstrated by HPLC analysis of the incubates. The ratio of daughter to parent metal species is time and GSH concentration dependent, as summarized in Fig. 2. In the representative radiochromatogram of Fig. 3 a 15 min incubate of **I** with 1 mM GSH at 37°C is shown, indicating **I** and daughter compounds eluting at 28 and 18 min respectively.

Tissue distribution studies in mice show retention of **I** in mice brain (Table 1). Given that rodent brain lacks the required esterases, retention of **I** due to intracellular brain GSH (2 mM) seems a valid option [1-4]. Moreover, retention in other tissues known for their high GSH content — such as the liver and the lungs — further supports this hypothesis. Studies

are presently conducted with the aim to elucidate the nature of the hydrophilic daughter compound of **I**, as well as to reveal if this is a common mechanism for the intracerebral trapping of ^{99m}Tc mixed ligand complexes of the SNS/S combination. On the other hand, preliminary in vitro experiments with esterase solutions followed by HPLC analysis indicate that hydrolysis of the ester function to the acid is fast and thus may be additionally involved in the retention of **I** in primate brain cells.

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Abstract

Labelling with ^{99m}Tc of fibrinogen, using DTPA anhydride as the bifunctional chelating agent, was studied in animals with venous thrombi. The parameters studied were: i) coupled reaction of ^{99m}Tc with the fibrinogen-DTPA-Sn(II) in lyophilised form; ii) biodistribution studies of fibrinogen-^{99m}Tc in animals with venous thrombi, and iii) imaging studies by scintigraphic methods. The present study showed that the radiochemical purity of fibrinogen-DTPA-^{99m}Tc is > 95% for a maximum of 5 mCi (185 MBq) radioactivity of ^{99m}Tc in the 1.5 - 2 ml volume. Above this level of radioactivity we found a drastic decrease in the radiochemical purity. The radioactivity ratio of the venous thrombi to the blood was 2.32 ± 0.45 . The scintigraphic images showed a significant accumulation of fibrinogen-DTPA-^{99m}Tc in 1-hour-old thrombi, 1 hour after injection.

From this results the diagnostic potential of fibrinogen-DTPA-Sn(II) in kit form was evaluated.

1. INTRODUCTION

The possibility of early diagnosis of thrombosis and free moving thrombi is offered by scintigraphic methods using fibrinogen, labelled with different radionuclides. Studies regarding the chemistry of technetium -^{99m}Tc and of macromolecule - bifunctional chelating agent complexes produced many scientific papers on the labelling of fibrinogen with ^{99m}Tc [1 - 8].

The intention of this study was to develop a kit for the preparation of fibrinogen labelled with ^{99m}Tc. Scintigraphic images of thrombosis models used for the evaluation of fibrinogen-DTPA-^{99m}Tc present a gradual accumulation of radioactivity with a maximum at 1 hour after intravenous injection.

2. MATERIALS AND METHODS

The bifunctional chelating agent DTPA - anhydride was synthesised as described in [1]. The IR spectra shown in Fig. 1 reveal the carbonyl bonds at 1820 cm^{-1} and 1773 cm^{-1} ; a strong bond at 1639 cm^{-1} reflected the ability of its central carboxyl group to ionise, thus it was amphoteric. The

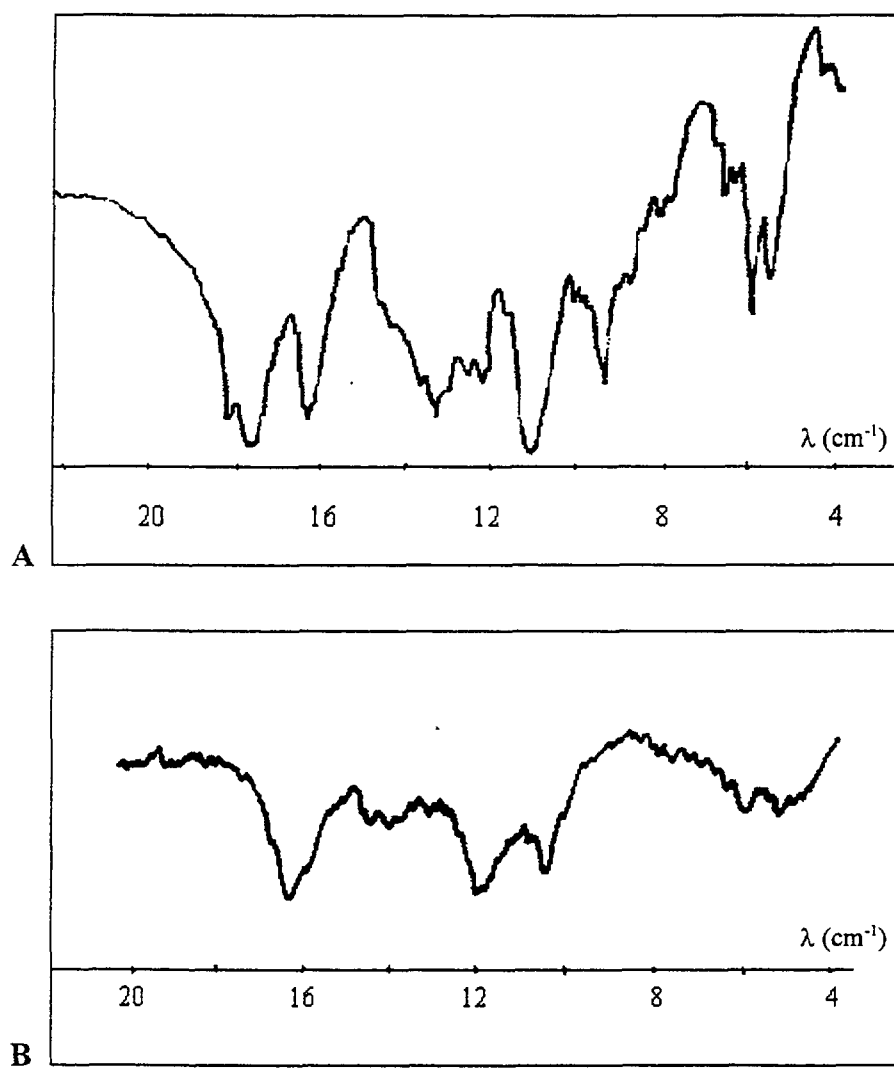


Fig. 1 IR Spectra of the DTPA-anhydride (A) and fibrinogen-DTPA conjugate (B)

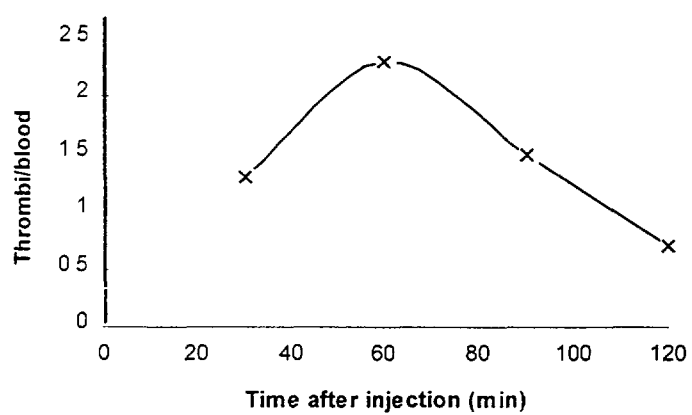


Fig. 2 Variation of the thrombi to blood ratio

application of stannite ions (SnO_2^{2-}) for the reduction of $^{99\text{m}}\text{TcO}_4^-$ in the preparation of fibrinogen-DTPA- Sn(II) has been investigated in our laboratory. The stannite solution was prepared by alkalisation of the stannous chloride solution with sodium hydroxide. The human fibrinogen was obtained in lyophilised form from the Haematological Institute, Bucharest. A $^{99}\text{Mo} - ^{99\text{m}}\text{Tc}$ extraction generator was used for labelling with $^{99\text{m}}\text{Tc}$.

All chemicals used were of reagent grade.

2.1. Preparation of Fibrinogen-DTPA-Sn(II) complex

Human fibrinogen (0.625g) and DTPA - anhydride (0.06 g) were dissolved in 80 ml HEPES buffer, pH = 8.6. Complete solution of anhydride was ensured by adding a few drops of chloroform, which was evaporated from the system by gentle agitation under a flow of nitrogen.

The solution obtained was incubated for 1 hour at 5°C . After heating the solution to room temperature, we added 20 ml Na_2SnO_2 solution (with a Sn O_2^{2-} content of 36 mg), in drops under a nitrogen atmosphere. The final pH of the solution was 8.8.

The solution obtained above was sterilised with a $0.4\ \mu\text{m}$ Millipore filter and divided into 1 ml portions. The lyophilisation was performed for 24 h under aseptic conditions, with the following parameters: -46°C eutectic point, -54°C optimal freezing point, $+25^\circ\text{C}$ drying temperature. The IR spectra of the fibrinogen - DTPA conjugate [Fig. 1(A)] showed a band at $1200\ \text{cm}^{-1}$ corresponding to $-\text{CO}-\text{NH}-$ vibrations in the conjugate, as can be seen by comparison with the IR spectra of DTPA-anhydride in Fig. 1 (B)

2.2. Labelling with $^{99\text{m}}\text{Tc}$ of Fibrinogen-DTPA-Sn (II) complex

The labelling was effected by injection into each vial of lyophilised fibrinogen-DTPA-Sn(II) (prepared as described above) of 1 - 1.5 ml $\text{Na}^{99\text{m}}\text{TcO}_4$ solution, containing 3 - 5 mCi (111-185 MBq) activity. The contents of the vial were dissolved using agitation by rotation, resulting in a clear and colourless solution with pH = 8.5.

Radiochemical purification was effected by chromatography with Whatman No.1 paper, using the solvents: (A) acetone and (B) acetic acid 0.1 M: sodium acetate 0.1 M (3:2). In the (A) solvent at $R_f = 0$, the percentage of fibrinogen-DTPA- $^{99\text{m}}\text{Tc}$ complex which was radioactively labelled was $> 95\%$ while in (B) solvent at $R_f = 0$ the percentage was 92 - 94%. At $R_f = 0.68 - 0.67$ in solvent (B), there is a radioactive spot representing DTPA- $^{99\text{m}}\text{Tc}$, with 2-3% of the total activity. It is possible that in the polar solvent, the appearance of DTPA - $^{99\text{m}}\text{Tc}$ was due to hydrolysis.

2.3. Biodistribution and imaging studies

For the biodistribution studies we used Wistar London rats with 130-150 g weight. Venous thrombi were induced by perivascular application of formalin to the femoral vein of rats [8]. One hour after the formation of thrombi, the rats were injected intravenously with 30 μCi in 0.1 ml fibrinogen-DTPA- $^{99\text{m}}\text{Tc}$. The animals were sacrificed at 30, 60, 90 and 120 minutes after injection. The target organs were dissected out and measured.

For the imaging studies was used rabbits with 2.5 kg weight. Venous thrombi were induced in the rabbits as just described. One hour after the formation of thrombi, the rabbits were injected i.v. with 200 μCi (7.4 MBq) in 0.2 ml fibrinogen-DTPA- $^{99\text{m}}\text{Tc}$. The scintigrams was taken 15, 30 and 60 minutes after i.v. administration, using a Pho-Gamma camera (Nuclear Chicago, USA).

3. RESULTS AND DISCUSSIONS

The time course of fibrinogen-DTPA- $^{99\text{m}}\text{Tc}$ distribution is shown in Table 1. Biodistribution studies showed a relatively fast and high uptake of fibrinogen-DTPA- $^{99\text{m}}\text{Tc}$ in the thrombi. The variation in the thrombi-to-blood ratio is noticeable, as shown in Fig. 2. The maximum accumulation of radioactivity in thrombi was at 60 minutes after injection. This high thrombi-to-blood-ratio would be a positive factor in the use of fibrinogen-DTPA- $^{99\text{m}}\text{Tc}$ for imaging of thrombosis. Scintigrams in a rabbit with thrombi are presented in Fig. 3

The localisation of thrombi improves gradually, with clear delimitation at 60 minutes after injection.

Table 1
Distribution of fibrinogen-DTPA- $^{99\text{m}}\text{Tc}$ in tissue

Time ORGAN	Distribution (percentage of dose per gram)			
	30 min	60 min	90 min	120 min
Thrombi	3.92 \pm 0.92	6.99 \pm 1.00	6.22 \pm 0.20	5.01 \pm 0.40
Blood	2.92 \pm 0.50	3.01 \pm 0.10	4.11 \pm 0.40	7.07 \pm 0.30
Liver	7.81 \pm 1.00	5.62 \pm 0.30	4.54 \pm 0.20	3.73 \pm 1.00
Spleen	0.19 \pm 0.20	0.17 \pm 0.03	0.15 \pm 0.04	0.12 \pm 0.01
Lung	0.62 \pm 0.10	0.61 \pm 0.03	0.59 \pm 0.04	0.34 \pm 0.06
Kidney	3.71 \pm 0.20	5.44 \pm 0.10	5.88 \pm 0.90	4.23 \pm 1.10

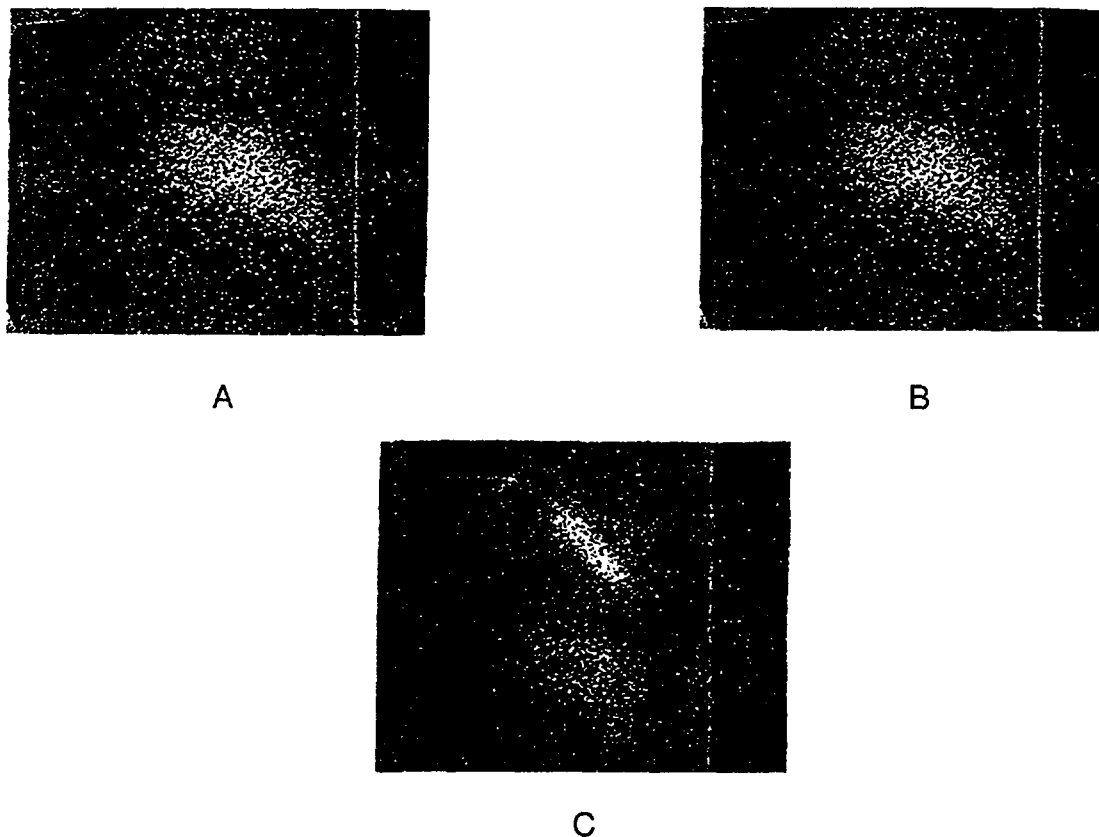


Fig. 3. Scintigrams in the rabbit with thrombi at 15 min (A), 30 min (B) and 60 min (C) after injection

4. CONCLUSIONS

In this investigation we have attempted to obtain a fibrinogen-DTPA-Sn(II) kit for labelling with ^{99m}Tc . All studies were effected with fibrinogen-DTPA-Sn(II) in lyophilised form, sterile and pyrogen-free. The results of our investigation suggest that fibrinogen-DTPA- ^{99m}Tc is a promising clinical diagnostic agent for the detection of thrombi.

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PREPARATION OF MONOCLONAL ANTIBODIES AGAINST CARDIAC MYOSIN AND SOME RADIOLABELLING STUDIES

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Abstract

Monoclonal antibodies were raised against myosin, a specific indicator of myocardial infarction and labelled with ^{125}I and $^{99\text{m}}\text{Tc}$. Human cardiac myosin was isolated from normal human heart and was used for raising the monoclonal antibodies by the hybridoma technique. Antibody producing clones were identified by ELISA and cloning was done by the limiting dilution technique. Of the 13 clones obtained, 4 were deemed suitable for further studies. The antibodies were grown in ascites, purified, isotyped and their cross reactions with other forms of myosin were estimated. All the clones showed negligible cross reaction with rabbit myosin, but reacted to different extents with bovine skeletal myosin. The most avid antibody Mab-4G4 was chosen for further labelling studies.

Mab-4G4 was labelled with ^{125}I using different oxidising agents such as iodogen, chloramine-T and lactoperoxidase. Purified radioiodinated antibody with radiochemical purity >95% could be obtained by gel filtration. Immunoreactivity was retained as tested by binding to myosin immobilised on a solid support. Mab-4G4 was also labelled with $^{99\text{m}}\text{Tc}$ using stannous tartrate as the reducing agent. Radiolabelling yield was ~60%, the purity was >95% and the immunoreactivity was retained. Both the labelled preparations were tested for bio-distribution in normal and infarcted rats. The activity accumulation in the infarcted region was ~ 1.5 and 3.5 times as that in normal heart muscle for ^{125}I and $^{99\text{m}}\text{Tc}$ labelled Mab-4G4 respectively. The major problem with the iodinated antibody was the *in vivo* deiodination resulting in very high

percentage of activity in the thyroid. Although the fraction of the total activity associated with the infarcted heart is not very impressive, the fact that the activities with the infarcted and normal hearts are significantly different is heartening. With further optimisation of labelling and use of F(ab)₂ fragments, better delineation of the infarct sites is aspired.

1. INTRODUCTION

Myocardial ischemia leading to damage and necrosis of myocytes or acute myocardial infarction (MI) is one of the major causes of morbidity. Myocardial ischemia and infarction are often monitored *in vitro* by the presence of certain serum proteins/enzymes released from the damaged myocytes [1]. Cardiac myosin is one such intracellular protein which is exposed due to sarcolemmal disruption occurring in MI. Immunoradiometric assays (IRMA) for light and heavy chains of myosin have been used for *in vitro* detection of MI. Heavy chains of myosin can serve as specific markers to detect MI by immunoscintigraphy [2-3]. These can be accomplished using anti-myosin antibodies and monoclonal antibodies specific for human cardiac myosin (HCM) would be ideal for this purpose. Attempts were therefore made to generate monoclonal antibodies against HCM and their suitability for immunoscintigraphy was assessed. Of the several clones obtained, four were selected and used for further studies on radiolabelling. Of these four, the monoclonal antibody Mab-4G4 which had the maximum relative affinity was used for *in vivo* studies in Wistar albino rats.

2. MATERIALS AND METHODS

All chemicals and biochemicals for hybridoma work, bovine serum albumin (BSA), lactoperoxidase, chloramine-T, Tween-20, iodogen, goat-antimouse polyvalent

antibody conjugated with alkaline phosphatase and Sephadex-G 25 were purchased from Sigma Chemical Co., USA. Hybridoma subtyping kit was from M/s. Boehringer Mannheim, Germany. All chemicals and salts for buffer preparations were purchased from M/s. Sarabhai Chemicals, India. $^{99m}\text{TcO}_4^-$ was eluted from a column generator. Na^{125}I (NEZ 033A) was from Dupont Canada. All the plastic wares used for the hybridoma work was from Nunc Inc., Denmark.

All the major equipment such as -80°C freezer, CO_2 incubator etc. in the hybridoma lab were from Forma Scientific Inc., USA. NaI(Tl) scintillation counter was used for measurement of radioactivity.

BALB/c mice were bred in the animal house of our institute. Human heart was obtained from the morgue of K.E.M. Hospital, Mumbai, India.

2.1. Isolation of human cardiac myosin(HCM)

Myosin was isolated from normal human hearts as per reported procedure [4]. The heart was stored at -70°C before processing and processed at 4°C . The purity of the myosin preparation was checked by 12.5% SDS polyacrylamide gel electrophoresis.

2.2. Anti-myosin monoclonal antibodies

2.2.1. Hybridoma technique

In brief, male BALB/c mice (10-12 weeks old) were immunised with 100 μg of HCM emulsified with Freund's complete adjuvant injected intraperitoneally. A single booster dose was given after a fortnight with the same amount of antigen emulsified in Freund's incomplete adjuvant. Three days after the booster, the splenocytes from the immunised mice were used for generation of Mab as per the reported method [5].

Spleen cells (1×10^6) and Sp2/0 myeloma cells (2×10^6) were centrifuged together and were fused by using 30% PEG as the fusogen. Fused cells were suspended in complete DMEM with HAT (5 mM hypoxanthine, 20 μ M aminopterin, 800 μ M thymidine). Supernatant of Sp2/0 culture was added (25%) to the medium instead of feeder cells [6]. The cells were initially plated at the cell density of 10^5 cells/mL. After observing the growth of the hybrids the cells producing antimyosin antibodies were identified by ELISA technique. In brief, Immulon-1 microtitre wells were coated with 5 μ g of HCM in 0.05 M Tris-KCl buffer, pH 8.5 for 18 h at 4°C. After washing the reaction wells with 0.05 M phosphate buffered saline, pH 7.5 (PBS) containing 0.05 % tween-20, the wells were blocked with PBS, 1% BSA for one hour at 25°C. The wells were again washed as before. 100 μ L supernatant from each of the plated wells was added to a corresponding HCM coated well and was allowed to incubate for 3 h at 25°C. After repeating the washing step, 0.1 mL 1:500 goat-antimouse polyvalent Ig conjugated with alkaline phosphatase was added to each well and incubated for 18 h at 4°C. These wells were once again washed as before and 0.1 mL (100 μ g) p-nitrophenylphosphate was added and the O.D. measured at 410 nm. The cells from the positive wells were pooled and were cloned by limiting dilution (0.3 cells /well). The individual clones were identified, tested for production of specific antibodies, and further recloned by limiting dilution to confirm the monoclonality.

2.2.2. Antibody production from ascites

The identified clones were propagated in BALB/c mice and the antibodies were collected from the ascitic fluids. One week prior to the injection of the desired hybridoma cells, the mice were primed (*i.p.*) with 0.5 mL of Freund's incomplete adjuvant [7]. The antibody from the ascitic fluid was purified using caprylic acid followed by ammonium sulphate precipitation [8].

The monoclonal antibodies were characterised for their isotype, titre, cross reactivities and relative affinities towards HCM. Isotyping was done by using the isotyping kit. Essentially the antibodies in the culture supernatant and ascites were captured on sheep-antimouse antibody coated wells and were identified by peroxidase labelled mouse subclass specific antibodies. Cross reactions of these antimyosin monoclonal antibodies with bovine skeletal myosin (BSM), rabbit myosin light (RMLC) and heavy chains (RMHC) were estimated by the ELISA technique. The relative affinity of Mabs for myosin was estimated by a competitive assay in terms of amounts of myosin required for 50 % inhibition of binding of the Mab to the solid phase bound myosin.

2.3. Radiolabelling of Mab with ^{125}I

The purified monoclonal antibodies were labelled with ^{125}I using different oxidants such as chloramine-T, iodogen and lactoperoxidase [9, 10]. 30 μL of 0.1 M phosphate buffer, pH 7.5 and ~ 30 μg of antibody solution were taken in a glass tube and 18–30 MBq (500–800 μCi) of Na^{125}I was added and the contents were mixed thoroughly. In the case of iodogen, the reaction was carried out in a glass tube coated with 10 μg iodogen for 5 minutes and stopped by removing the solution from the tube for purification. In case of chloramine-T, 25 μg of chloramine-T (2.5mg/mL) was added, mixed for 2 minutes and arrested by the addition of 25 μL of sodium metabisulphite (7.6 mg/mL). In the case of lactoperoxidase, 4 μg lactoperoxidase with 2 μL 1:10,000 H_2O_2 was used. After 2 minutes at ambient temperature, the reaction was arrested by dilution with buffer. In all the cases, purification was carried out by gel filtration over Sephadex G-25 column (1 \times 25 cm) using phosphate buffer (0.025 M, with 0.25% BSA, pH 7.5) as the eluant. The reaction yield and radiochemical purity were estimated by paper

electrophoresis using Whatman- 3 chromatography paper, in phosphate buffer (0.025 M, pH 7.5) for 1 h at 10 V/cm. The immunoreactivity of the peak fractions was checked.

One of the monoclonal antibodies raised, Mab-4G4, assessed to be the best of the lot, was labelled with ^{125}I using chloramine-T and bio-distribution studies in Wistar albino rats were carried out.

2.4. Radiolabelling of Mab with $^{99\text{m}}\text{Tc}$

Mab-4G4, was labelled with $^{99\text{m}}\text{Tc}$ using stannous tartrate as the reducing agent. 16 MBq ($\sim 420\ \mu\text{Ci}$) of $^{99\text{m}}\text{Tc}$ was mixed with 0.1 mL of a 5 mg/mL solution of stannous tartrate. To this was added $\sim 80\ \mu\text{g}$ of antibody in normal saline and reacted for 30 minutes. The radiolabelling was also attempted after reduction of the antibodies with 2-mercaptoethanol [11]. The radiochemical yield was determined by paper chromatography using saline as the mobile phase. The mixture was purified through Sephadex G-25 (1 \times 10 cm) column. The immunoreactivity of the peak fractions was tested and the appropriate fraction was used for bio-distribution studies in Wistar albino rats.

2.5. Bio-distribution of $^{99\text{m}}\text{Tc}$ and ^{125}I labelled Mab-4G4

Myocardial infarction was induced in Wistar albino rats by two subcutaneous injections of isoproterenol (75 mg/kg) on two consecutive days[12]. About 4 hours after the second injection, radiolabelled Mab-4G4 (4–5 μCi , $\sim 1\ \mu\text{g}/\text{rat}$) was intravenously injected. After 24 hours, the animals were sacrificed and radioactivity in different organs was determined.

3. RESULTS AND DISCUSSION

The HCM obtained was highly pure and was very similar to the BSM as confirmed by the SDS PAGE pattern. HCM was stored in 0.6 M KCl diluted with equal volume of glycerol at -20°C .

**TABLE I : ISOTYPES, TITRE, AFFINITY AND CROSS REACTIVITY OF
HCM MONOCLONAL ANTIBODIES**

Clone (Mab)	Isotype	Titre	Relative Affinity - IC ₅₀ *	% Cross reaction with BSM
4G4	IgG2a, λ	1:780	4.9	117
8G5	IgM, κ	1:450	N.D.	107
1G4	IgG2b, λ	1:50	3	143
8C4	IgG1, λ	1:70	1	115

* Relative affinity was measured in terms of amount of myosin in $\mu\text{g/mL}$ required to achieve 50% (IC₅₀) inhibition of the antimyosin activity assessed by ELISA,

N.D.- Not carried out since it was IgM

All the immunised BALB/c mice exhibited the presence of antibodies against HCM after the booster. After fusion and initial plating in 131 wells, antimyosin antibody activity was detected in the culture supernatants of 46 wells. After the two limiting dilution steps, 13 clones could be identified. Of these, four clones were selected on the basis of their reasonable titre values. Table I shows the isotypes, titres, affinities of the different antibodies and their cross reaction with bovine skeletal myosin (reaction with HCM is taken as 100%) of the selected antibodies. Barring one clone of IgM type, the clones were of IgG type, belonging to different subtypes. Being pentavalent, IgM was not used for further studies. The titres of these antibodies however, are not too high, perhaps because myosin is a complex sequestered protein of all vertebrates. None of these clones reacted with murine Coxsackie B virus, B1 to B6, or the light and heavy chains of rabbit myosin. It is seen that all the antibodies exhibited strong cross reactivity against bovine skeletal myosin, some even stronger than that for HCM. Several epitopes on HCM may

be "self" epitope for mouse and only a few epitopes may get exposed. It has been reported that complex antigens result in low affinity antibodies as compared to small antigens[13]. Such moderate affinity combined with the striking similarity between HCM and BSM could have resulted in high cross reactions for BSM. The affinity for HCM expressed in terms of HCM required to inhibit the solid phase binding by 50 % is seen to vary for the Mabs. The greater the amount required to inhibit the binding, the better the affinity and Mab-4G4 had the maximum affinity for HCM as well as maximum titre value. Mab-4G4 was hence used for labelling with ^{125}I and $^{99\text{m}}\text{Tc}$.

The ascitic fluids were purified satisfactorily by a two step method using caprylic acid precipitation to remove the non-IgG fractions, followed by the precipitation of the IgG fractions with ammonium sulphate and dialysis to remove salt.

^{125}I -Mab-4G4 was prepared and studied for its localization with a view to explore the possibility of using ^{123}I -Mab-4G4 as an immunoscintigraphic agent for infarct localisation. Irrespective of the oxidant used, the labelling yield of Mab-4G4 with ^{125}I was ~ 60% by paper electrophoresis and the radiochemical purity of the labelled antibody purified over G-25 gel was >95%. Similar results were obtained with other anti-HCM Mabs also. Although there was no significant difference when different oxidants were used in the quality of labelled Mab obtained in terms of purity, shelf life etc., the yields were consistently around 60% with chloramine-T while it fluctuated with the other two. A typical gel chromatography pattern is seen in Fig.1.

Radiolabelling yield with $^{99\text{m}}\text{Tc}$ was observed to be ~ 61-65%. The use of other transchelating agents such as citrate or reduction of antibodies with 2-mercaptoethanol did not improve the yield. Despite the variations in the amounts of stannous tartrate used for reduction of TcO_4^- , the yields were around 60%. On the other hand, similar experiments with purified immunoglobulins gave >90% labelling yield.

TABLE II: BIO-DISTRIBUTION OF ^{125}I -Mab-4G4 IN WISTAR RATS (n=3)

Organ	% Activity/organ \pm s.d.		% Activity/g \pm s.d.	
	Normal	Infarcted	Normal	Infarcted
Blood	20.2 \pm 5.2	25.8 \pm 6.2	1.2 \pm 0.4	1.2 \pm 0.25
Heart(perfused)	0.3 \pm 0.02	0.51 \pm 0.1	0.3 \pm 0.03	0.5 \pm 0.1
Lungs	0.8 \pm 0.2	0.6 \pm 0.1	0.4 \pm 0.2	0.5 \pm 0.2
Liver	5.5 \pm 2.1	3.5 \pm 1.1	0.4 \pm 0.1	0.3 \pm 0.1
Spleen	0.4 \pm 0.2	0.5 \pm 0.1	0.6 \pm 0.2	0.9 \pm 0.2
Kidneys	3.0 \pm 0.3	2.9 \pm 0.1	2.2 \pm 0.3	1.8 \pm 0.1
Muscle	-	-	0.3 \pm 0.1	0.5 \pm 0.3
Thyroid	74 \pm 8	81 \pm 0.5	-	-
Carcass	2.7 \pm 1.2	3.8 \pm 0.3	0.02 \pm 0.01	0.01 \pm 0.001

Only % act./g was taken in the case of muscle primarily to compare with the accumulation in other organs; while in the case of thyroid only % activity in the whole organ was alone calculated as a measure of in-vivo deiodination..

TABLE III: BIO-DISTRIBUTION OF $^{99\text{m}}\text{Tc}$ -Mab-4G4 IN WISTAR RATS (n=3)

Organ	% Activity/ organ \pm s.d.		% Activity/ gram \pm s.d.	
	Normal	Infarcted	Normal	Infarcted
Blood	6.9 \pm 2.2	3.3 \pm 1.2	0.8 \pm 0.3	0.35 \pm 0.15
Heart (perfused)	0.1 \pm 0.05	0.5 \pm 0.03	0.2 \pm 0.1	0.7 \pm 0.04
Lungs	0.4 \pm 0.3	1.1 \pm 0.6	0.6 \pm 0.4	1.1 \pm 0.1
Liver	45 \pm 7.8	57 \pm 6.8	8.6 \pm 2.0	6.9 \pm 0.4
Spleen	1.8 \pm 0.2	3.9 \pm 0.6	2.4 \pm 0.4	4.8 \pm 1.6
Kidneys	24.3 \pm 5.5	14.1 \pm 2.0	15.0 \pm 3.1	8.9 \pm 1.4
Intestine	9.7 \pm 4.6	12.2 \pm 4.1	0.7 \pm 0.4	0.7 \pm 0.3
Muscle	-	-	0.03 \pm 0.01	0.13 \pm 0.1
Stomach	7.7 \pm 2.8	2.3 \pm 0.6	1.1 \pm 0.2	0.4 \pm 0.1
Carcass	11.6 \pm 0.6	8.6 \pm 3.5	0.05 \pm 0.03	0.07 \pm 0.04

Only % act./g was taken in the case of muscle primarily to compare with the accumulation in other organs.

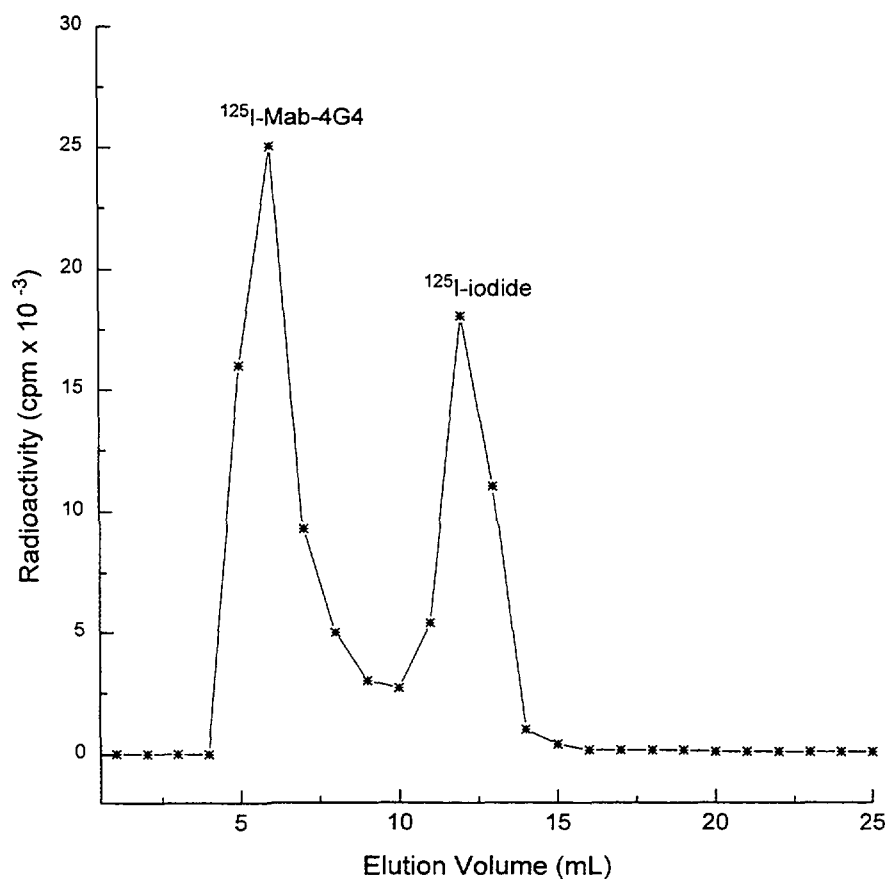


Fig. 1. Elution pattern of radioiodinated Mab-4G4 over sephadex G-25 column

Table II and III show the bio-distribution pattern of the labelled Mab-4G4, in normal and MI induced rats when ^{125}I -Mab-4G4 and $^{99\text{m}}\text{Tc}$ -Mab-4G4 were used respectively. The induction of MI was confirmed by histopathological examination and the damage to the myocardium could be clearly visualised. The net total activity retained at the end of 24 hours was taken as the total activity, since the prime aim was to study the distribution pattern of the retained activity in various organs. From the table II, it can be seen that severe *in-vivo* deiodination of ^{125}I -Mab-4G4 has occurred leading to accumulation of ~ 70 -80% activity in the thyroid in 24 hours. It was rather surprising to note that the amount of activity accumulated in the thyroid is much higher than even a normal thyroid uptake. High thyroid uptake has been a major problem with radioiodinated antibodies, especially when the tyrosyl moiety is labelled [14-16] and

alternative methods have been suggested [17]. Attempts are now being made to radioiodinate the Mabs through modified linkers such as alpha methyl tyrosine. In this study, it is encouraging to find a 1.5 fold increase in activity accumulation in the infarcted heart compared to the normal heart, although increased uptake is seen in other organs such as spleen, lungs, muscles etc. of the infarcted animal. It is not known if this is due to the effects of isoproterenol, the agent used for artificially producing the infarct. We are also attempting to produce infarcts in experimental animals by other means. But, use of a radioiodinated antibody for scintigraphy requires a lot of efforts to improve the performance at this stage.

In the case of ^{99m}Tc -Mab-4G4, 3.5 fold increase in activity was seen in hearts where MI was induced. In this case, the activity has also accumulated in several non-target organs such as liver, kidneys and spleen to a considerable extent. The increased liver uptake in the case of ^{99m}Tc -Mab-4G4 which is not seen in the case of ^{125}I -Mab-4G4 is perhaps an indication of aggregation of antibody molecules. In a similar manner, the activity in stomach and intestines were also appreciable for ^{99m}Tc -Mab-4G4 while they were negligible for ^{125}I -Mab-4G4 (not tabulated). Attempts to label this antibody with ^{99m}Tc through a suitable bifunctional chelating agent are also being made. As mentioned earlier, use of isoproterenol for infarct induction could be causing changes in the organs such as spleen, muscles etc. On comparing the two labelled antibodies, it is seen that in the case of radioiodinated antibody, the activity retained in thyroid and blood are much higher while that in liver, kidneys and carcass are much lesser than those for ^{99m}Tc -Mab-4G4. As discussed before, these effects could be due to multiple reasons such as *in-vivo* deiodination, aggregation etc.

In conclusion, of the various Mabs tested, Mab-4G4 had high affinity for HCM. Bio-distribution studies using ^{99m}Tc -Mab-4G4 and ^{125}I -Mab-4G4 in Wistar albino rats

with induced MI have given results that are encouraging but needing improvement. Although a product that could be used for infarct imaging has not been achieved in the present studies, monoclonals specific to HCM could be prepared and their use for development of *in vitro* or *in vivo* diagnostic agents appears possible. We propose to extend this work by using F(ab)₂ fragment of the Mabs.

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Abstract

Preparation of ^{99m}Tc-labelled tetrapeptides, namely acetyl-Gly-Gly-Cys-Gly (*I*), acetyl-Ser-Ser-Cys-Gly (*II*) and acetyl-Gly-Gly-Cys-Lys (*III*), analysis of their radiochemical purity and biodistribution were investigated in rats. The aim was to determine the relationship between structure and biological behaviour of ^{99m}Tc-labelled peptides which are formed by amino-acid sequences capable of chelating technetium useful as universal chelators in „hybrid“ peptides composed of receptor-specific part and the part chelating technetium. For labelling with ^{99m}Tc, a conventional transchelation from ^{99m}Tc-gluconate was used and radiolabelled peptides were purified by filtration on Whatman microfilters 12 kD. Radiochemical purity was higher than 98%. Biodistribution studies in rats showed that all agents are rapidly cleared from the body mostly via urine, but some part of administered radioactivity also in the faeces was found. The later route of elimination way increased in the order *III* < *II* < *I*. On the other hand, blood radioactivity-time decrease was more rapid for *I* and the slowest for *III*. No specific accumulation of radioactivity in different organs and tissues was found, the high radioactivity in the kidneys and liver at the shortest time interval (5 min after administration) was due mainly to partial elimination of the agents by the kidneys to urine and by the liver to bile. At 1 hour after dosing, a major part of administered agents was excreted by elimination through urine and bile and only tracer radioactivity remained in blood and other organs. An analysis of renal elimination mechanisms of radiolabelled peptides under study by using of perfused rat kidney in situ showed that all agents are eliminated by both glomerular filtration and tubular secretion,

but the intensity of their elimination by tubular secretion was lower than that for ^{99m}Tc -MAG3. The results obtained will assist with design of optimal biocompatible tetrapeptides as chelators for formation of hybrid receptor-specific peptides.

1. Introduction

Receptor scintigraphy with radiolabelled neuropeptides is a very sensitive method for the demonstration of receptor-positive tumors and their metastases (1). One class of such neuropeptides is based on radiolabelled somatostatin analogues (2). Somatostatin receptors are peptide hormone receptors located on the cell membrane of tumors of neuroendocrine origin, but also on other neoplasms, including several intracranial tumors such as meningiomas, gliomas and pituitary tumors (3). Somatostatin receptor scintigraphy has been performed with octreotide analogs labelled with different radionuclides such as cyclotron-produced I-123, In-111 and Ga-67. Considering that these radiopharmaceuticals share the ability to target tumors very quickly and thus diagnostic images can be theoretically obtained early after intravenous administration, a radiolabel with a shorter half-life and more favourable radiation dosimetry would be preferred to these radionuclides. The prime candidate for single photon emission tomography is readily available and low price ^{99m}Tc due to its nearly ideal nuclear characteristics, high count rate and high quality image, which translate into good diagnostic efficacy. For radiolabelling with metallic radionuclides, a coupling of peptide with bifunctional chelates such as DTPA (diethylenetriaminepentaacetic acid) and DFO (desferoxamine) is usually used. Such modification of peptide structure with foreign chelator results in change of both lipophilicity and pharmacokinetics of the agent (4). Another strategy lies in the design of a hybrid peptide which incorporates both the amino-acid sequence responsible for receptor-binding and a sequence capable of chelating technetium, separated by a short linker sequence (5). A radionuclide-binding sequence is formed by a biocompatible tetrapeptide

which is able to form a stable bound with the suitable radionuclide. Diagnostic value of these agents, containing aminoacids only, is dependent on the affinity to specific receptor sites on the one hand and on the rate of the organ background clearance (decisive for administration-to-imaging time) on the other hand. Ideally, the radionuclide-binding part would not affect the receptor affinity and specificity of the peptide; its effect on the rate and route of elimination of the peptide is, however, hard to predict. For ^{99m}Tc -radiopharmaceuticals, the administration-to-imaging time is limited by the physical half-life of the radionuclide (6 hrs). For this reason, the development of agents with a more rapid rate of elimination is still in progress.

The biological behaviour of such radiolabelled hybrid peptides is influenced by both the receptor-specific chain and the radiometal-binding part. The present paper evaluates the advantages and limitations of three potential chelators, namely preparation, biodistribution and elimination in rats of ^{99m}Tc -labelled tetrapeptides acetyl-Gly-Gly-Cys-Gly (*I*), acetyl-Ser-Ser-Cys-Gly (*II*) and acetyl-Gly-Gly-Cys-Lys (*III*).

2 Materials and Methods

2.1. Radiolabeling and quality control

For ^{99m}Tc -labelling two methods were used: the direct labelling and ligand exchange method.

2.1.1. Direct labelling

To 100 μl of peptide solution (conc. 2 mg/ml) in 0.5 M phosphate buffer pH 11, two μl of stannous chloride (conc. 0.5 M) in 1 M HCl and 100 μl of sodium [^{99m}Tc]pertechnetate activity approximately 300 MBq were added, followed by 50 μl of 0.5 M sodium triphosphate. The solution was heated at 90°C in water bath for 10 minutes and cooled to room temperature.

2.1.2. Ligand exchange method of labeling

A commercial glucoheptonate kit (Glucoheptosint, Fleurus, Belgium) was reconstituted with 2ml of ^{99m}Tc -pertechnetate eluate (activity approximately 300MBq). 0.3mg of the peptide was dissolved in 150 μl 0.5M sodium phosphate buffer pH 11 and 150 μl of reconstituted glucoheptonate kit was added. The solution was heated at 90°C for 10 min and cooled to room temperature. The solutions from both labelling procedures were then filtered by Microcentrifuge tube filter (Cellulose Triacetate) 12k MWCO and analysed by HPLC and ITLC-SG (in saline solution).

2.1.3. HPLC analysis

^{99m}Tc -labelled tetrapeptides under study were analysed by HPLC with UV and radiometric detection. 10 μl of labelled peptide was diluted 100 times with 0.01M sodium phosphate buffer pH 6.0 and 10 μl of diluted solution was subjected to HPLC analysis on C-18 reverse phase column. Mobil phase: methanol - 0.01M sodium phosphate pH 6.0

gradient elution: time 0 min 95% buffer in methanol

time 3 min 95% buffer to 5% buffer in methanol over 10 min (linear gradient)

time 13 min 5% buffer in methanol for 10 min

time 23 min 5% buffer to 95% buffer over 5 min (linear gradient)

time 28 min 95% buffer for 2 min

2.1.4. ITLC-SG

Solution of labelled peptide was spotted on to two strips of ITLC-SG (1x12cm) and chromatographed in 0.15M NaCl or MEK. Activity on start and front of chromatograms was detected on radiochromatograph (Berthold).

2.2. Biological experiments

2.2.1. Biodistribution studies

Groups of four male Wistar rats were injected through a lateral tail vein with the ^{99m}Tc -labelled agents containing 2 μg of peptide. At selected time intervals (5 min, 2hrs and 24 hrs) after administration, the carotid artery was exposed under ether anaesthesia and a blood sample was collected in glass tubes containing heparin. After exsanguination, selected organs were taken out and weighed using an analytical balance. Concomitant radioactivity was then measured in a Wallac 1480 Wizard 3 automatic gamma counter with three standard radioactivity samples prepared at the time of injection.

2.2.2. Elimination studies

The agents were administered to rat as described previously. Following administration, the animals were placed singly in glass metabolic cages, the construction of which allows reliable separation of urine and solid excrements. The animals had free access to standard diet and water. Two hours after dosing the rats were forced to empty their urinary bladders by handling (immobilization) and urine and faeces were collected. The procedure was repeated at 24 hr and 48 hr intervals after administration.

3 Results

The HPLC profile of radiolabeled peptides is shown in Fig. 1. It is evident that the retention time is related to the lipophilicity of the agents under study, the highest hydrophobicity of **II** is in agreement with its shortest retention time. The labeling efficiency (radiochemical purity higher than 95%) was sufficient for the biological experiments.

The distribution of radioactivity in selected organs and tissues of rats after intravenous administration of ^{99m}Tc -labelled tetrapeptides at different time intervals is presented in Table 1.

Table I Distribution of radioactivity in selected organs and tissues after intravenous administration of ^{99m}Tc -labelled tetrapeptides to rats

Organ	^{99m}Tc -Ac-Gly-Gly-Cys-Gly			^{99m}Tc -Ac-Ser-Ser-Cys-Gly			^{99m}Tc -Ac-Gly-Gly-Cys-Lys		
	5 min	60 min	120 min	5 min	60 min	120 min	5 min	60 min	120 min
<i>Percent dose in whole organ</i>									
Liver	8.57 ± 1.12	0.18 ± 0.01	0.13 ± 0.02	10.78 ± 1.39	1.99 ± 0.24	0.66 ± 0.12	5.65 ± 0.70	1.17 ± 0.08	0.25 ± 0.04
Kidney	7.23 ± 1.60	2.26 ± 1.81	1.63 ± 0.64	13.34 ± 2.06	2.23 ± 0.43	1.10 ± 0.23	12.55 ± 0.79	1.91 ± 1.20	0.97 ± 0.30
Lung	0.74 ± 0.18	0.03 ± 0.01	0.02 ± 0.01	0.76 ± 0.16	0.10 ± 0.03	0.03 ± 0.01	1.08 ± 0.22	0.10 ± 0.01	0.03 ± 0.01
Heart	0.30 ± 0.06	0.01 ± 0.01	< 0.01	0.22 ± 0.02	0.02 ± 0.01	< 0.01	0.34 ± 0.04	0.03 ± 0.01	< 0.01
Stomach	1.19 ± 1.34	0.07 ± 0.03	0.19 ± 0.01	0.74 ± 0.32	0.25 ± 0.08	0.28 ± 0.34	0.79 ± 0.14	0.66 ± 0.91	0.17 ± 0.18
Small intestine	9.79 ± 2.73	28.71 ± 2.73	41.18 ± 4.01	4.36 ± 1.17	18.63 ± 1.03	20.24 ± 2.57	2.40 ± 0.32	12.55 ± 3.26	9.98 ± 1.76
Colon	0.70 ± 0.18	0.05 ± 0.03	0.13 ± 0.12	0.86 ± 0.10	0.10 ± 0.02	0.94 ± 1.76	1.14 ± 0.19	0.14 ± 0.06	5.25 ± 4.96
Brain	0.07 ± 0.01	< 0.01	< 0.01	0.05 ± 0.01	< 0.01	< 0.01	0.08 ± 0.01	0.01 ± 0.01	< 0.01
Thyroid	0.11 ± 0.05	< 0.01	< 0.01	0.05 ± 0.01	< 0.01	< 0.01	0.07 ± 0.01	< 0.01	< 0.01
Adrenals	0.02 ± 0.01	0.01 ± 0.01	< 0.01	0.01 ± 0.01	< 0.01	< 0.01	0.02 ± 0.01	< 0.01	< 0.01
Spleen	0.12 ± 0.03	0.01 ± 0.01	0.02 ± 0.02	0.09 ± 0.01	0.01 ± 0.01	< 0.01	0.14 ± 0.03	0.02 ± 0.01	< 0.01
Testes	0.25 ± 0.06	0.03 ± 0.01	0.01 ± 0.01	0.28 ± 0.03	0.06 ± 0.01	0.02 ± 0.01	0.33 ± 0.05	0.08 ± 0.01	0.02 ± 0.01
<i>Percent dose per 1% body weight</i>									
Blood	1.86 ± 0.48	0.04 ± 0.02	0.01 ± 0.01	1.76 ± 0.17	0.17 ± 0.02	0.04 ± 0.01	2.27 ± 0.17	0.22 ± 0.03	0.05 ± 0.01
Skin	0.86 ± 0.21	0.08 ± 0.06	0.02 ± 0.01	1.05 ± 0.06	0.14 ± 0.04	0.03 ± 0.01	1.25 ± 0.15	0.18 ± 0.02	0.03 ± 0.01
Muscle	0.36 ± 0.05	0.01 ± 0.01	< 0.01	0.42 ± 0.02	0.04 ± 0.01	< 0.01	0.52 ± 0.03	0.05 ± 0.01	< 0.01
Fat	0.51 ± 0.10	0.01 ± 0.01	< 0.01	0.51 ± 0.05	0.06 ± 0.01	0.01 ± 0.01	0.51 ± 0.15	0.07 ± 0.01	0.01 ± 0.01

The high radioactivity in the kidney and liver found at the initial time interval (5 min after dosing) was evidently due to the elimination of agents into urine and bile, respectively. The time decrease of radioactivity in blood and tissues with the exception of the gastrointestinal tract was relatively rapid, 1 hour after administration less than one tenth of initial radioactivity in most of organs and tissues was determined. Increasing radioactivity in a small intestine and later also in a colon is due to a partial bile elimination of the agents. These findings were in good agreement with the results of elimination studies, presented in Fig. 2. Even if the radioactivity was eliminated mostly by urine, a significant portion of radioactivity excreted by faeces was also found.

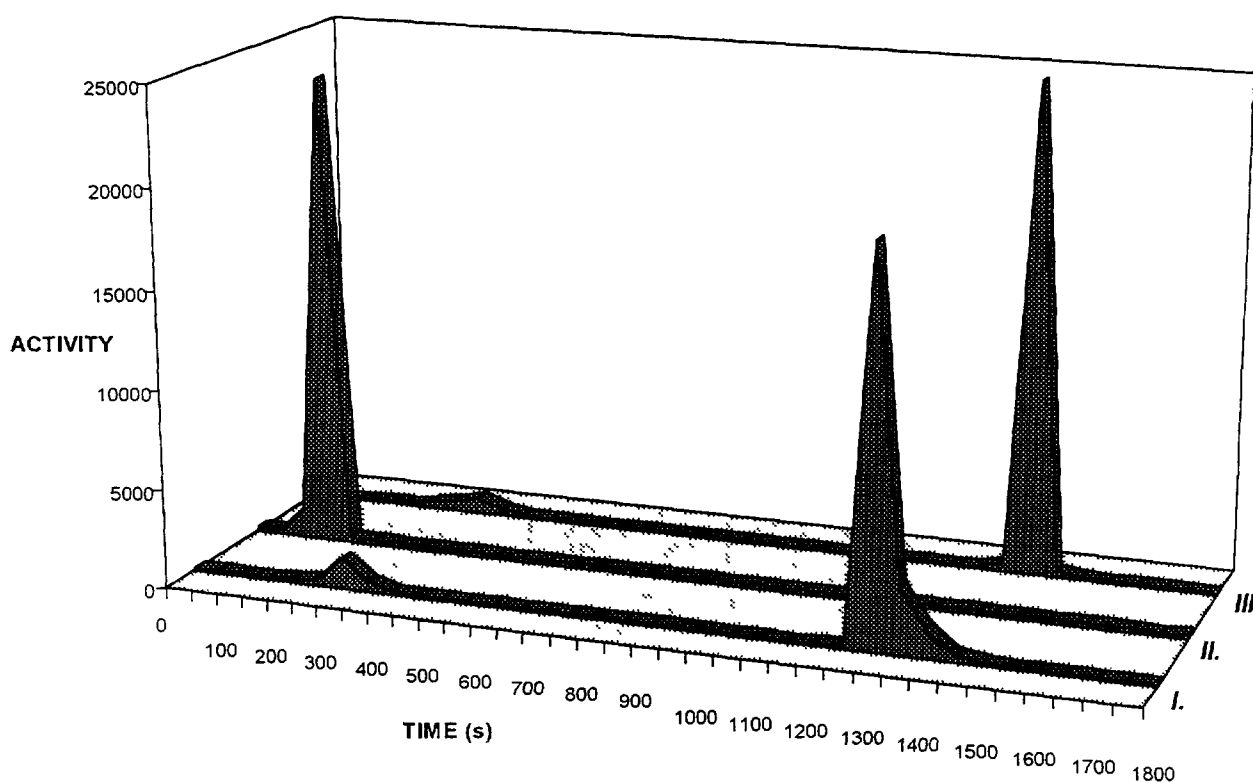


Fig 1 HPLC profiles of ^{99m}Tc -tetrapeptides

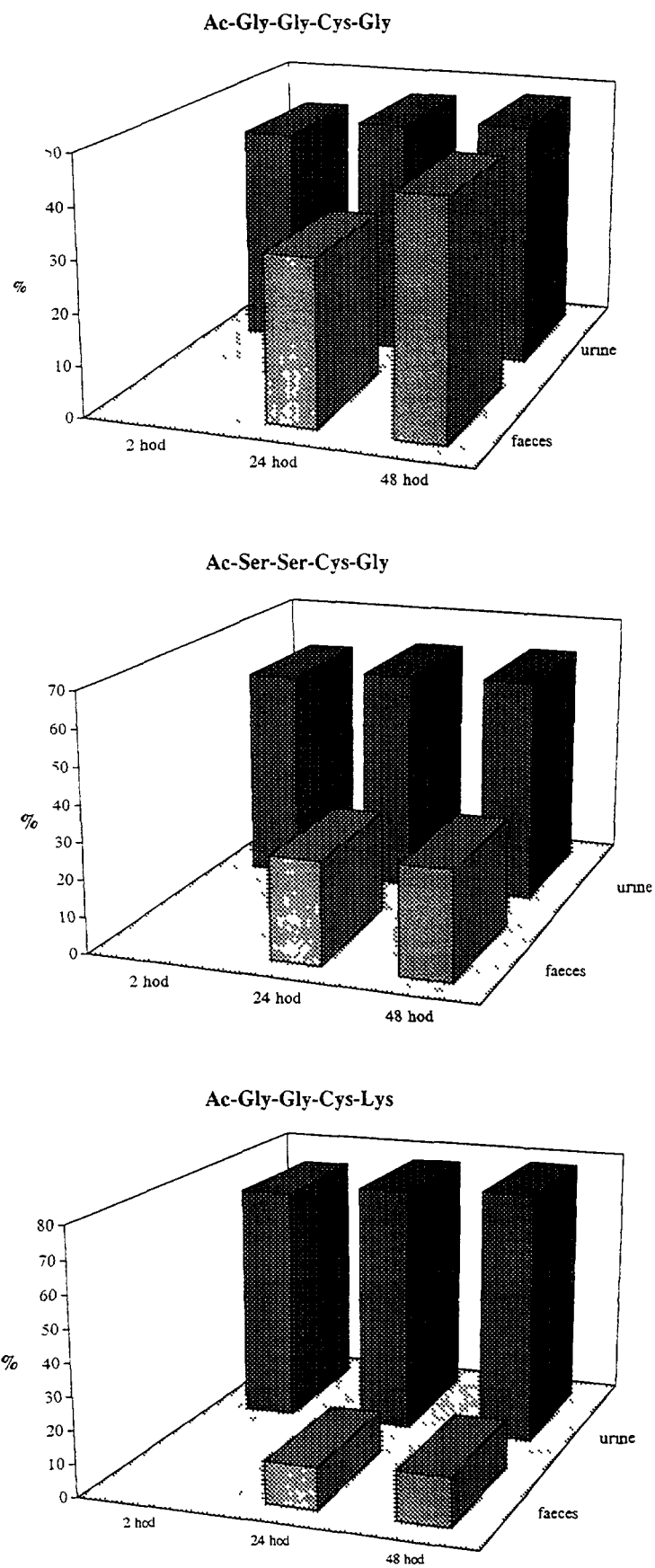


Fig 2 Cumulative excretion of radioactivity into urine and faeces after ^{99m}Tc -tetrapeptides

4. Discussion

An ideal somatostatin receptor specific agent would exert high and long term retention in receptor expressing tissues (especially in tumor) and rapid and efficient clearance from blood and non-tumorous tissues. While the affinity to receptor sites is primarily affected by the structure of the receptor-specific part of hybrid peptide, the free-peptide concentration around the receptors is influenced by both plasma concentration and plasma protein binding of agents, as at equilibrium the free drug concentration in tissues equals to that in plasma. The rate of the radioactivity-time decrease (and also administration-to-imaging time) is dependent primarily on the mechanisms of agent elimination. All these pharmacokinetic parameters of hybrid peptides are affected by both the receptor-specific and chelating parts. As different tetrapeptides able to bind ^{99m}Tc have been proposed as the chelators of hybrid peptides (5), comparison of their pharmacokinetics and biodistribution should make more clear advantages and limitations of individual tetrapeptides in using for intended purpose. Having this aim in mind it can be concluded that a significant bile elimination is an undesirable pathway as it may interfere with cancer visualization in abdominal region. Rapid elimination to urine is preferable. All agents in this study are eliminated in the kidney by both glomerular filtration and tubular secretion, but their renal clearance is significantly lower than that for ^{99m}Tc -MAG-3 (7). The highest renal clearance and total rate of elimination of *I* is preferable, but its highest bile excretion is a disadvantage in comparison with *II* and *III*. It can be concluded that none of the tetrapeptide exhibits markedly preferable pharmacokinetic behaviour in comparison with the others, the results, however, may assist in the future development of hybrid receptor specific peptides.

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INVITED LECTURE

DEVELOPMENTS in $^{99}\text{Tc}^{\text{m}}$ COMPLEXES FOR FUNCTIONAL IMAGING

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Abstract

Technetium-99m coordination complexes constitute the backbone of diagnostic nuclear medicine. Early exciting advances in products for excretory organs / pathways were followed by arduous research efforts to design and optimise $^{99}\text{Tc}^{\text{m}}$ compounds for imaging renal tubular function and mapping blood flow to myocardium and brain. A variety of neutral, cationic and anionic complexes of technetium, mostly in +5 or +3 oxidation states and usually involving N, S, P, O as coordinating atoms, have dominated the field. Blending the well-known versatile coordination chemistry of technetium with biochemical principles and pharmacology of some functional groups has helped achieve desirable properties in at least some of the resultant $^{99}\text{Tc}^{\text{m}}$ complexes. Fascinating developments to tap the merits of $^{99}\text{Tc}^{\text{m}}$ tracer for more sophisticated targeting approach involving biological substrates have yielded promising results. Use of appropriate ligands as bifunctional chelating agents (BCA) to form $^{99}\text{Tc}^{\text{m}}$ labelled radiopharmaceuticals has also led to development of several new $^{99}\text{Tc}^{\text{m}}$ complexes. Although $^{99}\text{Tc}^{\text{m}}$ complexes for metabolism or receptor imaging may still be far from a clinical reality, many useful efficacious clinical applications have become feasible with the advent of some new $^{99}\text{Tc}^{\text{m}}$ complexes, e.g. imaging infection / inflammation, certain tumours and even hypoxia. A strong synergism between academic universities and industries has evolved, amidst the rush for patenting all products and processes, despite low chances of success in developing a clinically useful product. The enormous research costs have made the new products very expensive and, in turn, driven many developing countries and large hospital radiopharmacies to seek alternate means of formulating equivalent products in-house or evolve modified protocols with commercial products for better economy. This review covers the major investigations of the last decade (but by no means exhaustive) after touching upon the milestones of the earlier period and also outlines the efforts at author's laboratory.

1.0 Introduction

Technetium-99m continues to occupy the centre stage in clinical nuclear medicine and accounts for well over 80% of all products used in this field. It may not be an exaggeration to state that the progress in nuclear medicine almost parallels the development of new $^{99}\text{Tc}^{\text{m}}$ radiopharmaceuticals. The early requirement to image the excretory organs / pathways or other similar clinical needs could be met by the technetium compounds developed at that time. With the advent of alternate imaging modalities, the emphasis to provide unique or distinctly additional information in various clinical conditions has become the need of the hour, challenges to be met by development of $^{99}\text{Tc}^{\text{m}}$ tracers capable of providing such data, e.g. image the blood flow to vital organs like myocardium and brain, quantify organ function, mark specifically lesions like an abscess or tumour etc. More sophisticated demands to study metabolism, receptor involvement in health and disease, imaging based on molecular recognition etc. have subsequently emerged, posing further challenges for the development of specific $^{99}\text{Tc}^{\text{m}}$ compounds to serve as radiopharmaceuticals.

Extensive research efforts have been devoted to develop ligands to form stable technetium complexes both for direct use and to serve as bifunctional chelates (BCA) to link technetium with substrates of potential utility, mostly of biological origin and in recent times of synthetic origin also. A strong multi-disciplinary approach has thus become essential in the development of new products, as the principles applicable encompass a number of subjects, such as chemistry, radiochemistry, bio-chemistry, pharmacology, biology amongst others. Due to the fact that technetium is completely alien to physiology, blending the well-known versatile co-ordination chemistry features of technetium (a second row transition element, $Z=43$, $[\text{Kr}] d^5 s^1$ electronic configuration) with known biochemical principles and pharmacology of some functional groups has helped achieve (of course after arduous efforts) desirable biological properties in at least some of the resultant complexes.

Over the years, a strong synergism has evolved between academic university researchers and interested industrial manufacturers of radiopharmaceuticals for sustaining the efforts to obtain clinically useful products from basic research works, no mean achievement by any standards. The enormous research costs involved have triggered a rush for patenting every product, though the chances of success in obtaining a clinically useful product are quite low, as the statistics would reveal. Against this backdrop, it would be virtually impossible to attempt to cite all the developments in $^{99}\text{Tc}^{\text{m}}$ complexes for functional imaging in this review. The author has chosen to trace some of the early milestones, cover those developments which have led to either clinically useful products or shown promise for clinical utility, compare the radiopharmacology features of products of similar nature, discuss practical aspects in the preparation and use, comment on the discrepancies in the case of some products, describe the efforts and accomplishments at author's laboratory (both independently and through a CRP of IAEA) and indicate emerging trends.

By design, this article does not cover $^{99}\text{Tc}^{\text{m}}$ labelled antibodies and peptides *per se*. While every effort to include major developments has been taken, it can be by no means claimed exhaustive. The literature coverage spreads over the past decade, in particular over the 3-4 year period of IAEA's CRP on "Bulk reagents for production of $^{99}\text{Tc}^{\text{m}}$ radiopharmaceuticals and kits" [1] and the subsequent recent years.

2.0 Milestones and Concepts in the Evolution of New Products

In the genesis of the growth of $^{99}\text{Tc}^{\text{m}}$ compounds, the introduction of DTPA chelate of technetium, use of stannous tin for reduction of Tc(VII) in pertechnetate and lyophilisation of pre-mixed stannous tin - ligand formulation would merit the first mention despite the passage of time. The subsequent major breakthrough could be cited as the studies on technetium complexes of derivatised lidocaine analogues, viz. acetanilido iminodiacetic acid based ligands (IDA, LIDA, HIDA). Suitable variation(s) in the functional groups on the ligand backbone to influence the pharmaco-kinetic behaviour of the resultant technetium complexes, while retaining the same chelating environment for technetium, was a major development. This eventually led to introduction of the most preferred hepatobiliary tracer, $^{99}\text{Tc}^{\text{m}}$ -mebrofenin [2]. Such systematic investigations of structure - activity distribution relation (SADR) provided a fresh approach for the development of many other new products (Table-I). The concept of bifunctional nature of ligands was also propounded after this work, for in LIDAs, IDA groups participate in complexing technetium, while the phenylcarbamoyl moiety bestows some of the required biological features. Although in the present sense of the term BCA, this may not be strictly correct, the way was paved for a new approach to develop $^{99}\text{Tc}^{\text{m}}$ compounds.

2.1 Use of ligand designs - Existing and derivatised analogues

The design of N,N substituted derivatised tetradentate ligand denoted as diamide-disulphide (DADS) based on the well-known ethylene diamine (en) ligand, by the Boston professor pair, Jones and Davison [3], helped explore the advantages of a multidentate ligand chelating technetium to form a more stable complex of the type $[\text{TcOL}]$, cf. labile nature of some complexes of type ML_n . The use of macroscopic quantities of the long lived ^{99}Tc for elucidation of the structure of the technetium complex of DADS as $[\text{Tc}^{(\text{V})}\text{O}(\text{DADS})_2]$ and application of HPLC technique to correlate the characterization of the carrier-added (ca) ^{99}Tc product with that of the no-carrier-added (nca) $^{99}\text{Tc}^{\text{m}}$ product, thanks to the ability to use a radiometric detector in series with a mass based detector in HPLC system, were important approaches. These major trends have been extensively followed thereafter. The biological behaviour of the well characterized product of Tc-DADS in 'nca' form obtained using generator produced $^{99}\text{Tc}^{\text{m}}$, indicated possibilities for progress towards a clinically useful product for renal function studies and finally led to development of $^{99}\text{Tc}^{\text{m}}$ - MAG_3 by Fritzberg et al. [4] as renal tubular function agent (Table-I). Another similar ligand design, denoted as diamine-dithiol (DADT, BAT) [5], was used in the research efforts to develop technetium complexes capable of crossing the blood brain barrier (BBB).

The use of well known or previously investigated ligands of inorganic and analytical chemistry provided another concept to develop new technetium complexes, though whether any desired biological role would be available in the product was a question mark. The need for monocationic complex of technetium to help trace the myocardial blood flow, a concept well advocated by Prof. Deutsch [6], based on the known in-vivo handling of some organic cations, opened up this avenue. The early euphoria of success of $[\text{Tc}^{(\text{III})}(\text{DMPE})_2\text{X}_2]^+$ for myocardial perfusion imaging in animal models, failure to achieve similar results in humans, recognition of species dependent bio-distribution patterns, chemical and biochemical investigations to understand the fate of the product in-vivo and eventual evolution of mixed ligand non-reducible Tc(III) complex denoted as Tc-Q_n (Tc(III) complex of tetradentate Schiff base H₂L ligands and of alkoxy phosphines) leading to clinically promising Tc-furifosmin (TcQ₁₂) are noteworthy sequence of developments [7]. The development of the other myocardial perfusion tracer Tc-tetrofosmin, a Tc dioxo bis-alkoxyphosphine complex, $[\text{TcO}_2(\text{RP}_2)_2]^+$ [8] could be also traced to the same structural requirement in a lipophilic mono cationic complex for myocardial uptake and could be cited as an off-shoot of the above evolution, but which in fact took precedence to development of Tc-Q₁₂.

However, the first successful technetium based myocardial perfusion imaging in humans was accomplished still earlier by the Boston professor pair, Jones and Davison [9], based on the known inorganic chemistry principles of the ability of isonitrile ligands (neutral, monodentate) to stabilise lower valent metal ions in forming coordination complexes, $[\text{M}^{(\text{I})}(\text{CNR})_n]$ e.g. Tc(I)-t-butylisonitrile, $[\text{Tc}(\text{TBI})_6]^+$ or more generally $[\text{Tc}^{(\text{I})}(\text{CNR})_6]^+$. The ensuing SADR approach gave birth to Tc-sestamibi (Tc-MIBI, $[\text{Tc}(\text{MIBI})_6]^+$), a product now clinically well accepted [10]. Similarly investigations on technetium complexes of vicinal dioximes led to the realisation of the need for boronic acid group as a cap to provide stability to the complexes and to the development of the series denoted as Tc-BATO products (boronic acid adducts of technetium dioximes) [11]. Instead of the intended cationic species [12], the neutral complex of Tc-BATO obtained, nevertheless, led to two useful products, $[\text{Tc}^{(\text{III})}(\text{CDO})_3\text{X.BR}]$ (Tc-teboroxime) and $[\text{Tc}^{(\text{III})}(\text{DMG})_3\text{X.BR}']$, the former for myocardial imaging and the latter for brain imaging [11,13] (CDO=cyclohexanedionedioxime, DMG=dimethylglyoxime, X=Br/Cl, R=methyl & R'=methylpropyl).

The potential of amine-oxime ligands of H₄L type capable of tetradentate chelation of [Tc(^{99m}Tc)O]³⁻ core through (HL)³⁻ group was exploited by the UMC-Columbia professor pair, Troutner and Volkert, in the search for small, neutral, lipophilic complex of technetium capable of crossing the BBB [14,15], the earlier studies of Co and Ni complexes of propylene amine - oxime (PAO, PnAO) by Murmann et al. [16] of the same University, providing the basis. The ultimate successful development of Tc-exametazime in the hands of an industrial manufacturer [17] who bought the patent rights and carried out extensive studies revealed the difficulties in introducing the desired biological properties in a technetium compound. The functional group or structural feature needed to render satisfactory the tracer retention inside the brain was a major challenge.

2.2 Role of metabolite behaviour

The in-vivo fate of tracer studied in animal models and the understanding of nature of metabolite(s) have helped achieve desirable biological behaviour of new products in some cases; e.g. Tc-CPI, an ester-isonitrile ligand showing hydrolysis of ester in-vivo [1] (an undesirable feature in a myocardial tracer), providing the possible trigger for the inclusion of ester group in a DADT ligand leading to the development of Tc-bicisate (Tc-L,L-ECD) as brain perfusion agent (which needs a suitable functional group to undergo an intra-cellular interaction to a polar species) or explaining the rationale for extended experiments of Tc-ECD in over 9 species of animal models, till finally brain retention in primates could be demonstrated [18,19]. The classical case is of the renal tubular agent Tc-L,L-EC developed by Verbruggen et al. [20] as a direct outcome of the study of the metabolite of Tc-ECD, since over 75% of Tc-ECD clearance was through urinary excretion. The two metabolites of Tc-ECD are Tc-EC monoacid-mono ethylester and Tc-EC diacid; the latter has been independently developed into a useful renal tubular function tracer [20]. Since EC is the precursor in the synthesis of ECD, the advantage of a single synthesis process to obtain two desired ligands could thus be availed [1].

2.3 Use of biochemicals and drugs

The other main concept of attempts to link technetium to known biochemicals or drugs without vitiating the original pharmacological role, has not been much successful, in spite of sound approaches to retain the pharmacological action intact in the final product. Probably due to the relatively smaller molecular sizes and changes in stereo chemistry involved in most cases, the technetium complexation appears to severely alter the original biological behaviour, even when the structural perturbations have been kept to the minimum. A classical failure is the inability to retain the free fatty acid properties in any derivatised fatty acid substrate required for complexation with technetium, despite excellent chemistry efforts [21,22], which could have otherwise provided a sound rationale for myocardial imaging tracers (by metabolic trapping, though not for study of metabolism *per se*). A successful case has been a recent work from England on a technetium complex of an antibiotic, ciprofloxacin, the product being called Tc-infecton, for imaging infections [23]. Another instance of sound success appears to be the case of retention of the biochemical ability to mark hypoxic tissues with the marker functional group 2-nitroimidazole, after linking the group as a side arm to an amine-oxime ligand in order to enable formation of [TcO(PnAO)-1-(2-nitroimidazole)] [24]. This is a promising product for marking hypoxia (e.g. imaging ischemia), the distribution following initial blood flow and retention by only hypoxic metabolism; retention being similar to that of classical markers of glucose metabolism and hypoxia, ¹⁴C-2-deoxyglucose and ¹⁴C-misonidazole, respectively, as proven by autoradiography [24,25].

TABLE I : CONCEPTS IN THE DEVELOPMENT OF NEW GENERATION $^{99}\text{Tc}^{\text{m}}$ PRODUCTS

<u>Approach</u>	<u>Product(s) developed</u>
Use of known ligands & SADR	
Isonitriles and monocationic complex	Tc-MIBI, Tc-sestamibi
vic-dioximes & use of boronic cap, neutral complexes	Tc-BATO : Tc-CDO-MeB & Tc-DMG-2MP
Amine-oxime and neutral, lipophilic complex	Tc-HMPAO, Tc-exametazime
Alkyl dithiocarbamates and neutral, lipophilic complex	TcNNOET, Tc-nitrido complex
Use of designed ligands and SADR	
Derivatised 'en', Triamidemonosulphide and / Derivatised DTPA, dimethylester-DTPA and Anionic hydrophilic complex	Tc-MAG ₃ , Tc-MAGAG Tc-DMDTPA
Lipophilic monocationic complex containing alkoxy-phosphine group	Tc-tetrofosmin, Tc-furifosmin
Derivatised 'en', Diamine-dithiol containing ester group, Small, neutral, lipophilic complex	Tc-bicisate
Labelled biochemical / drug	Tc-infecton, Tc-HlgG
Substrate - BCA conjugate	Tc-PnAO-2-nitroimidazole

3.0 Technetium radiopharmaceutical chemistry aspects

The use of ^{99}Tc for investigations of chemistry of technetium radiopharmaceuticals and the active contributions of several research groups across the world have tremendously enriched the understanding of the nature and structure of a large number of technetium coordination complexes [26,27,28]. While the lack of prior knowledge of the exact structure of many first generation products did not prove to be a specific handicap, the entire development of subsequent products has been critically dependent on the understanding of the structural features of the new products [27,28]. It is in fact the knowledge that Tc(V) is the predominant form upon reduction of pertechnetate in the presence of suitable ligands, mostly resulting in $[\text{TcO}]^{3+}$ core complex with 5 or 6 as the coordination number, that led to development of many new products, as well as formulation procedures involving transchelation of a stronger coordinating group replacing a weaker ligand, and even with further reduction of oxidation state of technetium [29]. Tc(III) and Tc(I) are the other oxidation states exploited for complexation.

TABLE II : FEATURES OF NEW GENERATION TECHNETIUM COMPLEXES USED AS RADIOPHARMACEUTICALS

Tc compound / USAN name / Popular acronym	Technetium complex features		
	Oxidation state	Complex type	Core atoms
Tc(V)-DMSA	V	$[\text{TcOL}_2]^-$	TcOS_4
Tc-Isonitriles, Tc-Sestamibi	I	$[\text{TcL}_6]^+$	TcC_6
Tc-BATO : Tc-Teboroxime (Tc-CDO-MeB), Tc-DMG-2MP	III	$[(\text{TcL}_3\text{X})\text{BR}]^0$	TcN_6X
Tc-MAG ₃ (Tc-Mertiatide)	V	$[\text{TcOL}]^-$	TcON_3S
Tc-d,I-HMPAO (Tc-exametazime)	V	$[\text{TcO}(\text{LH})]^0$	TcON_4
Tc-L,L-ECD (Tc-bicisate)	V	$[\text{TcO}(\text{LH})]^0$	TcON_2S_2
Tc-MRP20	V	$[\text{TcOL}]^0$	TcON_3O
Tc-L,L-EC	V	$[\text{TcOL}]^-$	TcON_2S_2
Tc-Tetrofosmin (Tc-P53)	V	$[\text{TcO}_2\text{L}_2]^+$	TcO_2P_4
Tc-Furifosmin (Tc-Q ₁₂)	III	$[\text{TcLL}'_2]^+$	$\text{TcN}_2\text{O}_2\text{P}_2$
Tc-DACH	V	$[\text{TcO}_2\text{L}_2]^+$	TcO_2N_4
TcN - alkyl (alkoxy)- dithiocarbamate, Tc-NOET	V	$[\text{TcNL}_2]^0$	TcNS_4
TcN - alkyl (alkylester)- dithiocarbamate, Tc-NOET	V	$[\text{TcNL}_2]^0$	TcNS_4

Table-II gives details of the nature of complex in many of the important new generation compounds. It can be seen that there are two instances of proven favourable biological features noted with Tc dioxo core complexes, viz. $[\text{TcO}_2(\text{Tetrofosmin})_2]^+$ and $[\text{TcO}_2(\text{DACH})_2]^+$, while there are a number of clinically useful anionic and neutral complexes containing Tc monooxo core. The presence of alkoxy-phosphine group in the chelates seems to aid myocardial uptake. The role of HPLC to characterize the product in relation to ca vis-à-vis nca formulations, as well as to validate simple alternatives, by way of instant TLC (ITLC) and paper chromatography (PC) for day to day practice in any hospital radiopharmacy, has been well established. In the product development stage, it is required to establish that the radiochemically pure formulation (represented by single elution peak in HPLC) and the kit made formulation are comparable in pharmaco-kinetic studies in animal models. The possibility to investigate chemistry of technetium radiopharmaceuticals even in 'nca' level (10^{-7} - 10^{-8}M) using ^{99}Tc (to avoid any radiolysis effects with $^{99}\text{Tc}^m$) and liquid scintillation counting technique has been nicely demonstrated in the case of Tc-HMPAO [30].

The new generation products have also brought forth some other aspects like incompatibility with a large excess of reductant, e.g. Tc-exametazime, and presence of relatively less excess of ligand to Tc and reductant to Tc molar ratios in the kit / final formulation. The formation of some products at highly alkaline pH (e.g. Tc-EC) also renders formulation of easy to use lyophilised kits (the need of hospital radiopharmacy) quite difficult in many cases. The attendant effects on stability have, in turn, posed challenges in the regular utility of a number of new generation products. There have been successful attempts to segregate the reductant tin(II) from the ligand by resorting to a 2 components kit and taking advantage of transchelation process [29,31], rather than adapt extremely stringent preparatory conditions otherwise needed for reliable kit formulation [1,32]. The carrier effect of ^{99}Tc in generator produced $^{99}\text{Tc}^{\text{m}}$ assumes greater significance with the new generation products, wherein the content of reductant and/or ligand could be quite low, compared to early $^{99}\text{Tc}^{\text{m}}$ products. It can be shown that the technetium content in $^{99}\text{Tc}^{\text{m}}$ is approximately 0.8 ng/mCi of $^{99}\text{Tc}^{\text{m}}$ in typical daily eluates. At 20-50 mCi/ml radioactive concentration level, this would represent 0.16-0.4 μM solution only. Thus the need to use less excess of reductant adds to the already known complications of deleterious effects due to dissolved oxygen or trace ionic impurities in water. Stringent specifications for pertechnetate 'quality', additive free saline eluent, use of de-ionised water etc. are precautions recommended.

An alternate approach to form technetium complexes involving Tc(V) nitrido core, $[\text{Tc}\equiv\text{N}]^{2-}$, has presented interesting possibilities. Tc(V) nitrido group is iso-electronic with Tc(V) oxo group and could be beneficially used especially when the easy to reach Tc(V) oxo core might lead to multiple species formation. The preparation of nitrido compounds involves multi-step formulation and an intermediate species. At least one product each of the same type of complexes (Table-II) has shown promise for myocardial and brain perfusion imaging, $\{\text{TcN}[\text{Et}(\text{OEt})\text{NCS}_2]_2\}^0$ and $\{\text{TcN}[\text{R}(\text{CH}_2\text{COOMe})\text{NCS}_2]_2\}^0$, (R=Me/Et), respectively [33,34]; design feature of ether group in the former and of ester group in the latter are benefits from earlier findings. $[\text{TcN}(\text{EC})]^-$ has been recently reported [35] and compared with the previously well studied renal tubular tracer $[\text{TcO}(\text{EC})]^-$. Ligands containing P,N / P,S / P,N,S donor atoms have also been investigated extensively, but no clinical utility has been as yet demonstrated [36].

Application of advanced biochemical techniques has helped unravel the interaction of the technetium compounds at cellular level and invariably extensive in-vitro biological evaluation of the well characterized products has followed the initial demonstration of their efficacy. Elaborate studies to elicit mechanism(s) involving in-vitro models and isolated organ systems, interventional procedures etc. are quite common [1,19,20,23,25,27,37]. The new generation technetium products have thus undergone not only extensive chemical characterization, but also biochemical investigations. It is this multi-disciplinary evaluation of the biological behaviour that has helped acquire new information on radio-pharmacology, (e.g. Tc-sestamibi metabolism in liver, sequential hydrolysis of methoxy groups, accounting for liver clearance) as well as render feasible new applications in the case of some products (e.g. P glycoprotein involvement in uptake of Tc-sestamibi making tumour studies meaningful).

4.0 Myocardial tracers

Tc-sestamibi (Tc-MIBI, $[\text{Tc}(\text{MIBI})_6]^+$) is nearly 10 years old and though now clinically accepted [10], has neither replaced $^{201}\text{TlCl}$ nor arrested the search for new myocardial perfusion tracers. The need for new tracers arises not just from commercial considerations related to patent rights, but more so to be close to the ideal agent for the clinical purpose. Superior features consistent with intended clinical goals require to be available in any new substitute for acceptance.

TABLE III : COMPARISON OF $^{99}\text{Tc}^{\text{m}}$ TRACERS FOR MYOCARDIAL PERFUSION IMAGING @

Product Name				
i) USAN	Tc-Sestamibi	Tc-Teboroxime	Tc-Tetrofosmin	Tc-Furifosmin
ii) Other names	Tc-MIBI	Tc-CDO-MeB	Tc-P53	Tc-Q ₁₂
iii) Trade Name	Cardiolite / Miraluma	Cardiotec	Myoview	Technecard
Mol. Formula	$[\text{Tc}^{(0)}(\text{MIBI})_6]^+$	$\{[\text{Tc}^{(\text{III})}(\text{CDO})_3\text{Cl. BMe}]^{\circ}\}$	$\{\text{Tc}^{(\text{V})}\text{O}_2[\text{RP}_2(\text{ROR}')_4]_2\}^+$	$\{[\text{Tc}^{(\text{III})}]\{[\text{R}(\text{NR}'\text{O})_2][\text{P}(\text{R}''\text{OMe})_3]\}_2\}^+$
Molecular Weight	777	579 (Log P = 4.78)	895	961
Myocardial uptake (% i.d.)	2.5, 3 (Rest, Stress)	3.5 - 4	1.2 (Rest)	2.2 (Rest)
T _{1/2} (myocardium)	~ 6 h	4-5 min	~4 h	~ 6h
Preparation	Δ , 10 min.	Δ , 15 min	Room temp., 15 min	Δ , 15 min
Imaging Time	60 min	2 min	30-60 min	15-60 min
Heart / Liver (1h)	0.6 \pm 0.1	-----	1.2 \pm 0.8	1.4 \pm 0.5
Salient Features of myocardial kinetics & HB clearance rate	Good Myocardial Uptake & Retention. Fair	High Myocardial Uptake. Rapid Washout. Very High Liver Activity	Adequate Myocardial Uptake & Washout. Fast	Good Myocardial Uptake & Retention. Very Fast
1 Day Protocol for two imaging studies	++	+++++ (Multi-head SPECT)	++++	++

@ [7, 8, 10, 11, 27]

Table-III gives comparative data of the features of myocardial perfusion tracers. Tc-tetrofosmin would appear to have the edge presently, in view of its greater compatibility for same day protocol of rest and stress perfusion study (a distinct advantage in clinical setting), thanks to relatively lower uptake in myocardium (1-1.2%) and relatively faster wash out from myocardium [8] cf. Tc-sestamibi. In all cases hepatobiliary involvement is an attendant feature due to similarities in biochemical requirements of the tracers for these two organs; but both faster clearance from liver introduced by design in the new tracers and SPECT mode of acquiring images help reduce interference from liver uptake. Tc-Furifosmin is reported to be closer to Tc-Sestamibi in uptake and retention, but with much less liver interference [7].

There are fine examples of applications evolved thanks to better understanding of the radiopharmacology of the products. The new myocardial perfusion tracers render feasible practice of additional techniques to provide more information e.g. on cardiac pumping action, LVEF data reliably and comparable to conventional MUGA technique. In view of the higher activity and unaltered prolonged residence in myocardium, gated myocardial images can be acquired using these new tracers, from which one can deduce the LV volume and therefore calculate LVEF. Further, patients arriving in emergency cardiac care units could also benefit from the perfusion study with these tracers by simply being given a dose of the tracer soon after admission, as the images acquired much later, after the patient is well stabilised, would still represent the perfusion as at the time of tracer administration. In fact a new branch of emergency care nuclear cardiology is emerging. The non-cardiac applications with these tracers are noteworthy and consistent with the biochemical findings of P glycoprotein involvement in the in-vivo handling of Tc-sestamibi. A rather unique situation of the same kit product for Tc-Sestamibi being described as CardioliteTM for myocardial imaging and MiralumaTM for tumour imaging is notable. Tc-tetrofosmin has also shown utility for tumour studies, especially in breast cancer.

The use of Tc-Teboroxime is not attractive except for the highest myocardial extraction aspect [11,13], as the very rapid wash-out from myocardium requires expensive multi-head SPECT to acquire images in short time. A Tc-nitrido compound being developed by Pasqualini et al. [33] has shown promise for myocardial perfusion imaging, but is not yet in regular clinical use.

A simple new tracer for imaging myocardial infarcts, a technetium complex of 6 carbon dicarboxylic acid sugar, Tc-glucarate [37], is reported to be superior to Tc-PYP and Tc-GHA. The more efficacious Tc-antimyosin antibody is covered in another article in this Volume. The other important development, Tc-PnAO-2-nitroimidazole as a promising marker for hypoxia [24,25], e.g. myocardial ischemia, has been covered in Section 2.3.

5.0 Brain perfusion agents

Tc-d,l-HMPAO and Tc-L,L-ECD are now in regular use. Both exhibit about 5-6% brain uptake and prolonged retention, but the latter has been shown to have slightly more favourable pharmaco-kinetic features, in particular more rapid blood clearance and lesser muscle uptake [17-19]; in turn enabling early imaging feasibility, an advantage in handling some types of patients of neurological disorders. The lipophilic nature of the tracers drives the products through the hepatobiliary pathway. The relatively much higher renal excretion of Tc-ECD is thus an added advantage from the angle of radiation dosimetry to the patients [18,19].

Tc-exametazime is the first FDA approved brain perfusion agent. It is also the first in a number of other ways, such as recognition of importance of stereospecificity for biological efficacy [Tc-d,l-HMPAO, Tc complex of racemic form of HMPAO being useful, but not the meso

form, still later l-HMPAO has been shown as the active component], unclear mechanism of brain retention (despite a number of hypothesis, including a detailed theoretical calculation of a stability index called solid angle factor sum (SAS) based on the structure of complexes [38]), inherent instability of the compound, both in-vitro and in-vivo, very extensive investigations on radiopharmaceutical aspects even after approval for regular use, 'stringent' specifications for the pertechnetate to be used etc. A lot of information acquired as a result of much studies is summarised below. The Tc-exametazime is inherently unstable [30,39] and is converted into a less lipophilic species with time, even in the absence of high levels of radiation [30]. This decomposition is hastened by radiolysis [40], stannous tin [30] and higher pH [39]. Remedial measures [39] such as addition of gentisic acid soon after formulating Tc-exametazime, reducing the pH to 6.5-7, addition of KI to pertechnetate [41] and $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ [42] or methylene blue [43] to Tc-exametazime to prolong the shelf-life, refrigerated storage of Tc-exametazime [30,38,40] etc. have been suggested. The commercial kit product is unique in that it contains the lowest amount of stannous tin (7.6 μg) in any lyophilised kit. The salient finding that the highest stability of Tc-HMPAO is observed with the least excess of stannous tin [30,38], in turn, demanding a good match between the content of total technetium in the pertechnetate and the quantity of reductant to be used, has led to a simple recipe for hospital radiopharmacy involving the use of saturated stannous tartrate solution (10^{-4}M) [31].

Although many other amine-oxime and related ligands have been systematically evaluated, none of them have shown useful biological features except potential as BCA [44-46].

Tc-bicisate (Tc-L,L-ECD) is the more recently approved brain perfusion tracer and is both species specific and stereo specific. This product has been well adapted in many countries even earlier [1], thanks to more clearly understood mechanism of brain retention involving enzymatic hydrolysis and absence of any stability problems [1,18,19]. The complex has to be formulated at pH ~7.5 and the commercial kit product comprising two components has been refined over the years, in that the need to keep the tin-ligand component at $<0^\circ\text{C}$ has been later modified to room temperature storage. A single component lyophilised kit requiring to be prepared according to an extremely stringent protocol has also been proposed [1,32]. Difficulties in reliable kit formulation, attributable to the vulnerable -SH group in the ligand have been reported [1,31].

One of the technetium complexes of aminoalkyl diamine-dithiol (DADT, BAT) derivatives denoted as Tc-NEP-DADT [47] and a Tc-BATO member, Tc-DMG-2MP have also shown brain retention [11,13], but have no specific additional merits. The same is the case with Tc complex of a derivative of pyrrole - azomethine called Tc-MRP20 [48]. Technetium nitrido complex of alkylesterdithiocarbamate, $\{\text{TcN}[\text{R}(\text{COOR}')\text{NCS}_2]\}^0$, has been proposed for brain perfusion imaging, an off shoot of the attempts to use technetium complex of derivatives of alkyl dithiocarbamates [49] and using ester group in the ligand for brain retention based on intracellular hydrolysis [34].

6.0 Renal tubular function agents

A true replacement to hippuran- $^{99\text{Tc}}$ has been a challenging task in the development of technetium complexes, as no Tc compound is completely extracted and secreted into urine. It has, however, been possible to develop compounds which are handled by the renal tubules and actively secreted into urine. Structural feature requirements for recognition by renal tubules and for delivery by serum protein bound transport propounded way back by Despopoulos [50], $[-\text{C}(=\text{O})-\text{NH}-\text{CH}_2-\text{COOH}]$, have been sought in the technetium complexes to achieve some degree of success. For most clinical purposes, a renogram agent based on renal tubular handling would be

very much more useful, apart from being superior to purely GFR based agent such as $^{99}\text{Tc}^{\text{m}}$ - DTPA, and hence the intense research efforts. Tc-MAG3 complex contains the structure referred to above, while Tc-EC has a structural mimic, 3 oxygen atoms at 3-4 Å to one another in $\text{Tc}(=\text{O})\text{-NH-CH}_2\text{-COOH}$, cf. $\text{-C}(=\text{O})\text{-NH-CH}_2\text{-COOH}$. Both Tc-MAG3 and Tc-EC show less excretion than hippuran, but Tc-EC has relatively superior features [1,20,31]. The room temperature formulation recipe of Tc-EC is another practical advantage.

Tc-MAG3, the first successful technetium based renal tubular agent, also belongs to the category of products continued to be much investigated even after approval for regular use. The early apprehensions of differences in the purity of kit formulated and chromatographically purified product [51] were removed with refinements in kit formulation procedures. However, due to inherent nature of possible trace impurities in MAG3 synthesis as well as different types of Tc - MAG3 complexes feasible [1,52], interference from hepatobiliary involvement during renography studies has not been ruled out. One study is in fact devoted to the anomalies in Tc-MAG3 behaviour [53]. Modifications to MAG3 ligands to overcome the drawbacks have been sought, replacing glycine by another amino acid, introduction of chiral centre to influence the stereochemical role etc.; some superior results have been achieved, e.g. $^{99}\text{Tc}^{\text{m}}$ -D-MAGAG [54].

Tc-L,L-EC requires to be prepared at highly alkaline pH of 11-12 and it is consequently difficult to present in a reliable single component lyophilised kit form, though a commercial kit has been cited in literature. Detailed stringent protocol for kit formulation has been suggested [1,31,32]. A multi-component kit recipe would be generally necessary [1,31], but advantage of ease of transchelation (using GHA) based kit has also been reported [31]. As discussed earlier, the development of Tc-EC for renal tubular function, is an outcome based on the excretory pattern of Tc-ECD and the study of its metabolite(s).

The attempts to utilise cysteine, cystine and analogues for complexing technetium for obtaining renal agents had shown mixed findings [55], but the same group from India has recently demonstrated a new product for renal tubular function imaging. $^{99}\text{Tc}^{\text{m}}$ complex of dimethyl ester of DTPA denoted as Tc-DMDTPA, has shown promising results including in human volunteers [56,57]. Analogous to Tc-DTPA, the Tc-DMDTPA complex is anionic, but has predictably less (~50%) electrophoretic mobility. Ease of reliable, stable, single component lyophilised kit formulation, room temperature preparation of Tc-DMDTPA in high yield, purity and stability and similarity in biological behaviour to hippuran- ^{131}I and Tc-MAG3 in both normal and probenecid (renal tubular transport inhibitor) treated mice are the salient advantages reported [31,57].

A cationic pathway renal tubular agent has also been reported from UK involving the complex of 1,2-diaminocyclohexane (DACH) [58]. The product, $[\text{Tc}^{(\text{V})}\text{O}_2(\text{DACH})_2]^+$, is formulated using stannous tartrate reduction of pertechnetate and has shown utility for eliciting renal tubular function, when anionic pathway is not freely accessible due to high concentrations of circulating anions, e.g. during chemotherapy.

7.0 Products for imaging infection / inflammation

The ability to image infection either directly or by marking the inflammatory response of the body to infection is a very useful procedure for patient management, especially when associated with high specificity. Technetium complex of the antibiotic, ciprofloxacin called Tc-infecton, is the main recent development for imaging infection [23]. The use of this product appears to be, however, confined to UK. Amongst the technetium complexes useful for labelling WBC (leucocytes) in order to target Tc-leucocytes to sites of infection, Tc-HMPAO has been the

most well established [59]. In fact, much of the effort for stabilisation of Tc-HMPAO has stemmed from this need [42,43]. Tc-ECD, being tissue specific, has not been mentioned for this purpose, except in a recent report [60]. Tc-citrate, Tc-glutathione etc. have also been shown to concentrate in inflammatory lesions and in some tumours.

$^{99}\text{Tc}^{\text{m}}$ - human immunoglobulin G (HlgG, HIG) is a serendipitous discovery for imaging inflammation. The labelling of HIG with $^{99}\text{Tc}^{\text{m}}$ is achieved directly by mild reduction of disulphides to liberate free sulphydryl groups or through a BCA, hydrazino nicotinamide (HYNIC) [61]. Isonicotinyl hydrazine with an activated ester reacts with an amino group of the protein/ peptide and then with pertechnetate to give a Tc complex uncharacterised as yet. In the attempts to harness the efficacy of chemotactic peptides as substrates for imaging infection (covered elsewhere in this Volume), as they act by binding to receptors on the WBC accumulated at the site of infection, HYNIC is the preferred bifunctional route for labelling with $^{99}\text{Tc}^{\text{m}}$.

8.0 Tumour imaging agents

Small technetium complexes capable of specifically imaging tumours have eluded the researchers except in a few cases like Tc(V)-DMSA, particularly useful for medullary thyroid cancer [62,63] and in recent times Tc-citrate to a limited extent [64]. The development of Tc(V)-DMSA as a clinically useful product, though perhaps a case of chance success, has been followed by chemical characterisation studies and the structure has been proven to be $[\text{Tc}^{\text{(V)}}\text{O}(\text{DMSA})_2]^-$ [65]. Considerable work on the radiochemical and biological properties of this product, especially in comparison with the more widely used renal cortical imaging agent $^{99}\text{Tc}^{\text{m}}(\text{III})$ -DMSA (Tc-succimer), has been reported [12].

The utility of Tc-Sestamibi and Tc-Tetrofosmin for imaging tumours, especially metastasis of breast and thyroid cancer, and of Tc-HMPAO for some brain tumours can be next cited [66-68]. It is reported that the ability to elicit metastases in thyroid cancer patients using Tc-isonitriles (Tc-TBI, Tc-MIBI) without having to take the patients off eltroxine would provide a convenient screening test prior to the use of large dose ^{131}I for scanning to locate metastases, which requires to take the patients off eltroxine [68]. Similarly, the use of the well-known renal agent Tc-glucaptate as a ^{18}F -FDG mimic for brain tumours like glioma has been shown to be beneficial [69]. Reports of other technetium complexes for tumour imaging also abound in literature, e.g. Tc-citrate [64], Tc-glutathione [70].

The major thrust approach for efficacious tumour imaging is based on monoclonal antibodies and small synthetic peptides (covered in another article in this Volume). An exciting new advance is the investigations on the use of molecular biology based substrates, such as oligo nucleotides and later more suitable peptide nucleic acids (PNA), for labelling with $^{99}\text{Tc}^{\text{m}}$ for possible tumour imaging [71,72]. The possibility of intracellular localisation of such radiolabelled substrates through hybridization technique after making use of a protein unique to the target cell holds promise, but many hurdles have to be overcome prior to realising clinical applicability.

9.0 Receptor based products

$^{99}\text{Tc}^{\text{m}}$ based receptor radiopharmaceuticals are not yet a clinical reality. The only successful case is that of $^{99}\text{Tc}^{\text{m}}$ - neogalactosyl glycoalbumin (NGA) [73] for binding hepatocyte binding protein (HBP) receptor, a plasma membrane bound hepatocyte specific receptor that binds galactose terminated glyco proteins. Tc-NGA would be useful for staging certain liver diseases (since HBP is implicated in cirrhosis, hepatoma and liver metastases) and for monitoring response to therapy.

The concept of BCA to attach receptor specific molecules with $^{99}\text{Tc}^{\text{m}}$ has been extensively investigated [74], but with limited success. Arduous chemical studies followed by receptor binding experiments have revealed poor specificity in most cases, e.g. $[\text{Tc}^{(\text{V})}\text{O}(\text{DADS})\text{-Progesterin}]$, $[\text{Tc}^{(\text{III})}(\text{CO})(\text{diethylthiocarbamate-Spiperone})_3]$, $[\text{Tc}^{(\text{III})}\text{-BATO-QNB}]$ & $[\text{Tc}^{(\text{V})}\text{O}(\text{DADS})\text{-QNB}]$. It appears that in all cases the complexation with technetium severely alters the bio-activity and precludes receptor binding. Attempts to overcome steric effects by increasing the distance between the essential functional groups have not been much successful. The important aspects to be reckoned with are molecular weight & size, lipophilicity changes, stereochemical effects and non-specific binding [74,75]; two approaches called tridentate-monodentate (3+1) scheme (the former for facile chelation with Tc and the latter for presenting the receptor avid moiety) and pendant scheme have been pursued.

The novel concept of molecular mimics, i.e. Tc-chelate simulating a regular ring structure in a native receptor binding molecule [75], especially in a steroid (e.g. progesterone) / drug (e.g. morphine), is being pursued to target receptor sites for imaging using $^{99}\text{Tc}^{\text{m}}$. Radiolabelled peptides have been recognised as the most likely successful candidates for imaging receptors (covered in another article in this Volume), based on the promising experience with ^{111}In labelled octreotide. The earlier stated problems in disguising and presenting technetium to the receptors persist, but scope for optimism is seen in this approach. In view of the importance of receptor imaging capability in health and disease, research efforts are continuing in more than one way.

10.0 Epilogue

As the search continues for new products and newer areas of applications, ^{123}I compounds would provide the vital bridge between truly biological PET tracers based on ^{11}C / ^{18}F labelled compounds and the much more easily accessible SPECT tracers based on $^{99}\text{Tc}^{\text{m}}$ and ^{111}In products, thereby rendering a transition from PET to SPECT, that is from medical research to clinical utility, a reality. ^{111}In products would provide a fine complement to $^{99}\text{Tc}^{\text{m}}$ compounds, especially for imaging process(es) involving slower kinetics of tracer and in the cases where conjugation of In-BCA with the bio-active substrate causes less alterations of the biological activity. Though the question "After $^{99}\text{Tc}^{\text{m}}$, what next?" is posed time and again, the well-known attractive advantages of $^{99}\text{Tc}^{\text{m}}$ are not likely to be matched by any other tracer in the foreseeable future and consequently the impetus to develop $^{99}\text{Tc}^{\text{m}}$ based radiopharmaceuticals will continue. It could be safely predicted that the future of radiopharmaceuticals and in turn, clinical nuclear medicine, will continue to be dominated by $^{99}\text{Tc}^{\text{m}}$ products, as has been the case in the past.

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STUDY ON THE FORMATION OF MIXED LIGAND OXORHENIUM AND OXOTECHNETIUM COMPLEXES (SNS/S COMBINATION)

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Abstract

When a mixture of the tridentate aminedithiol ligand $\text{EtN}(\text{CH}_2\text{CH}_2\text{SH})_2$, L_1H_2 , and a monodentate thiol $p\text{-ClC}_6\text{H}_4\text{SH}$, L_2H , is added to a suitable Re^{VO} or $^{99\text{m}}\text{Tc}^{\text{VO}}$ precursor, the following complexes are theoretically possible : (i) neutral mixed ligand complexes MOL_1L_2 (*syn*, **1** and **1'** for rhenium and technetium respectively, and *anti*, **2** and **2'**) (ii) binuclear complex of the tridentate ligand, $(\text{MO})_2(\text{L}_1)_3$, **3** and **3'**, (iii) anionic complex of the monothiol $[\text{MO}(\text{L}_2)_4]^-$, **4** and **4'**. All of the above types of complexes have been synthesized by exchange reaction using $\text{ReOCl}_3(\text{PPh}_3)_2$ as precursor and selectively monitoring reaction stoichiometry. The crystal structures of **1**, **3**, and **4** are determined by X-ray crystallography. All the above types of complexes have been prepared at technetium-99m level by exchange reaction using Tc-99m glucoheptonate as precursor and the appropriate ligand(s), as shown by high performance liquid chromatographic (HPLC) analysis. The major reaction product is the *syn* isomer, while none of other

complexes forms during the reaction, when a L_1H_2/L_2H 1/1 molar ratio is retained. This fact satisfies a very important prerequisite for the further evaluation of the above mixed ligand system, SNS/S, in biological systems at tracer level, Tc-99m.

1. Introduction

The widespread use of compounds of the γ -emitting radionuclide Tc-99m in nuclear medicine for the scintigraphic imaging of internal organs, as well as the recent introduction of the β -emitting radionuclides Re-188 and Re-186 in radiotherapy have led to a rapid development of their chemistries, in order to produce novel useful radiopharmaceuticals. Concerning the particular class of brain perfusion agents many efforts have been focused on the synthesis of neutral, small size, lipophilic complexes containing the Tc=O core with two linear tetradentate ligand systems: (a) propylendiamine dioxime (PnAO), N_4 , backbone, and (b) the diaminedithiol (DADT), N_2S_2 system [1,2].

An alternative concept for the synthesis of neutral oxotechnetium and oxorhenium complexes is based on the 3+1 mixed ligand approach involving the SNS+S donor atom set [3]. According to this concept, the simultaneous action of a tridentate ligand (SNS) and a monodentate thiol (S) as coligand on a suitable $M=O^{3+}$ ($M=Tc, Re$) precursor leads to the formation of neutral lipophilic metal species. The major advantage of the mixed ligand system lies in its flexibility. Thus, a great variety of tridentate/monodentate ligand combinations is possible by use of this system, thereby facilitating the production of homologous series. So far, interesting radioagents have been developed from this system and evaluated either as brain perfusion agents, or

even as specific radiopharmaceuticals for the mapping of brain receptors [4,5].

Our goal in the present study is to elucidate the chemical structure of the mixed ligand complex of the general formula ReOL_1L_2 , formed by reacting equimolar amounts of L_1H_2 [= $\text{EtN}(\text{CH}_2\text{CH}_2\text{SH})_2$] and L_2H [= $p\text{-ClC}_6\text{H}_4\text{SH}$] with a ReO^{3+} precursor. The conditions of formation of some potential side products and their chemical characterization have also been investigated. Eventually, the conditions under which each one of the analogous technetium-99m complexes forms at tracer level are studied, since they are candidates for a future application in biological systems. The latter study is possible by chromatographic comparison of the technetium-99m with their well characterized rhenium analogs, given that rhenium is a surrogate for technetium.

2. Experimental section

2.1 Materials and methods

IR spectra were recorded on a Perkin-Elmer 1600 FT-IR spectrophotometer, while elemental analyses were performed on a Perkin-Elmer 2400/II analyzer. High performance liquid chromatography (HPLC) analysis was performed on a Waters chromatograph equipped with a 600E delivery system and a μ -Bondapak C-18 column using 85 % methanol as the mobile phase at a 1.0 mL/min flow rate.

The tridentate ligand, 2,2'-dimercaptotriethylamine, was synthesized as described previously [6]. $\text{Na}^{99\text{m}}\text{TcO}_4$ was obtained from a commercial $^{99}\text{Mo}/^{99\text{m}}\text{Tc}$ generator. Commercial glucoheptonate kits containing a lyophilized mixture of 0.2 mg of SnCl_2 and 200 mg of calcium glucoheptonate (Gluco/Demoscan, NCSR, "Demokritos") were used.

2.2 Synthesis of $\text{ReO}[\text{CH}_3\text{CH}_2\text{N}(\text{CH}_2\text{CH}_2\text{S})_2][p\text{-SC}_6\text{H}_4\text{Cl}]$, **1** and **2**.

To a stirred suspension of trichlorobis(triphenylphosphine)rhenium(V) oxide (166 mg, 0.2 mmol) in methanol (10 mL), 1 N CH_3COONa in methanol (2 mL, 2 mmol) was added. A mixture of 2,2'-dimercaptotriethylamine, (L_1H_2), (33 mg, 0.2 mmol) and 29 mg (0.2 mmol) of 4-chlorothiophenol (L_2H) was added under stirring. The solution was refluxed until the green-yellow color of the precursor turned to dark-green. After cooling, the reaction mixture was diluted with CH_2Cl_2 (30 mL) and then washed with water. The organic layer was reduced to 5 mL and then 5 mL of methanol were added. Analysis of the solution by HPLC demonstrated the formation of two complexes in a ratio of 25/1. Slow evaporation of the solvents at room temperature afforded the major product of the reaction as a green solid (**1**, *syn* isomer, 50% yield). Crystals suitable for X-ray crystallography were obtained by recrystallization from $\text{MeOH}/\text{CH}_2\text{Cl}_2$. The minor complex was separated as an orange solid from the filtrate on standing at -20°C for several days (**2**, *anti*, 1% yield).

Complex **1** (*syn*): R_f 0.8 (silica gel, $\text{CH}_3\text{OH}/\text{CH}_2\text{Cl}_2/\text{Et}_2\text{NH}$, 8/1.5/0.5), FTIR (cm^{-1} , KBr pellet): 940 ($\text{Re}=\text{O}$), Anal. % Calcd for $\text{C}_{12}\text{H}_{17}\text{ClINOS}_3\text{Re}$: C, 28.31, H, 3.37, N, 2.75, S, 18.19. % Found: C, 28.35, H, 3.45; N, 2.67; S, 18.53.

Complex **2** (*syn*): R_f 0.85 (silica gel, $\text{CH}_3\text{OH}/\text{CH}_2\text{Cl}_2/\text{Et}_2\text{NH}$, 8/1.5/0.5), FTIR (cm^{-1} , KBr pellet): 963 ($\text{Re}=\text{O}$).

2.3 Synthesis of $[\text{ReO}]_2[\text{CH}_3\text{CH}_2\text{N}(\text{CH}_2\text{CH}_2\text{S})_2]_3$, **3**.

To a stirred suspension of trichlorobis(triphenylphosphine)rhenium(V) oxide (166 mg, 0.2 mmol) in methanol (10 mL), 1 N CH_3COONa in methanol (2 mL, 2 mmol) was added. Then, a solution of 2,2'-dimercaptotriethylamine

(L₁H₂), (33 mg, 0.2 mmol) in methanol (2 mL) was added. The same procedure as above was followed and HPLC analysis demonstrated the formation of one complex. Slow evaporation of the solvents at room temperature afforded the product as green crystals. Yield : 60%.

Complex **3** : R_f:0.7 (silica gel, CH₃OH/CH₂Cl₂/Et₂NH, 8/1.5/0.5), FTIR (cm⁻¹, KBr pellet): 944 (Re=O), Anal. % Calcd for C₁₈H₃₉N₃O₂S₃Re : C, 24.18; H, 4.40; N, 4.32; S, 21.51. % Found: C, 23.80; H, 4.75; N, 4.32; S, 21.11. Crystals suitable for X-ray crystallography were obtained by recrystallization from MeCN/MeOH/CH₂Cl₂.

2.4. Synthesis of [ReO(SC₆H₄Cl)₄][PH(C₆H₅)₃], **4**.

To a stirred suspension of trichlorobis(triphenylphosphine)rhenium(V) oxide (166 mg, 0.2 mmol) in methanol (10 mL), 1 N CH₃COONa in methanol (2 mL, 2 mmol) was added. Then, a solution *p*-HS-C₆H₄Cl, (L₁H₂), (29 mg, 0.2 mmol) in methanol (2 mL) was added. The same procedure as above was followed and HPLC analysis demonstrated the formation of mainly one complex. Slow evaporation of the solvents at room temperature afforded the product as green crystals. Yield : 45%.

Complex **4** : R_f:0.9 (silica gel, CH₃OH/CH₂Cl₂/Et₂NH, 8/1.5/0.5), FTIR (cm⁻¹, KBr pellet): 963 (Re=O), Anal. % Calcd for C₄₂H₃₁Cl₄OPS₄Re : C, 48.51; H, 3.10; S, 12.51. % Found: C, 48.29; H, 3.35; S, 12.71. Crystals suitable for X-ray crystallography were obtained by recrystallization from EtOH/CH₂Cl₂.

2.5 Synthesis at ^{99m}Tc level. General method.

A Gluco/Demoscan kit was reconstituted with 10 mL water, and then a 1.0 mL aliquot was mixed with 0.5-1.0 mL of ^{99m}Tc-pertechnetate solution (5-10 mCi). The ^{99m}Tc(V)O-glucuheptonate solution was added to a centrifuge tube

containing i) equimolar quantities (0.02 mmol) of the tridentate ligand, L_1H_2 , and the monodentate ligand, L_2H , complex **1** and **2**, ii) 0.02 mmol of the tridentate ligand, L_1H_2 , complex **3**, and iii) 0.02 mmol of the monodentate ligand, L_2H , complex **4**. The mixture was agitated in a vortex mixer and left to react at room temperature for 10 min. The complexes were extracted with CH_2Cl_2 (3 x 1.5 mL) and the combined organic extracts were dried over $MgSO_4$ and filtered. The extractions were nearly quantitative for all three preparations.

The identity of the ^{99m}Tc complexes was established by HPLC comparison with samples of the corresponding Re complexes, well characterized by classical methods.

2.6 X-ray crystallography

Diffraction measurements for **1** and **4** were made on a Crystal Logic Dual Goniometer diffractometer using graphite monochromated Mo K α radiation, but data collection for complex **3** was performed on a P2₁ Nicolet diffractometer with Ni-filtered Cu K α radiation. Unit cell dimensions were determined by using the angular settings of 25 automatically centered reflections in the range $11 < 2\theta < 23^\circ$ (for **1** and **4**) and $24 < 2\theta < 54^\circ$ (for **3**). Intensity data were recorded using a θ - 2θ scan. ($2\theta_{max}$ = 50.7° , 130° and 54° for **1**, **3** and **4** respectively). Three standard reflections monitored every 97 reflections showed less than 3% variation and no decay. Lorentz, polarization and psi-scan absorption corrections were applied using Crystal Logic software. For **1**: reflections collected/unique/used 2989/2755 [Rint=0.0125]/2755. For **3**: reflections collected/unique/used 6081/5713 [Rint=0.0179]/5712. For **4**: reflections collected/unique/used 9826/9379

[Rint=0.0125]/9363. The structures were solved with direct methods using SHELXS-86 [7] and refined by full-matrix least-squares techniques on F^2 with SHELXL-93 [8]. All non-hydrogen atoms were refined anisotropically.

3. Results and Discussion

When a mixture of the tridentate aminedithiol ligand $\text{EtN}(\text{CH}_2\text{CH}_2\text{SH})_2$, L_1H_2 , and a monodentate thiol $p\text{-ClC}_6\text{H}_4\text{SH}$, L_2H , is added to a suitable Re^{VO} or $^{99\text{m}}\text{Tc}^{\text{VO}}$ precursor, the following complexes are theoretically possible (Fig. 1):

- i. neutral mixed ligand complexes $\text{MO}(\text{L}_1)_2$ (*syn*, **1** and **1'** for rhenium and technetium respectively and *anti*, **2** and **2'**). Formation of two isomers, *syn* and *anti*, during coordination of L_1H_2 and a monodentate thiol L_2H , is expected as a result of the different possible alternative orientations of the N-ethyl substituent of the tridentate ligand in respect to the MO^{3+} core. However, the *anti* isomer is not always formed [3c,d, 4].
- ii. binuclear complex of the tridentate ligand, $(\text{MO})_2(\text{L}_1)_3$, **3** and **3'**.
- iii. anionic complex of the monothiol $[\text{MO}(\text{L}_2)_4]^-$, **4** and **4'**.

All of the above complexes have been synthesized at carrier level by exchange reaction using $\text{ReOCl}_3(\text{PPh}_3)_2$ as precursor and selectively monitoring reaction stoichiometry. The major product of the reaction is the *syn* isomer, when a $\text{L}_1\text{H}_2/\text{L}_2\text{H}$ 1/1 molar ratio is applied, as demonstrated by crystal structure analysis. Only in some preparations HPLC analysis of the crude reaction mixture revealed the presence of a small amount of the *anti* isomer. HPLC analysis of the crude reaction mixture also revealed, that neither of the complexes **3** and **4** form during the reaction under the above conditions.

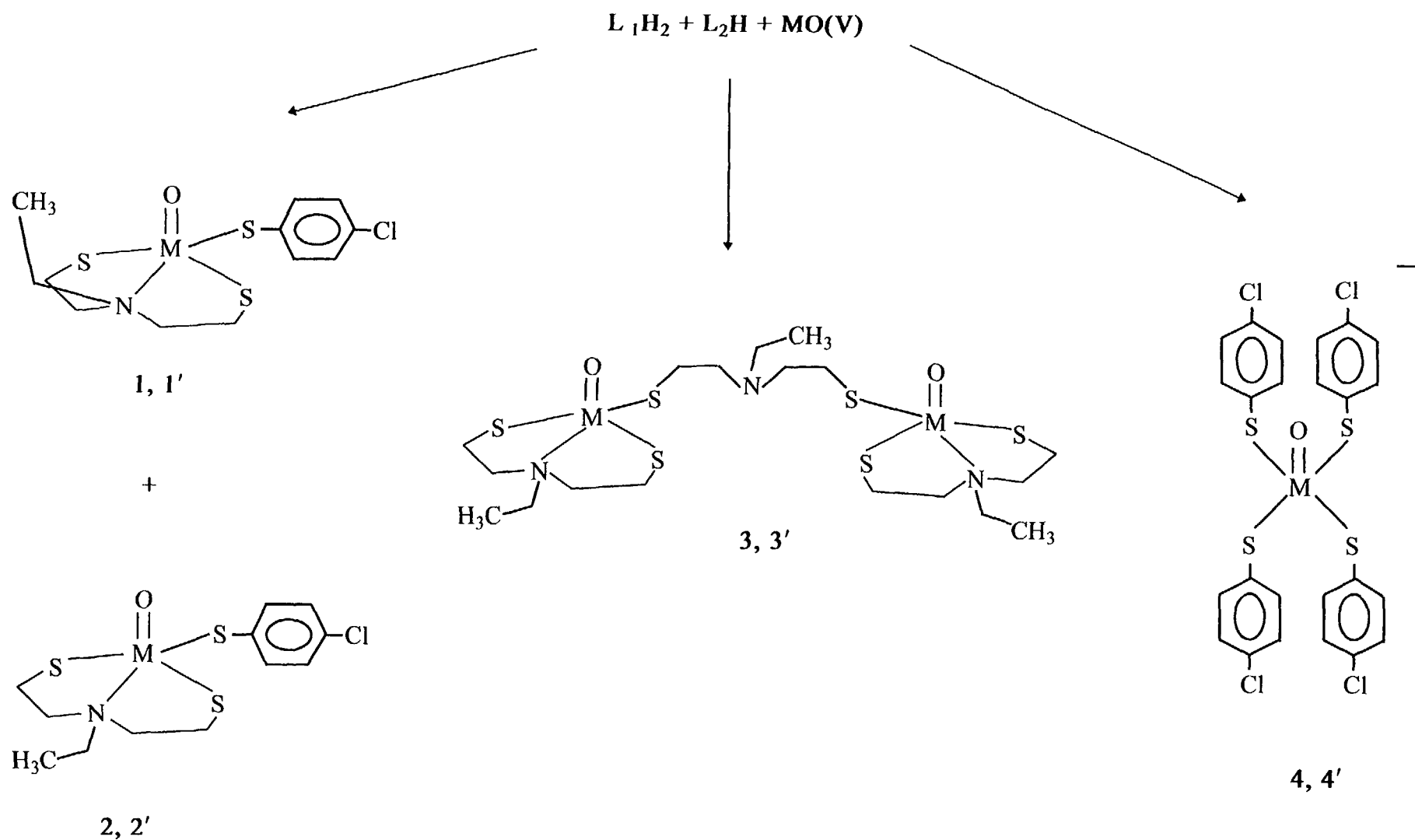


Fig.1. Possible complexes by joint action of $L_1H_2 + L_1H$ on $ReOCl_3(PPh_3)_2$ or Tc-99m-glucoheptonate. 1, 2, 3, 4 for $M = Re$ and 1, 2, 3, 4 for $M = Tc$.

All the above complexes have been prepared at tracer (technetium-99m) level by exchange reaction using Tc-99m glucoheptonate as precursor. Similarly the major reaction product is the *syn* isomer, complex **1'**, while neither of complexes **3'** and **4'** form during the reaction at tracer level, when a L_1H_2/L_2H 1/1 stoichiometry is maintained.

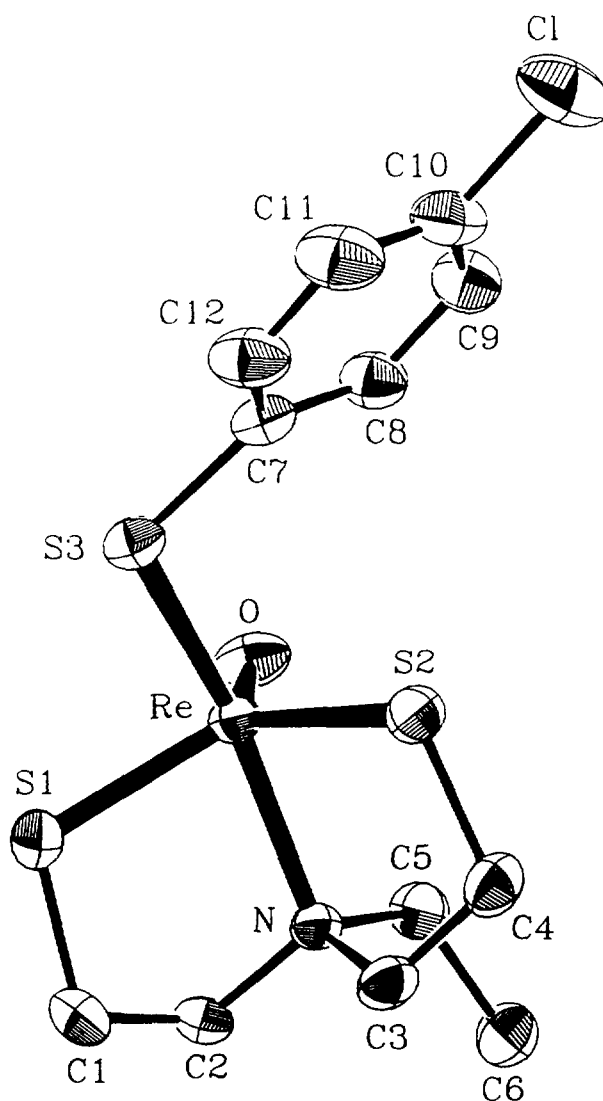


Fig. 2. ORTEP diagram of $\text{ReO}[\text{CH}_3\text{CH}_2\text{N}(\text{CH}_2\text{CH}_2\text{S})_2][p\text{-SC}_6\text{H}_4\text{Cl}]$, complex **1**

3.1 Description of the structures

An ORTEP diagram of complex 1 is shown in Fig. 2. As has already been mentioned, the formation of two isomers, *syn* and *anti*, is possible. Complex 1 is the *syn* isomer with the N-ethyl substituent and the oxo group pointing in the same direction. The coordination geometry around rhenium(V) is distorted trigonal bipyramidal. The basal plane of the trigonal bipyramid is formed by the atoms S(1) and S(2) of the tridentate ligand and the oxo group, while the

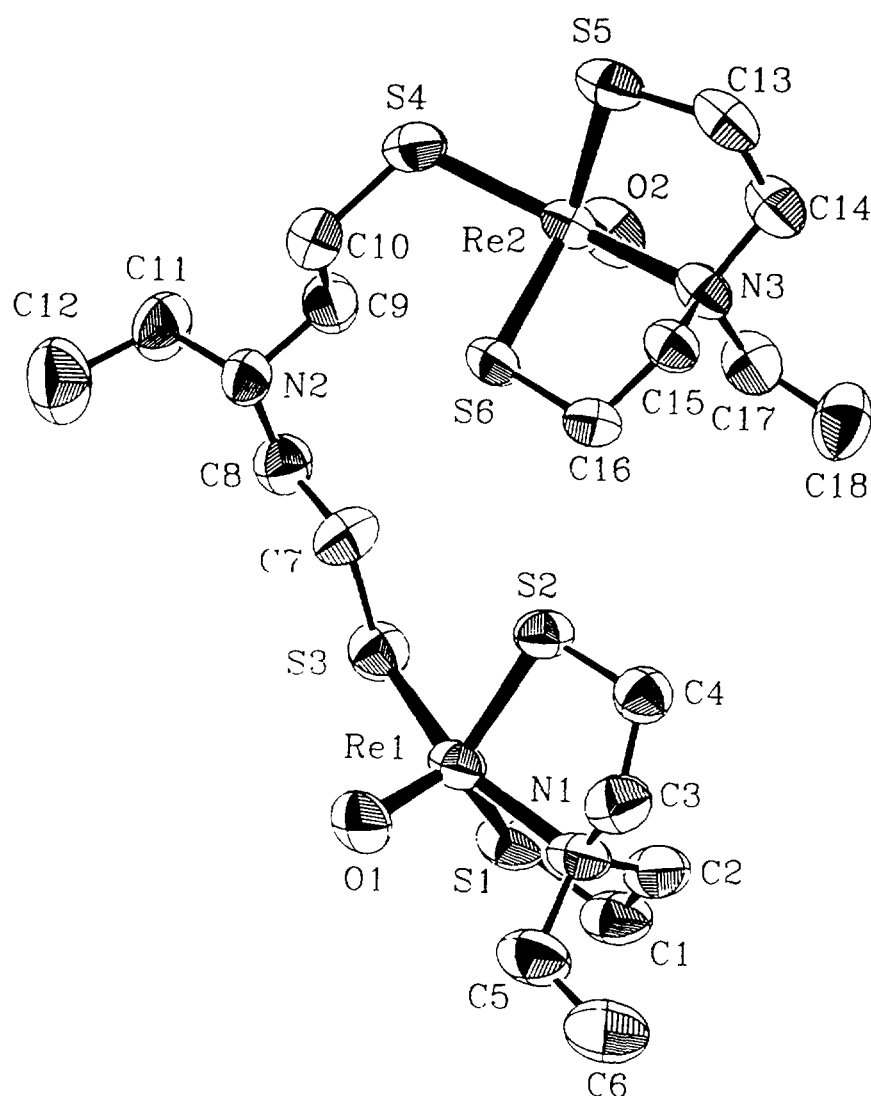


Fig. 3. ORTEP diagram of $[\text{ReO}]_2[\text{CH}_3\text{CH}_2\text{N}(\text{CH}_2\text{CH}_2\text{S})_2]_3$, complex 3.

apical positions are occupied by the nitrogen atom of the ligand (N(1)) and the sulfur of the monodentate thiol (S(3)). The angles between the atoms of the basal plane range from $116.9(2)^\circ$ to $121.5(1)^\circ$, values close to the ideal 120° , but the distortion of the trigonal bipyramid arises from the N-Re-S(3) angle of $160.3(1)^\circ$. The calculated trigonality index τ ($\tau = (\phi_1 - \phi_2)/2$, ϕ_1 , ϕ_2 the largest angles in the coordination sphere, $\tau = 0$ for perfect *sp*, $\tau = 1$ for perfect *trigonal bipyramidal*.) is 0.65 showing a degree of distortion [9]. Rhenium lies 0.10 Å out of the basal plane of the trigonal bipyramid towards the monodentate thiol.

The crystal structure of the novel binuclear complex **3** is given in Fig. 3. The tridentate ligand is coordinated to the metal in two different ways; as the usual chelating tridentate agent through the SNS donor set and as a bridging bidentate one through the sulfur atoms, while the nitrogen atom remains noncoordinated. We have so far widely used this type of tridentate ligands, but it is the first time, that this bridging bidentate arrangement is observed. The coordination geometry around each rhenium atom is distorted trigonal bipyramidal. The basal plane is defined by the sulfur atoms of the tridentate chelating ligand and the oxo group, while the apical positions are occupied by the nitrogen of the chelating ligand and the sulfur atom of the bridging ligand, instead of a monothiol. The calculated trigonality index τ is 0.64 and 0.63 for Re(1) and Re(2) respectively. Each rhenium atom lies 0.10 Å out of the basal plane towards the sulfur atom of the bridging bidentate ligand. The ethyl substituent on the nitrogen atom of the tridentate ligand is in *cis* position with respect to the oxo group resulting in *syn* configuration around each rhenium, but the Re=O groups are *trans* to each other.

The molecular structure of the anionic complex **4** is given in Figure 4. The coordination of rhenium is distorted square pyramidal with four sulfur

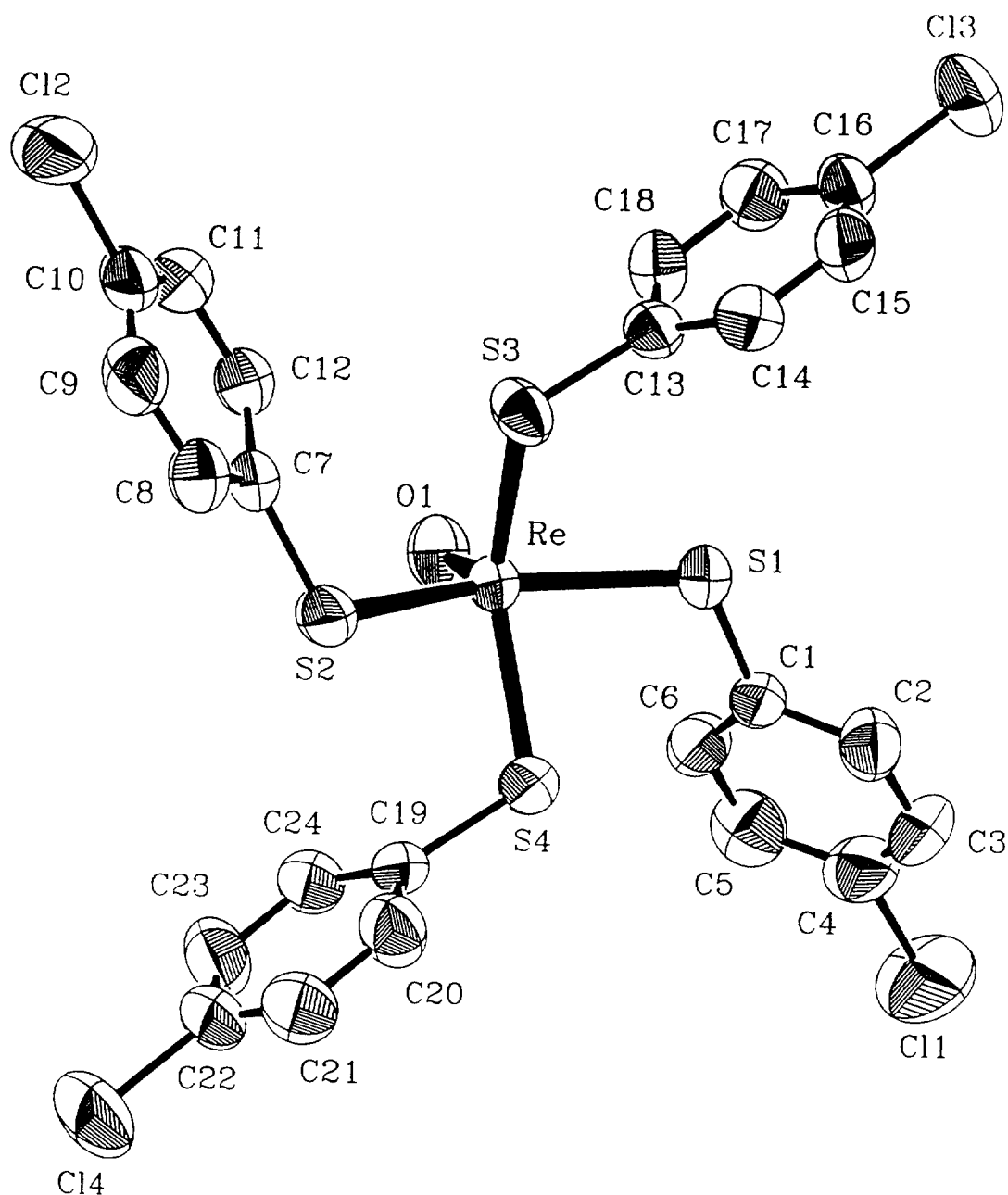


Fig. 4. ORTEP diagram of $[\text{ReO}(\text{SC}_6\text{H}_4\text{Cl})_4][\text{PH}(\text{C}_6\text{H}_5)_3]$, complex 4.

atoms in the basal plane and the oxo group at the apex. Rhenium lies 0.74 \AA out of the mean plane of the sulfur atoms towards the apical oxygen. The angle formed by the intersection of the S1S2S3 and S2S3S4 planes is 10.6° and its value was used previously as the measure of the distortion of the square pyramid. Blower and coworkers [10] reported that in complex $[\text{ReO}(\text{SPh})_4]^-$ this angle takes the value of 3.8° and in complexes

$[\text{ReO}(\text{TMT})_4]^-$ and $[\text{ReO}(\text{DIPT})_4]^-$ containing more substituted thiophenols as ligands, the calculated values are 13.6° and 5.4° respectively. They also conclude, that the distortion of the square pyramid may be due to intermolecular and crystal packing effects. McDonell and coworkers [11] reported, that the anion $[\text{ReO}(\text{SPh})_4]^-$ is distorted significantly from C_4 symmetry ("turbine" conformation) by measuring the dihedral angles O-Re-S-C (67 - 108°) and the angles between the plane of the four sulfur atoms of the square pyramid and those of the phenyl rings of the thiophenol ligands (82 - 114°). In our case, variation in the O-Re-S-C dihedral angles is small (71.3° to 79.5°), but the range in the angles of the S1S2S3S4 mean plane with the mean planes of the phenyls is large (53.8° - 87.7°) leading to a significant distortion from C_4 symmetry. Finally, the trigonality index τ is 0.26.

The above studies demonstrate, that simultaneous action of a tridentate SNS ligand and a monodentate thiol in equimolar quantities on $\text{Re}^{\text{V}}\text{OCl}_3(\text{PPh}_3)_2$ or Tc-99m glucoheptonate leads to a single rhenium or technetium-99m species, the *syn* MOL_1L_2 . This fact validates the above mixed ligand system, SNS/S, for further study in biological systems at Tc-99m tracer level.

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**SYNTHESIS AND CHARACTERIZATION OF LIGANDS AND
BIFUNCTIONAL CHELATING AGENTS BY MODIFICATION OF
CYSTEINE FOR COMPLEXATION STUDIES WITH ^{99m}Tc**

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Abstract

The synthesis of four novel ligands using the amino-acid cysteine and its ethyl carboxylate derivative is described. The synthetic method involves a two-step procedure, wherein the intermediate Schiff base formed by the condensation of the amino group of the cysteine substrate and salicylaldehyde is reduced to give the target ligands. The intermediates and the final products are characterised by high resolution NMR spectroscopy. Complexation studies of the ligands with ^{99m}Tc are standardised using stannous tartrate as the reducing agent at varying reaction conditions. The complexes are characterised using standard quality control techniques such as TLC, paper electrophoresis and PC. Lipophilicities of the complexes are estimated by solvent extraction into chloroform. Substantial changes in net charge and lipophilicity in the ^{99m}Tc complexes are observed on substituting the carboxylic acid residue in ligand I and II with the ethyl carboxylate groups (ligands III and IV). All the ligands formed complexes in high yield. While the complexes of ligand I and II are observed to be hydrophilic in nature and are not extractable into CHCl_3 , ligands III and IV gave neutral and lipophilic complexes. Though the distribution ratios of the complexes of ligands III

and IV in CHCl_3 /saline system are observed to be very high, considerable differences in lipophilicities are also observed as evidenced by the difference in their respective extractabilities in chloroform. On storage, the complex of ligand III exhibit a tendency to get converted to a hydrophilic and non-extractable species. The bio-distribution of the complexes of ligands I and II showed that they have predominantly renal clearances whereas the complexes of ligands III and IV exhibited a significant hepatobiliary uptake and did not show much uptake in brain in spite of its favourable properties such as neutrality, lipophilicity and conversion into a hydrophilic species.

1. INTRODUCTION

Several reports describe the use of amino acids having sulphydryl groups in complexing $^{99\text{m}}\text{Tc}$ as a novel approach to the designing of radiopharmaceutical agents [1-4]. Considerable interest has been focused on the development of $^{99\text{m}}\text{Tc}$ labelled N_2S_2 type complexes incorporating functional moieties which enable metabolic trapping in the brain on crossing the blood brain barrier [5]. This has lead to the development of $^{99\text{m}}\text{Tc}$ -ECD (ethylcysteinate dimer) as a brain perfusion agent [6]. A few other radiopharmaceutical agents having N_2S_2 and N_3S_2 donor atom sets such as $^{99\text{m}}\text{Tc}$ labelled EC (ethylene dicysteine), MAG_3 (mercaptoacetyl triglycinate) respectively have also been developed for renal function imaging [7-8]. Prior to the use of $^{99\text{m}}\text{Tc}$ complexes of iminodiacetic acid derivatives, cysteine coupled with bromosulphonaphthalein has also been used [9] as a hepatobiliary agent.

The amino acid cysteine has been used without modification [10] for complexation with $^{99\text{m}}\text{Tc}$ in the development of new radiopharmaceuticals. In this direction, the designing of novel synthetic derivatives of cysteine as models for the radiopharmaceutical preparation of $^{99\text{m}}\text{Tc}$ mercaptide complexes constitute an interesting

study. Cysteine offers the possibility of synthetic modification at the amino, carboxylic acid and the sulphhydryl terminal. This structural versatility can be exploited to offer a variety of ligands which could be explored as potential radiopharmaceutical agents. Alterations in the biological functions of the complexes obtained from these ligands can be effected by suitable modification of the carboxylic acid group in designing either neutral and lipophilic or charged and hydrophilic complexes.

In the pursuit of novel ligands for use as diagnostic radiopharmaceuticals, Pillai et al. have reported the syntheses and radiochemical studies of a wide variety of amine phenol ligands [11-13]. These ligands were found to form neutral, lipophilic ^{99m}Tc complexes in high yields. It was therefore felt pertinent to synthesise novel ligands incorporating an amine phenol unit in the cysteine moiety. The apparently simple synthetic strategy which has been employed earlier could be envisaged in the present case also. However, the presence of reactive groups such as the carboxylate and the sulphhydryl groups could possibly call for standardisation of the usual reaction conditions[11]. In the present study, we report the syntheses of four novel ligands, wherein cysteine and ethyl cysteinate have been condensed with salicylaldehyde to give the corresponding imines (I, III) and subsequently their reduced products (II, IV) (Fig.1).

Ligands I and II could also be used as bifunctional chelating agents in covalent linking of the free carboxylate substituent with suitable functional groups of receptor specific substrates or antibodies for development of receptor or antibody based radiopharmaceuticals. The addition of more chiral centres to the molecule is possible by alteration of the aromatic moiety from salicylaldehyde to 2-hydroxy acetophenone. Lipophilicity of the resultant complex can also be varied thus or by substitution of other suitable groups. Such synthetic manipulations open up the possibility of arriving at a

spectrum of ^{99m}Tc complexes that could be explored as potential radiopharmaceutical agents.

^{99m}Tc complexes of all the four ligands were synthesised by reducing TcO_4^- in the presence of ligand with stannous tartrate at optimum pH. The yields of complexation were estimated by TLC, PC and paper electrophoresis. The lipophilicities of the complexes were estimated by solvent extraction into CHCl_3 .

Bio-distribution studies of the complexes were carried out in Swiss mice.

2. MATERIALS

L-Cysteine, salicylaldehyde and sodium borohydride were purchased from Aldrich Chemical Company, U.S.A. L-Ethylcysteinyl hydrochloride was purchased from Lancaster, England. Stannous tartrate was obtained from Sigma Chemical Company St. Louis, MO, U.S.A. $^{99m}\text{TcO}_4^-$ was eluted from a ^{99}Mo - ^{99m}Tc column generator prepared in our laboratory [14]. $\text{NH}_4^{99}\text{TcO}_4$ was obtained from New England Nuclear and diluted in 0.9% saline solution to the required concentration. Flexible silica gel plates IB-F(7.5 x 2.5 cm) were from J.T. Baker Chemical Company, N.J. Whatman 1 chromatography paper (30 x 2.5 cm) was used for paper electrophoresis.

FT-IR spectra were recorded in Nicolet Magna IR Spectrometer 550. Proton NMR spectra were obtained using a Bruker 500 spectrometer, 500 MHz for ^1H and Varian VXR 300S, 300 MHz for ^1H using d_6 -DMSO and CDCl_3 as solvents and TMS as the internal reference. Elemental analyses were done by Desert Analytics, Tucson, Arizona.

3. METHODS

3.1. Synthesis and spectroscopic characterisation of the ligands

3.1.1. Ligand I : The synthesis of ligand I was achieved via a two-step procedure (Fig. 1). 5 g (0.04 mol) of L-Cysteine and 5.0 g (0.04 mol) of salicylaldehyde were refluxed for 4 hours in ethanol (200 mL). The solution turned light yellow and precipitation was observed during reaction and on cooling. The pale yellow solid was collected by filtration, washed with cold water and dried to give 9.0 g (95 %) of the imine.

IR (KBr, ν cm^{-1}) : 3434 (OH), 2585, 2696 (SH), 1630 (C=N), 1597, 1578 (Ar);

^1H NMR (d_6 DMSO, δ ppm): 3.44 (1H dd, $J=12.0, 8.2$ Hz) $-\text{CH}_\text{A}\text{H}_\text{B}\text{SH}$, 3.21 (1H dd, $J=12.0, 8.2$ Hz) $-\text{CH}_\text{A}\text{H}_\text{B}\text{SH}$, 3.83 (1H dd, $J=10.8, 8.3$ Hz) $-\text{CH}(\text{N})(\text{CH}_2\text{SH})$, 5.65, 5.84 (1H s) Ar- $\text{CH}=\text{N}$ -(geometrical isomers), 6.73-6.83 (2H m) Ar, 7.03-7.16 (1H m) Ar, 7.28-7.36 (1H m) Ar.

Elemental analysis: %Calculated for $\text{C}_{10}\text{H}_{11}\text{NO}_3\text{S}$: C 53.33 H 4.89 N 6.22

%Found : 52.97 4.99 6.30

3.1.2. Ligand II : To a stirred solution of 3.6 g (0.02 mol) of the imine (I) in 200 mL dry ethanol was added 0.3 g of sodium borohydride in instalments (0.05 g) when the solid slowly dissolves. After 30 min the solution was gently heated for complete dissolution. An additional 0.7 g of sodium borohydride was added in two portions and stirring continued for two hours. The reaction mixture was concentrated under vacuum and 100 mL water was added and pH of the solution adjusted to approximately 5 when a precipitate was observed. The product could not be extracted with chloroform and hence the aqueous solution was filtered and concentrated *in vacuo* to give a white precipitate (3.3 g, 92 %). The crude product was recrystallized from ethanol: water: acetone (1:1:0.2) mixture.

IR (KBr, ν cm^{-1}) : 3460 (OH), 3151 (NH), 2539 (SH), 1624 (acid $>\text{C}=\text{O}$), 1564 (NH), 1597(Ar); ^1H NMR (d_6 DMSO, δ ppm): 2.92 (1H dd , J = 16.6, 5.8 Hz) - $\text{CH}_\text{A}\text{H}_\text{B}\text{SH}$, 2.80 (1H dd, J = 16.6, 7.3 Hz) - $\text{CH}_\text{A}\text{H}_\text{B}\text{SH}$, 3.38 (1H broad t, J = 6.5 Hz) - $\text{CH}(\text{NH})(\text{CH}_2\text{SH})$, 3.56-3.60 (1H m) - $\text{CH}(\text{NH})(\text{CH}_2\text{SH})$, 4.0 (2H d, J = 16Hz) Ar- $\text{CH}_2(\text{NH})$, 6.76-6.85 (2H m) Ar, 7.13-7.25 (2H m) Ar.

Elemental analysis: %Calculated for $\text{C}_{10}\text{H}_{13}\text{NO}_3\text{S}$: C 52.86 H 5.73 N 6.17

%Found : 51.80 5.67 6.00

3.1.3. Ligand III : L-cysteine ethyl ester was generated by basification (pH 8-9, 1M NaOH) of an aqueous solution of the hydrochloride salt (5 g in 5 mL water), extracted with chloroform, dried over anhydrous sodium sulphate and solvent removed to give 3.3 g of L-ethyl cysteinate. Freshly generated L-ethyl cysteinate (3.4 g, 0.02 mol) was reacted with 3 mL of salicylaldehyde (0.03 mol) in 20 mL dry ethanol at 0-5°C for 15 min. The reaction was monitored to completion using TLC (1 % methanol in chloroform). (It may be mentioned that under the refluxing condition employed initially the reaction mixture showed decomposition). The crude reaction mixture was concentrated *in vacuo* and 100 mL water was added. The product was extracted with chloroform (4 x 10 mL) after saturation with brine. The organic extracts were pooled, washed with brine (2 x 10 mL), dried over anhydrous sodium sulphate and evaporated in vacuum to yield the crude product. The crude product (0.7 g) was purified by column chromatography on silica gel using 0.3 % methanol in chloroform as the eluate to give (0.13 g, 19 %) of the pure imine as a pale yellow solid.

IR (KBr, ν cm^{-1}) : 2575 (SH), 1744 (COOEt), 1627 ($\text{C}=\text{N}$), 1583,1490 (Ar);

^1H NMR (CDCl_3 , δ ppm): 1.31, 1.32 (3H a pair of triplets, J=7.1 Hz) - $\text{CO}_2\text{CH}_2\text{CH}_3$ (geometrical isomers), 1.59 (1H broad singlet) - CH_2SH , 3.19-3.24 (1H m), - $\text{CH}_\text{A}\text{H}_\text{B}\text{SH}$,

3.40-3.46 (1H m) $\text{CH}_\text{A}\text{H}_\text{B}\text{SH}$, 4.13 (1H dd, $J = 5.5, 7.5$ Hz) $-\text{CH}(\text{COOEt})(\text{CH}_2\text{SH})$, 4.27, 4.28 (2H q, $J = 7.3$ Hz) $-\text{CO}_2\text{CH}_2\text{CH}_3$ (geometrical isomers), 5.62, 5.92 (1H s) $\text{Ar}-\text{CH}=\text{N}-$ (geometrical isomers), 6.79-7.12 (2H m) Ar, 7.16-7.25 (2H m) Ar.

3.1.4. Ligand IV : To a stirred solution of 0.13 g (0.5 mmol) of ligand III in 5 mL dry ethanol at 0°C was added 0.04 g of sodium borohydride in small portions and stirring continued for three hours. The reaction mixture was rotary evaporated and water (20 mL) was added. The product was extracted in chloroform (4 x 5 mL) after saturation with brine. The organic extracts were pooled, dried over sodium sulphate to give 0.07 g of the pure amine obtained as a viscous product and observed as a single spot on TLC (5 % methanol in chloroform). IR (KBr, $\nu \text{ cm}^{-1}$) : 3315 (NH), 2558 (SH), 1742 (ester $>\text{C}=\text{O}$), 1492, 1591 (Ar); ^1H NMR (CDCl_3 , δ ppm): 1.32 (3H, a pair of triplets $J = 7.14$ Hz) $-\text{CO}_2\text{CH}_2\text{CH}_3$, 1.45 (1H t, $J = 8.6$ Hz) $\text{CH}_\text{A}\text{H}_\text{B}\text{SH}$, 2.89 (2H dt, $J = 2.65, 5.6$ Hz) $\text{CH}_\text{A}\text{H}_\text{B}\text{SH}$, 3.53 (1H t, $J = 5.4$ Hz) $-\text{CH}(\text{NH})(\text{CH}_2\text{SH})$, 4.1 (2H t, $J = 13.7$ Hz) $\text{Ar}-\text{CH}_2(\text{NH})$, 4.21-4.30 (2H m) $-\text{CO}_2\text{CH}_2\text{CH}_3$ diastereomeric, 6.76-6.82 (2H m) Ar, 7.15-7.22 (2H m) Ar.

3.2. Complexation studies with $^{99\text{m}}\text{Tc}$

Complexation studies of $^{99\text{m}}\text{Tc}$ with the ligands were carried out using stannous tartrate as the reducing agent. Ligands I and II were prepared in 0.05 M bicarbonate buffer and ligands III and IV were prepared in methanol. Solutions of the ligands (0.1 mL), 0.5 mL of 0.5 M bicarbonate buffer of pH 9 and 1.0 mL of $^{99\text{m}}\text{TcO}_4^-$ (10 - 20 MBq/mL) was made up to a volume of 4.8 mL with nitrogen purged saline. Saturated solution of stannous tartrate (0.2 mL) was added to the above solution. The resultant complexes were characterised by PC, TLC, paper electrophoresis and solvent extraction.

Reaction conditions were optimised by varying the parameters such as ligand concentration, pH (buffers- acetate pH 3, phosphate pH 5 and 7 and bicarbonate pH 9 and 12) and time. Complexation yields were also studied using varying concentrations of $^{99}\text{TcO}_4^-$.

3.2.1. Thin layer chromatography

TLC was performed using flexible silica gel plates. A 5 μL aliquot of the reaction mixture was spotted 1 cm from the lower end of the plate. The strips were developed using solvents such as saline or acetonitrile, dried, cut into equal segments of 0.5 cm width and measured for radioactivity in a NaI(Tl) well counter.

3.2.2. Paper electrophoresis

Samples were spotted on Whatman 3 chromatography paper at 10-12 cm from the cathode and paper electrophoresis was carried out at 10 volts/cm for 1.5 hour in 0.02 M phosphate buffer, pH 7.5. The strips were dried, cut into 1 cm segments and measured for radioactivity.

3.2.3. Solvent Extraction

Solvent extraction was performed by mixing 1 mL of the reaction mixture with 1 mL of chloroform on a vortex mixer for about a minute. Equal aliquots of the aqueous and the organic layers were withdrawn and measured for radioactivity. The organic extract was back extracted repeatedly with saline to estimate the distribution ratio.

3.3. Biodistribution

Swiss mice weighing 20-25 g were injected through the tail vein with 0.1-0.3 mL of the complex solution containing ~370-550 kBq of activity. Tissues and organs excised from the mice following sacrifice at 10 min, 30 min 2 h and 4 h, post injection were weighed and the radioactivity estimated in a NaI(Tl) well counter. The percent injected dose in each tissue was calculated from the above data. The % ID in blood was calculated by measurement of the activity in 0.5 to 1 g of blood withdrawn by cardiac puncture immediately after sacrifice and assuming the whole blood volume as 6.5% of the body weight.

4. RESULTS AND DISCUSSION

4.1. Characterisation of the ligands

The formations of the imine ligands were indicated preliminarily by the observation of an IR band at $\sim 1630\text{ cm}^{-1}$ for ligands I and III. The spectral characteristics, peak multiplicities and integrations of the ^1H NMR spectra of the ligands were consistent with the expected features. The presence of the geometrical isomers in case of the imine ligands I and II were reflected in the appearance of a pair of singlets at δ 5.65, 5.84 for ligand I and δ 5.62 and 5.92 for ligand III corresponding to the imine proton. In case of the reduced products, the appearance of the NH band at 3151 and 3315 cm^{-1} in IR corresponding to the amine ligands II and IV respectively, indicated the reduction of the imine bond. The SH bands at 2539 and 2558 cm^{-1} IR spectra also confirmed that the thiol group remained unaffected during the course of the reaction. The formation of the diastereomeric products emerging from non-stereospecific reduction was evident in the ^1H NMR spectra of the reduced products. The results of elemental analyses for the imine ligands provide additional evidence to support the formation of the ligands.

TABLE I. COMPLEXATION YIELDS (%) WITH VARYING AMOUNTS OF $^{99}\text{TcO}_4^-$

Ligands	NCA*	$^{99}\text{TcO}_4^-$ (M/L)			
		10^{-7}	10^{-6}	10^{-5}	10^{-4}
I	93	93	93	93	30
II	90	88	83	50	30
III	90	90	90	48	25
IV	96	97	96	94	28

* No carrier added

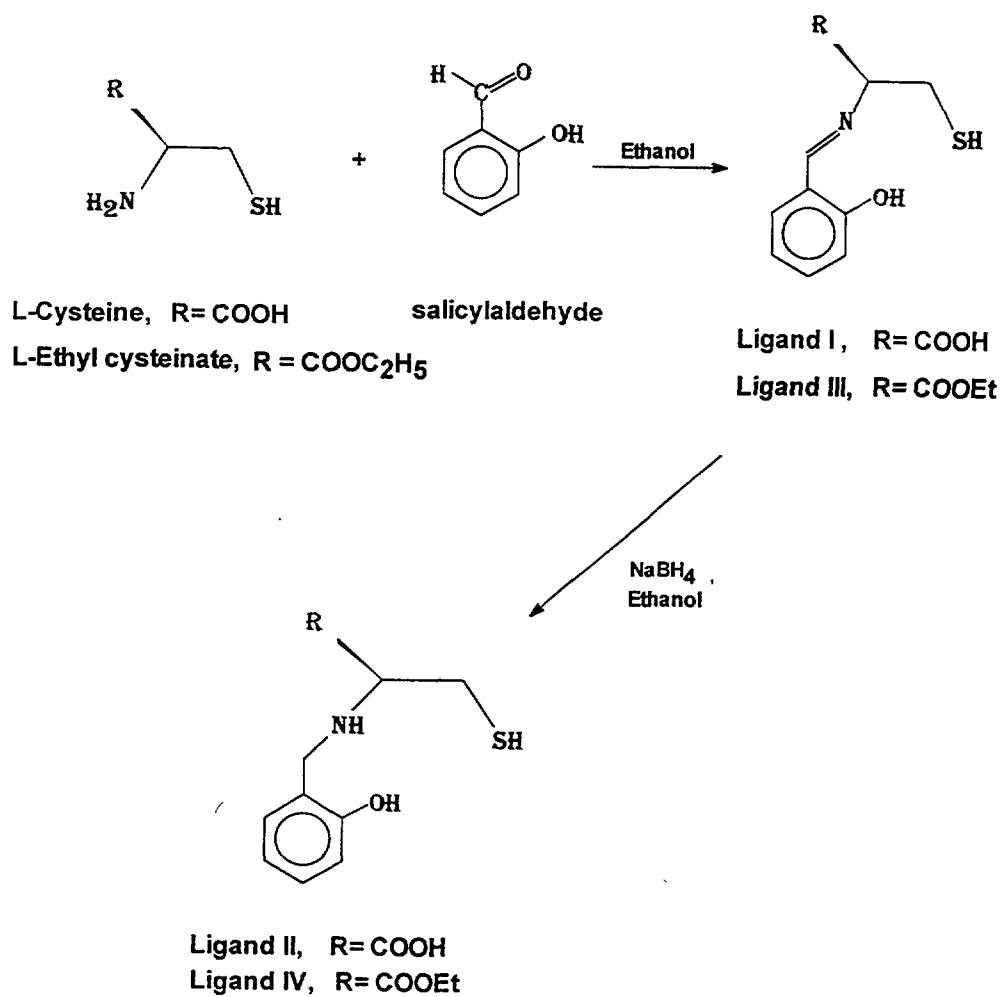


Fig.1: Scheme for synthesis of the ligands

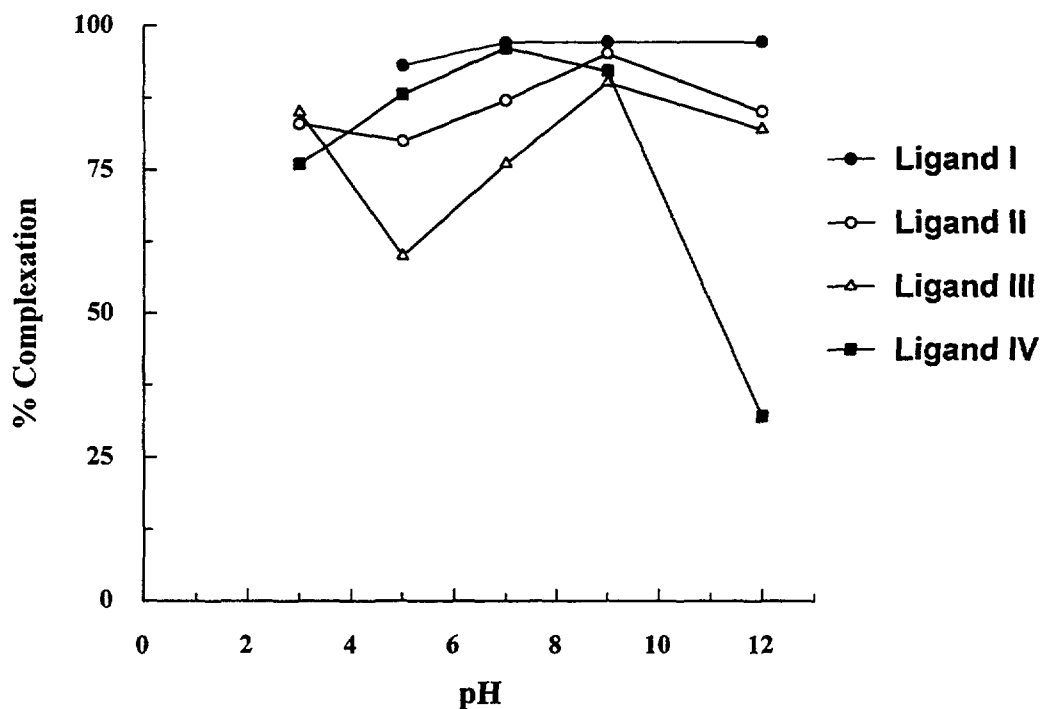


Fig. 2: Complexation yields at varying pH using different buffers (acetate pH 3, phosphate pH 5 and 7, bicarbonate pH 9, 12)

4.2. Radiochemical studies

The results of complexation studies with the ligands I - IV at various pH is shown in Fig. 2. While ligand I was found to complex with ^{99m}Tc in high yields at both alkaline as well as acidic pH, ligand II and III showed maximum complexation at pH 9. The complexation yields for ligand IV was observed to be maximum at pH 7 with a drastic reduction at pH 12. At the respective optimum pH, maximum complexation was achieved at concentrations of 5 mM for ligands I and II, 0.1 mM for ligand III and 0.5 mM for ligand IV.

Multiple quality control techniques were essential to obtain the complexation yields in the case of these ligands. The complexes of ligands I and II were found to be non-extractable in chloroform and were characterised by PC in saline and TLC in acetone (Fig. 3A and 3B). In PC/saline the complexes of ligands I and II were found to move

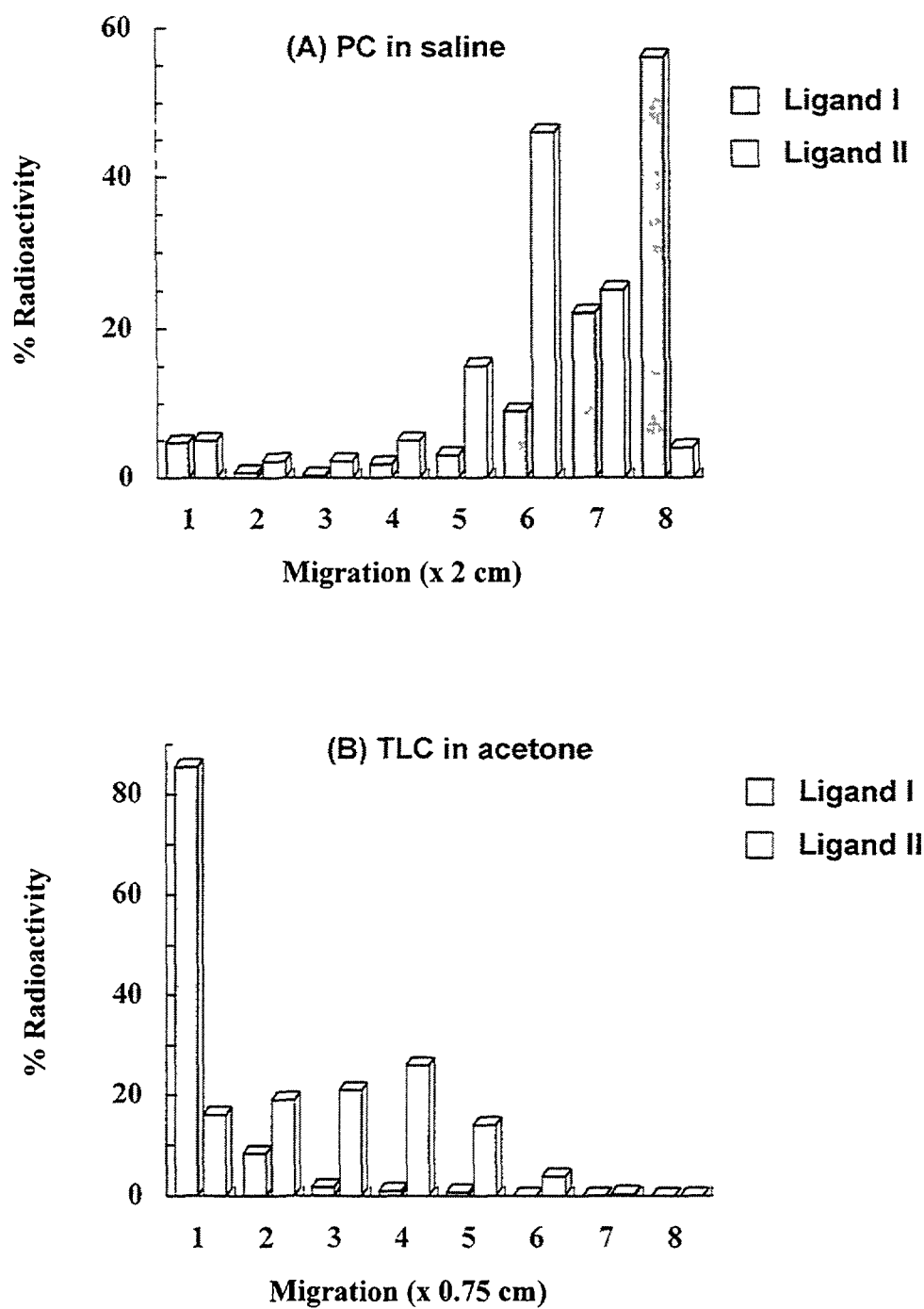


Fig. 3: Characterisation of ^{99m}Tc -complexes of Ligands I and II by (A) Paper chromatography in saline and (B) TLC in acetone

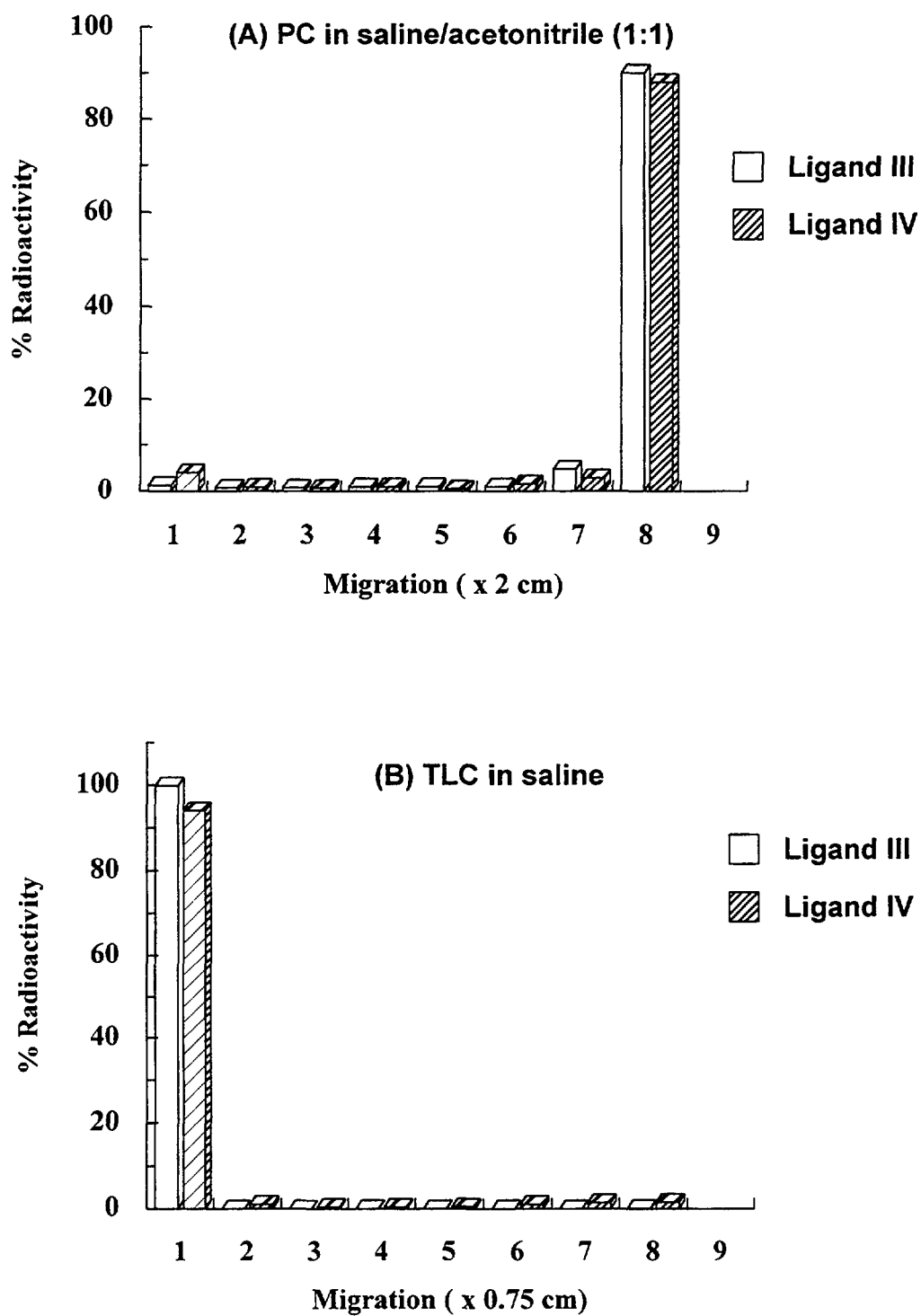


Fig. 4: Characterisation of ^{99m}Tc -complexes of Ligands III and IV by (A) Paper chromatography in saline: acetonitrile (1:1) and (B) TLC in saline

with the solvent front whereas in TLC/acetone, the complex of ligand I remained at the point of spotting but in case of the complex of ligand II, the activity was distributed on the chromatography plate. The above observations suggest that the complexes are hydrophilic and the presence of pertechnetate and TcO_2 in appreciable quantities in the reaction mixture are also ruled out. These results can be substantiated by considering the presence of carboxylic acid groups which results in the formation of charged hydrophilic complexes as observed earlier by Baldas et al [15]. The hydrophilic nature of the complexes of ligands I and II were inferred from their movement on paper electrophoresis. The complexes of ligand I and II moved towards anode, whereas complexes of ligand III and IV remained at the point of spotting. The complexes of both the ligands III and IV were extractable into chloroform. In PC/ saline:acetonitrile 1:1 (Fig.4A), the complexes moved towards the solvent front whereas in TLC/saline (Fig.4B), they did not exhibit any migration. The lipophilicity of the ligands were

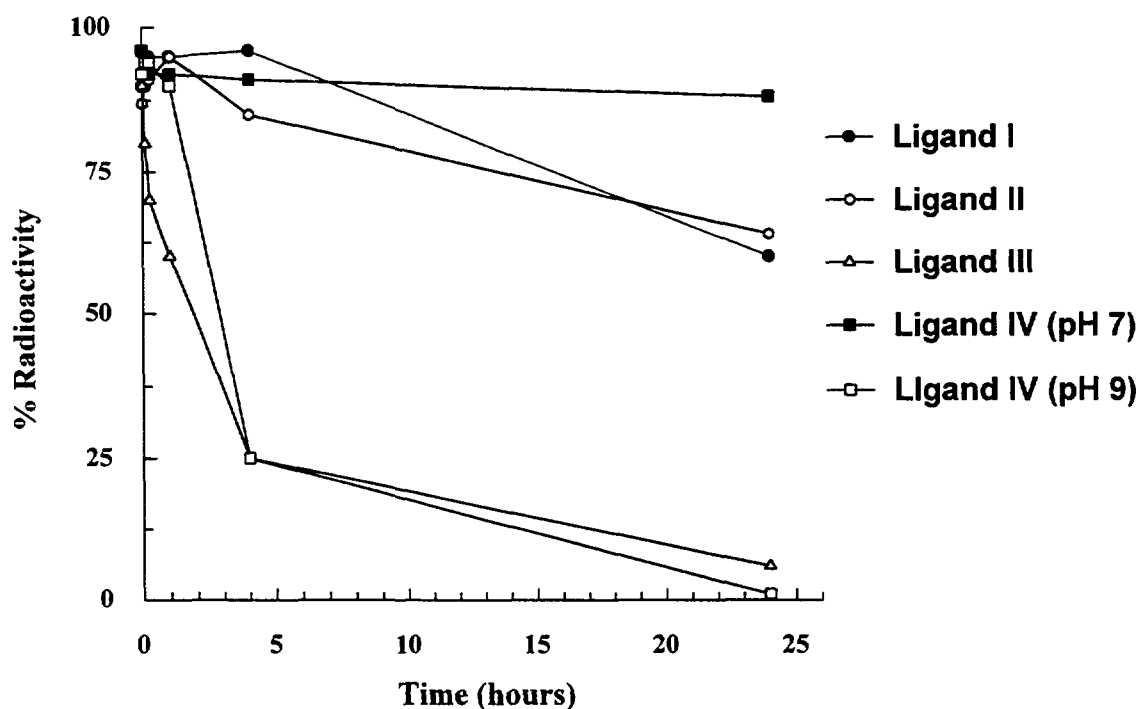


Fig. 5: Stability of $^{99\text{m}}\text{Tc}$ -complexes of Ligands I - IV as a function of time

estimated by determination of the distribution ratio of the ligands. Complexes of ligands I and II showed poor extractability (<1%) whereas those of ligands III and IV were observed to be 90 and 95%, respectively. The CHCl₃/saline distribution ratios were 99±8 and 70±7 for complexes of ligand III and IV, respectively.

To establish the equivalence of chemistry occurring at ⁹⁹Tc and at no carrier added ^{99m}Tc (~10⁻⁷ M) concentrations, ^{99m}TcO₄⁻ was added as a tracer in ⁹⁹TcO₄⁻ and complexation studies were carried out with the respective optimum concentration of the ligands. It is evident from the studies (Table I) that upto 10⁻⁵ M concentration of ⁹⁹Tc, there is no change in the complexation yield for ligands I and IV whereas for ligands II and III there is significant reduction as ⁹⁹Tc concentration is increased to 10⁻⁵ M.

The stability of the complexes with time was studied by the standardised quality control techniques and the results are given on Fig. 5. While the complex of ligand I exhibited remarkable stability upto 4 hours, that of ligand II showed slight reduction in stability with time. The complex of ligand IV showed an appreciable reduction in stability with time when prepared at pH 9 but was found to be stable when prepared at pH 7. Complex of ligand III was found to be significantly unstable and underwent rapid interconversion to a hydrophilic species as estimated by solvent extraction yields. The simultaneous estimation of TcO₄⁻ and TcO₂ by chromatographic techniques also confirmed the above observation.

4.3. Bio-distribution

The complexes of all the ligands showed rapid blood clearance. Results of the renal excretion and hepatobiliary uptakes for the complexes are given in Fig 6 and 7, respectively. The uptake in the liver for complexes of ligands I and II were significantly

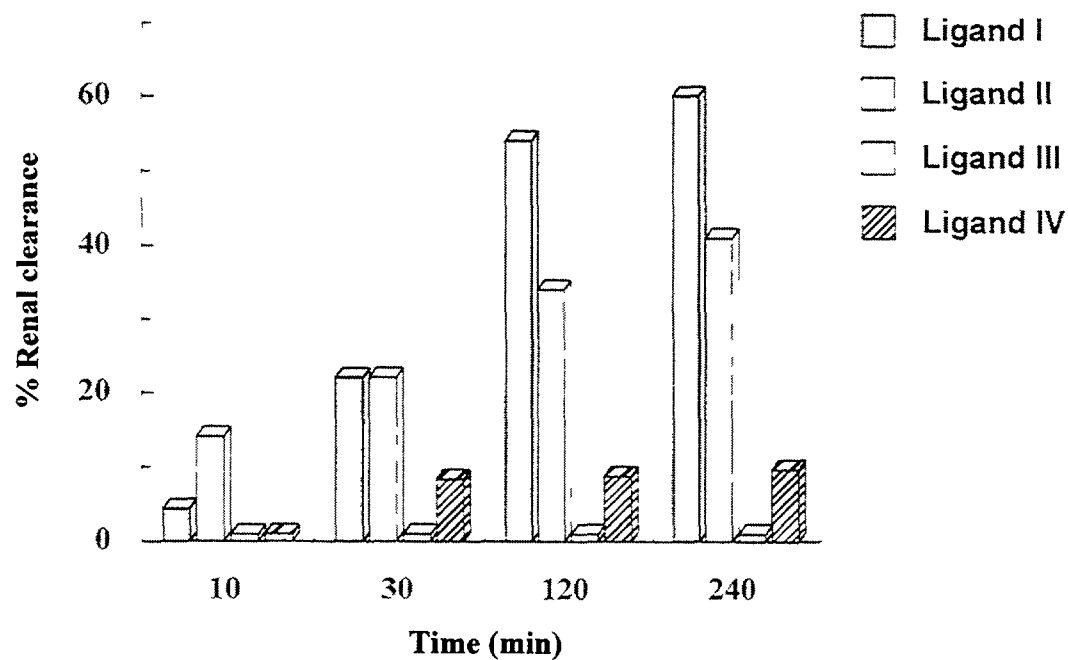


Fig. 6: Renal clearances of the ^{99m}Tc -complexes of Ligands I-IV :
10, 30, 120 and 240 minutes post injection

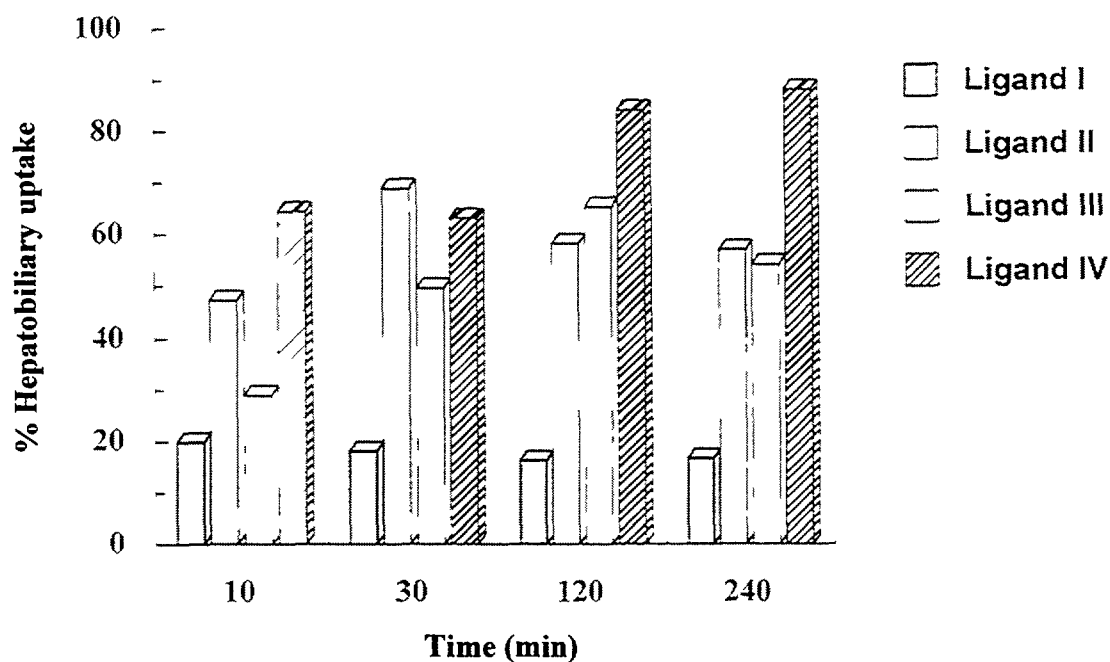


Fig. 7. Hepatobiliary clearances of the ^{99m}Tc -complexes of Ligands I-IV :
10, 30, 120 and 240 minutes post injection

low being 9 % and 16 % respectively. Histograms of the urinary clearances (Fig.6) at 120 and 240 minutes show that complex of ligand I exhibits predominantly renal excretion. The complex of ligand II though hydrophilic, exhibit both hepatobiliary as well as renal clearance. The rates of renal clearances for complexes of ligand III and IV were found to be negligible. The above results could be attributed to the presence of carboxylic acid groups in I and II imparting a negative charge to the complex thereby augmenting renal excretion properties. This observation is in concurrence with the results of Eshima et al. [16]. Esterification of the carboxylic acid residue increases the lipophilicity of the complexes of ligand III and IV and an increase in the hepatobiliary clearance could thus be expected. These observations are reflected in Fig. 7. However, it has also been documented earlier that increase in the lipophilicity decreases the rate of renal clearance at the expense of an increase in the slow hepatocellular transit times [17]. This has been observed in the present case wherein initial liver uptake of the complexes of ligands III and IV were found to be significant with relatively slower rates of hepatic clearances. Criteria such as rapid uptake by the liver, low renal excretion and slow transition time therefore contribute to possibility that the complex of ligand IV could be used as a liver-function evaluating agent. On the other hand, complex of ligand III exhibiting an initial lower liver uptake and a faster clearance rate compared to that of the complex of ligand IV therefore offers potential as an intra hepatic bile-duct imaging agent.

CONCLUSION

Four novel cysteine based ligands of varying lipophilicities were synthesised and characterised. The radiochemical studies of these ligands with $^{99m}\text{TcO}_4^-$ were standardised and the complexes were characterised by established quality control techniques. The complexes of the ligands containing the free carboxylic acid groups exhibited

predominant renal clearances. A significant increase in the lipophilicity of the complexes was achieved on preparing the corresponding ester derivatives and this was found to be reflected in the difference in the excretory patterns of the latter to hepatobiliary modes. The free carboxylic acid residue in two of the ligands could be derivatized for use as bifunctional chelating agents.

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Abstract


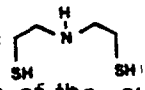
Computer-assisted techniques have found extensive use in the design of organic pharmaceuticals but have not been widely applied on metal complexes, particularly on radiopharmaceuticals. Some examples of computer generated structures of complexes of In, Ga and Tc with N,S,O and P donor ligands are referred. Besides parameters directly related with molecular geometries, molecular properties of the predicted structures, as ionic charges or dipole moments, are considered to be related with biodistribution studies. The structure of a series of oxo neutral Tc-biguanide complexes are predicted by molecular mechanics calculations, and their interactions with water molecules or peptide chains correlated with experimental data of partition coefficients and percentage of human protein binding. The results stress the interest of using molecular modelling to predict molecular properties of metal-based radiopharmaceuticals, which can be successfully correlated with results of *in vitro* studies.

1. INTRODUCTION

Molecular computer modeling describes the generation, manipulation and representation of 3D structures of molecules and their associated physicochemical properties. In addition, the simulation of intermolecular interactions between the optimized molecular structures and specific macromolecular targets, could be used to recognize biological potential binding sites, and leads to the understanding of biological phenomena.

This research area has been developed for largely on the search for new drugs in the pharmaceutical industry. However, its application is still relatively uncommon in the field of metal-based radiopharmaceuticals, mainly due to the difficulty in the determination of reliable force field parameters for transition metal ions. Reliable parameters were obtained by modeling metal complexes and varying the parameters involving in Me-L bonds until the agreement between the molecular generated geometry and the crystallographically observed structures was as close as possible. According to R.D.Hancock [1], optimized parameters

for octahedral complexes of In(III) and Ga(III) with N, S and O donors were applied to predict several structures which demonstrated good agreement with those crystallographically determined. In the case of Tc(V) oxo complexes, the apex of the Tc=O group is readily reproduced by defining appropriate ideal O-Tc-L angles. Some structures predicted by molecular mechanics are:

- N,N'-ethylene bis(3-ethyl-3-mercaptobutyl) oxo Tc(V), with root mean deviations relatively to the crystallographic structure of 0.196 Å, for the heavy atoms, and 0.347 Å, for all atoms [2].
- [TcO(DME)₂]⁺ (DME = ) , [TcO(DMEA)SPh] (DMEA = ) and [TcO(TCC)₂] (TCC=tetrachlorocatechol). For these compounds, the overlap of the sulfur p_z orbital with the d_{xz} orbital of Tc (π-bond) restrains the rotation about the Tc-S bond and the calculations were not able to reproduce exactly the crystallographic structures [1,3].

On the other hand, the structures of five oxo Tc(V) complexes bearing several ligands with Tc-N, Tc-S and Tc-P bonds were predicted by density-functional methods and the results compared with the corresponding crystal structures. The authors claim the advantages of this approach, in spite of the computational resources needed be considerable [4].

When reliable force field parameters are achieved and confidently tested, the use of molecular mechanics to predict molecular structures of Tc-complexes, that cannot be readily determined experimentally (such as ^{99m}Tc-complexes at carrier-free level, 10⁻⁸ - 10⁻⁹ M, in where inorganic classic analytical techniques are not easy to apply and the corresponding ⁹⁹Tc chemistry is complex), appears as a very powerful method of study. Indeed, when supported by both the available informations on the specific peculiarities of the ⁹⁹Tc and/or Re and the previously determined molecular structures of the ligands, molecular mechanics has been proved its great power in furthering our understanding of the chemical reactivity and fundamental structural features of Tc-radiopharmaceuticals [5,6]. Besides the bond lengths and valence or dihedral angles, a series of other relevant molecular properties can be evaluated by molecular mechanics, including dipole moments, surface areas, atomic charges, Van der Waals (VDW) energies, dipole-dipole or charge-charge interaction energies (DD/QQ) etc., which can then be correlated with experimental data. Hansen et al. [7], developed a series of a second-generation ^{99m}Tc renal radiopharmaceuticals based on the prototype agent [TcO(MAG₃)]²⁺ (MAG=mercaptoacetyltriglicine) on the basis of results provided by molecular mechanics studies. These authors tried to find a correlation between both the position of the carboxyl group and the oxo-carboxyl interatomic distances with biologic activity, and understand the relevance of the above molecular parameters to renal excretion. In a different molecular mechanics study, Reichert and Welch [8] established

correlations between dipole moments and ionic charges of several copper complexes of macrocyclic ligands (cyclen, cyclam, Etcyclam, DOTA, TETA), and their liver uptake by rats.

Another interesting problem, is the evaluation of specific molecular interactions between the predicted metal-based radiopharmaceuticals and biological molecules. Each complex has its own specific properties, such as electron donor/acceptor character, lipophilicity-hydrophilicity balance, redox potential, pK, chirality and isomerism. These specific properties are the key-base to molecular recognition by biological molecules. So, it is expected that simulations of intermolecular interactions can shed light, at a molecular level, to the specific binding involving the radiopharmaceuticals and the biological molecules.

In this paper, the computer generated optimized geometries of the oxo neutral complexes $[\text{TcOL}_2(\text{OH})]$ are reported, where L is the monoanionic form of biguanide $[\text{H}_2\text{NC}(=\text{NH})\text{NHC}(=\text{NH})\text{NH}_2]$ (Big) or the N1 substituted dimethyl (DMBig), phenyl (PBig) and phenethyl (PEBig) biguanides. The calculations were undertaken by *molecular mechanics*, being the reliability of the approach used first checked by detailed comparison between theoretically predicted geometrical parameters (atomic distances, valence and dihedral angles) and the correspondent experimental values obtained by X-ray crystallography for the cationic complex $[\text{TcO}(\text{DMBig})_2]^+$. In addition to the structural predictions, the computational method used also enabled a detailed evaluation of the main intermolecular interactions between the Tc-complexes and simple molecules, such as water or small peptide chains. Correlations were found between interaction energies and surface areas of the low energy aggregate species and the results of *in vitro* studies, as partition coefficients and percentage of human protein binding of the $^{99\text{m}}\text{Tc}$ -biguanide complexes.

2. MOLECULAR MECHANICS CALCULATIONS

The calculations were undertaken with the PCMODEL program (version 3.0) [9], in a 486DX4-100 PC. PCMODEL is a simple interactive molecular modeling program that can handle up to 296 total atoms. The MMX force field is an extension of Allinger's MM2 force field which, besides performing conventional *molecular mechanics* calculations on the molecular σ -system, undertakes a simultaneous π -system semi-empirical valence electron self consistent field calculation. This approach improves considerably both the qualitative and quantitative description of systems having delocalized π -electrons, such as the ligands studied in this work. General parameters for technetium are available in this force field, where the metal covalent radius is taken to be 135.5 pm [10]. To check the reliability of the PCMODEL/MMX calculations for the systems considered, a minimized structure of the analogous complex $[\text{TcO}(\text{DMBig})_2]^+$ was first generated and compared with available

experimental structural data[13]. Satisfactory agreement is observed, even when relevant dihedral angles, which are in general difficult to reproduce by *molecular mechanics*, are considered [11,12]. In particular, the calculations agree with the experimental data showing that the Tc(V) ion close a pseudoaromatic ring, due to extensive π electron delocalization along the N-C-N skeleton and that the Tc=O core lies slightly above the plane formed by the two ligands

The structures of the neutral oxo Tc-complexes, [TcO(Big)₂(OH)], [TcO(DMBig)₂(OH)], [TcO(PBig)₂(OH)] and [TcO(PEBig)₂(OH)], were analyzed by similar calculations. Finally, the intermolecular interactions between each one of the complexes and water (or peptide chain) molecules were evaluated by:

- i) optimizing the structures for each Tc-complex/water molecule or Tc-complex/peptide chain aggregate resulting from a systematic search in their configurational space, the whole process being systematically checked by inspection of relative energies of the final structures,
- ii) calculating a series of relevant structure-related properties (such as polar, apolar and total surface areas, VDW and DD/QQ interaction energies) that, together with the same properties obtained for the individual components of the aggregates, can be correlated with the degree of interaction between these species within the aggregates.

3. RESULTS AND DISCUSSION

3.1. Geometries

The values of the theoretically predicted parameters (bond distances, valence and dihedral angles) of the proposed structures of Tc-biguanide complexes: [TcO(Big)₂(OH)], [TcO(DMBig)₂(OH)] , [TcO(PBig)₂(OH)] and [TcO(PEBig)₂(OH)] are shown in Table 1. In all complexes studied the ligand molecules were found to be quasisplanar, with the C-N-C-N(R) torsion angles, (R = H₂ , (CH₃)₂, HPh, HCH₂CH₂Ph) varying from 169.7 to 177.5°. The ligand substitution effect is more relevant in the case of the phenyl and phenethyl derivatives, where the torsion angle O=Tc-O-H are 143.5° and 148.2°, respectively, while in the case of non-substituted and dimethyl substituted complexes this angle is 178.3° and 177.3°. The deviation from planarity observed in these complexes having ligands bearing a phenyl group, can be explained considering that the presence of the aromatic rings considerably increase the overall steric hindrance, thus forcing the axial ligands to assume the observed non-planar geometry. In the case of the phenethylene derivative this effect is less pronounced, since the presence of the ethylenic chains leads to reduce steric contacts.

Table 1. Relevant calculated geometrical parameters for the oxo ^{99m}Tc -biguanide complexes*

	TcOBig	TcODMBig	TcOPBig	TcOPEBig
bond distance(Å)				
Tc=O	1.72	1.71	1.73	1.73
Tc-OH	1.90	1.89	1.91	1.92
Tc - N	1.92-1.96	1.92-1.97	1.91-1.97	1.92-2.04
C - N	1.38-1.41	1.35-1.46	1.31-1.42	1.30-1.46
C - C	-	-	1.39-1.42	1.40-1.54
N - H	0.96-0.97	0.96-0.97	0.96-0.98	0.96-1.00
C - H	-	1.11	1.10	1.10-1.12
valence angle (°)				
O = Tc - O	156.5	154.6	171.6	157.0
O = Tc - N	91.6-111.4	90.1-106.4	82.0-102.8	77.6-124.1
N - Tc - OH	65.9-111.7	66.0-112.5	83.6-103.9	78.7-108.8
N - Tc - N	85.9-176.8	87.2-179.0	83.5-167.0	85.1-160.8
C - N - Tc	125.8-127.4	125.8-128.2	118.1-127.1	120.7-126.1
N - C - N	117.4-124.0	118.1-124.7	111.4-127.1	114.1-128.7
C - N - C	124.7;125.0	119.2-126.2	125.6-131.1	124.2-126.5
C - C - N	-	-	116.6-126.6	109.8;112.4
C - C - C	-	-	118.4-121.7	118.9-130.2
N - C - H	-	109.7-111.8	-	105.9-109.3
C - N - H	115.6-120.8	114.8-120.8	114.4-121.3	105.7-118.1
H - N - H	119.0-119.7	119.0;119.1	119.0;119.4	118.1;119.7
torsion angle (°)				
O = Tc - O - H	178.3	177.3	143.5	148.2
C - N - C - NH ₂	171.2-176.6	169.7;176.3	174.2;179.7	173.3;173.7
C - N - C - N(CH ₃) ₃	-	169.9;177.5	-	-
C - N - C - NH(Ph)	-	-	169.7;174.5	-
C - N - C - NH(EtPh)	-	-	-	171.6;176.9

*TcOBig, TcODMBig, TcOPBig and TcOPEBig are abbreviated notation of [TcO(Big)₂(OH)], [TcO(DMBig)₂(OH)], [TcO(PBig)₂(OH)] and [TcO(PEBig)₂(OH)]

3.2. Molecular interactions

Simulation of molecular interactions between water molecules and [TcO(Big)₂(OH)], [TcO(DMBig)₂(OH)], [TcO(PBig)₂(OH)] or [TcO(PEBig)₂(OH)] suggest the attachment of two water molecules to the complexes, by means of hydrogen bond formation between the nitrogen atoms of the guanidine imino groups and hydrogen atoms of the water molecules, as shown in Figure 1, for the specific case of [TcO(Big)₂(OH)]. Additional water molecules stay close to the Tc aggregate environment, but no more hydrogen bond formation was predicted.

The relative changes in polar surface areas (ΔSA), for each molecular aggregate (Tc-complex and two water molecules), relative to the sum of the polar surface areas of the individual components may be used as a measure of the relative stability of the various aggregates (or the relative water affinity to the complex). A greater water affinity would correspond to a greater surface area reduction and, consequently, to a greater ΔSA value.

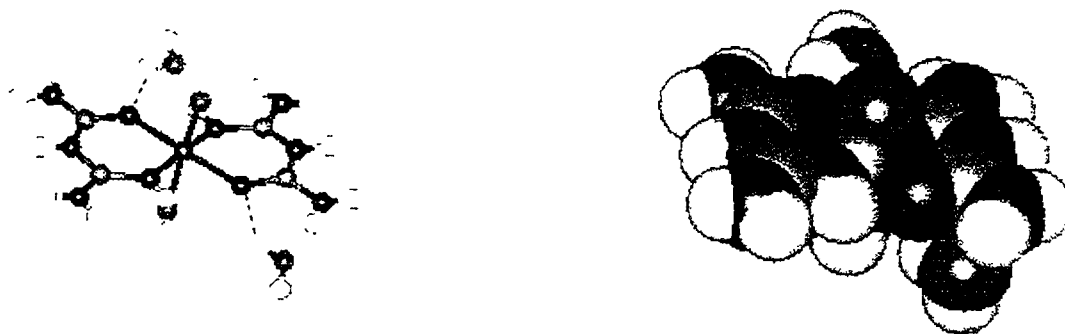


FIG. 1. Minimum energy aggregate resulting from interaction of $[\text{TcO}(\text{Big})_2(\text{OH})]$ with water (ball & stick and CPK space filling models; H bonds represented as dash lines)

In addition, the relative values of $\Delta\text{DD}/\text{QQ} = \text{DD}/\text{QQ} (\text{aggregate}) - \sum \text{DD}/\text{QQ} (\text{individual components})$ may be correlated with the partition coefficients experimentally evaluated for each complex. In fact, the minimum $\Delta\text{DD}/\text{QQ}$ value ($-33.15 \text{ kcal.mol}^{-1}$) corresponds to the more hydrophilic Tc-complex, and the maximum $\Delta\text{DD}/\text{QQ}$ value ($+0.81 \text{ kcal.mol}^{-1}$) to the less hydrophilic (or more lipophilic), which means that the electrostatic interaction energies could also be related to water affinity along the same series of Tc-complexes. The observed correlations of relative water affinity with both, ΔSA and $\Delta\text{DD}/\text{QQ}$, are consistent with the dipolar character of water molecules and consequently with the prevalence of Coulomb electrostatic interactions in these systems. Other structure-related parameters, such as relative changes in molecular apolar surface areas or VDW interactions, are not expected to play an important role in stabilizing the aggregates, and were not considered. The graphical representation of partition coefficients (PC), ΔSA and $\Delta\text{DD}/\text{QQ}$ along the series of studied Tc-complexes, gives a clear indication that calculated ΔSA and $\Delta\text{DD}/\text{QQ}$ follow the same pattern of variation of the experimentally determined partition coefficients (Figure 2).

Using the same methodology, the molecular interactions between the complexes, $[\text{TcO}(\text{Big})_2(\text{OH})]$, $[\text{TcO}(\text{DMBig})_2(\text{OH})]$, $[\text{TcO}(\text{PBig})_2(\text{OH})]$, $[\text{TcO}(\text{PEBig})_2(\text{OH})]$, and the simple dipeptide chain $^+\text{H}_3\text{N}-\text{CH}_2-\text{C}(=\text{O})-\text{NH}-\text{CH}_2-\text{COO}^-$ (whose structure was previously optimized as described for all other systems here considered) were also evaluated. The optimized geometry of the dipeptide has the relevant bond distances and valence angles similar to those described in literature (in parentheses), *i.e.*, a $\text{C}=\text{O}$ distance of 1.21\AA (1.24\AA), a $\text{C}(=\text{O})-\text{N}$ distance of 1.34\AA (1.32\AA), a $\text{N}-\text{C}$ distance of 1.46\AA (1.47\AA) and a $\text{O}=\text{C}-\text{N}$ angle of 125.3° (125°).

The observed multiple interactions between the complex $[\text{TcO}(\text{Big})_2(\text{OH})]$ and the dipeptide could be explained in terms of electrostatic interactions involving the different atoms of the system. Then, the hydrogen atoms of the NH_3^+ group of the peptide chain (charge $+0.22e$)

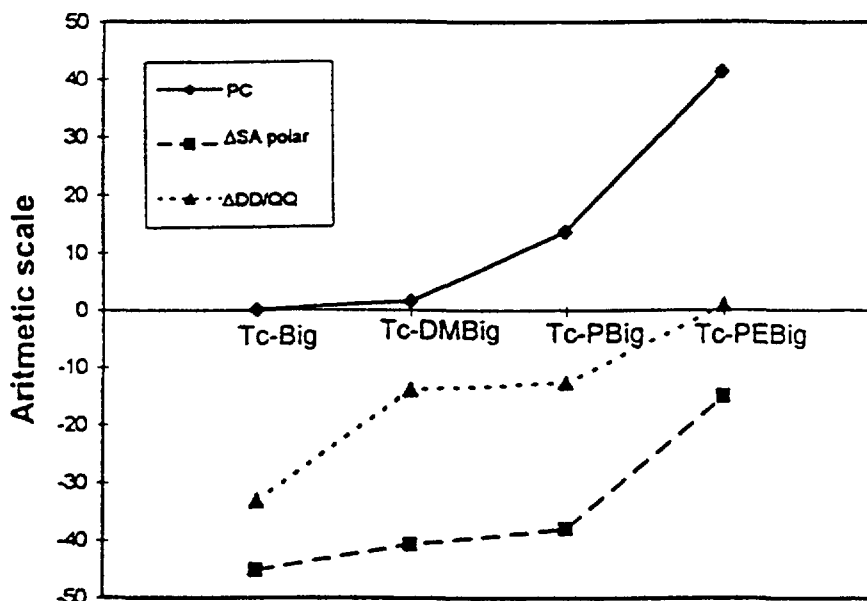


FIG. 2. Partition coefficients (PC), ΔSA polar and $\Delta DD/QQ$ for Tc-biguanide complexes.

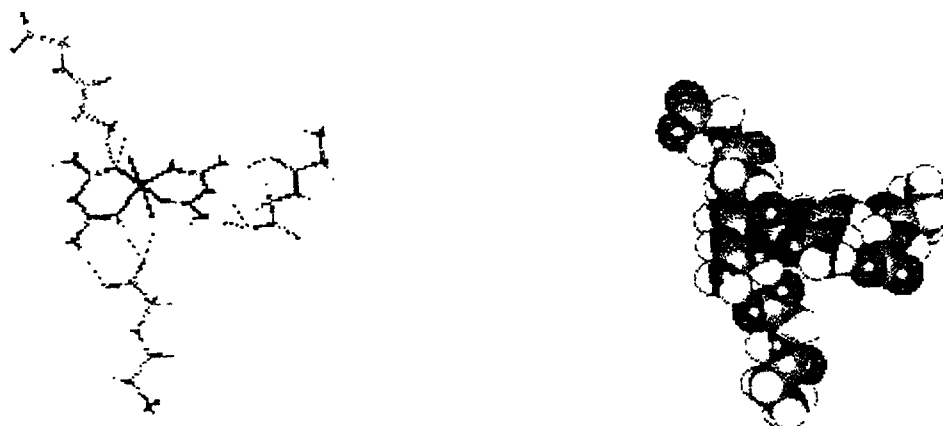


FIG. 3. Minimum energy aggregate resulting from interaction of $[TcO(Big)_2(OH)]$ with three peptide chains (ball & stick and CPK space filling models; H bonds represented as dash lines)

form hydrogen bonds with the electronegative nitrogen atoms of the Tc-complex (charges ranging from -0.19 to -0.46e), while the oxygen atoms of both the C=O and COO⁻ groups of the dipeptide (charges: C=O, -0.29e; C(=O)O⁻, -0.41e; C(=O)O⁻, -0.79e) form hydrogen bonds with the electropositive hydrogen atom of the Tc-OH group (charge +0.29e) and with the hydrogen atoms of the imino (+0.22e) and amino (+0.15e) groups of the biguanide ligands. The cyclization of the dipeptide can occur as shown in Figure 3, through hydrogen bond formation between the COO⁻ and C=O groups with imino and amino groups. The COO⁻ group of a second dipeptide, is also hydrogen bonded to the Tc-OH, imino and amino

groups. A third dipeptide is hydrogen bonded by its NH_3^+ group to an imino group of the complex.

The Tc-complexes of the remaining ligands studied do not have as many amino groups accessible as $[\text{TcO}(\text{Big})_2(\text{OH})]$. In addition, the effect of substitution of hydrogen atoms by the dimethyl, phenyl and phenethyl groups on the N1 atom leads also to a reduction of the charges on all nitrogen atoms, specially those that stay closer to the substitution site. This decreasing effect can be explained considering that coordination favors electronic delocalization involving the N atoms. On the other hand, the presence of non-polar groups leads to the appearance of essentially hydrophobic surfaces that limit the extension of the attractive interactions involving polar surfaces of the complex and the peptide chains. In the case of the N1 dimethyl derivative only one peptide chain interacts significantly with the complex (two hydrogen bonds, between the COO^- and Tc-OH groups and an imino group are predicted). Additional peptide chains are pushed out by the essentially non-polar dimethyl groups. In the $[\text{TcO}(\text{PBig})_2(\text{OH})]$ and $[\text{TcO}(\text{PEBig})_2(\text{OH})]$ complexes a similar effect is promoted by the phenyl and phenethyl groups.

The interaction of $[\text{TcO}(\text{Big})_2(\text{OH})]$ with the peptide chains is clearly distinct from the processes involving the other complexes studied. It is interesting to note that the percentages of human protein binding for the various complexes evaluated by gel filtration, are: $[\text{TcO}(\text{Big})_2(\text{OH})]=44.9\pm2.3$; $[\text{TcO}(\text{DMBig})_2(\text{OH})]=13.4\pm1.1$; $[\text{TcO}(\text{PBig})_2(\text{OH})]=10.6\pm0.9$ and $[\text{TcO}(\text{PEBig})_2(\text{OH})]=7.1\pm0.7$. Thus the percentage of human protein binding for $[\text{TcO}(\text{Big})_2(\text{OH})]$ is considerably higher than for the other complexes. This findings agrees with the theoretical results, which predict that $[\text{TcO}(\text{Big})_2(\text{OH})]$ is considerably more efficient in establishing energetically favorable hydrogen bonds with the peptide chains.

4. CONCLUSION

The elucidation, at a molecular level, of the way of functioning of pharmacologically relevant systems is one of the most important challenges in biomedical research. Molecular modeling has been playing an ever growing role in this field, and it appears nowadays as a very powerful method of study, whose relevance in the specific field of radiopharmaceuticals is very promising. The present study adopts this methodology to shed light on the molecular structures of pharmacologically relevant Tc-complexes of biguanides, and enabled us to establish fundamental relationships between some structure-related molecular properties and important physicochemical properties of the studied systems, such as partition coefficients and water affinity, as well as protein binding and peptide interactions. The success of this approach to study the kind of systems here considered opens good perspectives to start a series of systematic studies by this method directed both to the computer assisted design of

new Tc-radiopharmaceuticals and to the establishment of important correlations between their relevant molecular properties and results obtained from experimental *in vitro* studies.

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SYNTHESIS CHARACTERIZATION AND BIOLOGICAL EVALUATION OF A NOVEL MIXED LIGAND ^{99m}Tc COMPLEX AS POTENTIAL BRAIN IMAGING AGENT



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Abstract

One approach in the design of neutral oxotechnetium complexes is based on the simultaneous substitution of a tridentate dianionic ligand and a monodentate monoanionic coligand on a $[\text{Tc(V)O}]^{+3}$ precursor. Following this 'mixed ligand' concept, a novel ^{99m}Tc complex with N,N-bis(2-mercaptoethyl)-N,N-diethylethylenediamine as ligand and 1-octanethiol as coligand is prepared and evaluated as potential brain radiopharmaceutical. Preparation of the complex at tracer level was accomplished by using ^{99m}Tc -glucoheptonate as precursor. The substitution was optimized and a coligand/ligand ratio of 5 was selected. Under these conditions the labeling yield was over 80% and a major product (with radiochemical purity > 80%) was isolated by HPLC methods and used for biological evaluation. Chemical characterization at carrier level was developed using the corresponding rhenium complex as structural model. The Re complex was also prepared by substitution method and isolated as a crystalline product. The crystals were characterized by UV-vis and IR spectra and elemental analysis. Results were consistent with the expected ReOLC structure. X-ray crystallographic study demonstrated that the complex adopts a distorted trigonal bipyramidal geometry. The basal plane is defined by the SS atoms of the ligand and the oxo group, while the N of the ligand and the S of the coligand occupy the two apical positions. All sulphur atoms underwent ionization leading to the formation of a neutral compound. ^{99}Tc complex was also prepared. Although it was not isolated due to the small amount of reagents employed, the HPLC profile was identical to the one observed for the rhenium complex, suggesting the same chemical structure. Biodistribution in mice demonstrated early brain uptake, fast blood clearance, excretion through the hepatobiliary system and a brain/blood ratio that increased significantly with time.

1.- Introduction

The development of neutral lipid soluble ^{99m}Tc complexes capable of penetrating the intact blood brain barrier is still a major goal in radiopharmaceutical research, as brain perfusion imaging allows detection of various cerebral vascular abnormalities (stroke, transient ischemia) and

benefits management of neurological disorders (Alzheimer, epilepsy, Parkinson)

Two ^{99m}Tc brain perfusion agents are extensively used in clinical Nuclear Medicine: ^{99m}Tc - d,l-hexamethylpropyleneamineoxime (^{99m}Tc - HMPAO) (1,2) and ^{99m}Tc -l,l-ethylenecysteine- dimer (^{99m}Tc -ECD) (3). ^{99m}Tc - HMPAO is based on a N_4O_2 ligand, which loses two amine protons and an oxime proton on coordination to the Tc(V) monooxocore, resulting in a neutral complex. The complex is taken up by brain and then decomposes to a more hydrophilic species that is unable to diffuse back out of the brain. Instability, responsible of prolonged brain retention, occurs also "*in vitro*" and is considered a major disadvantage for routine assessment of brain perfusion (3). The ligand of ^{99m}Tc - ECD belongs to the family of the diaminedithiols (DADTS) (4). The $[\text{Tc(V)O}]^{3+}$ core coordinates with 2 nitrogen and 2 sulphur donor atoms to form a complex with 3 5-membered rings which is very stable "*in vitro*", easily crosses the blood brain barrier, and distributes in the brain according to regional blood flow. The retention mechanism of ^{99m}Tc -ECD has been identified as an specific enzymatic process occurring in the brain of the primates.

Both ^{99m}Tc -HMPAO and ^{99m}Tc -ECD are formed by the action of tetradentate ligands on the $[\text{Tc(V)O}]^{3+}$ core. Another approach in the design of neutral oxotechnetium complexes is based on the simultaneous substitution of a tridentate dianionic ligand and a monodentate monoanionic coligand on a $[\text{Tc(V)O}]^{3+}$ precursor (5,6). The advantage of the "mixed ligand concept" in the design of radiopharmaceuticals lies in the variety of possible tridentate/monodentate complexes that can be synthesized by modifying either the ligand or the coligand.

Various TcOLC complexes, where L is a tridentate aminothiols and C is a monodentate aromatic monothiols, have already been synthesized and

characterized (7,8) . In the present study a novel mixed ligand ^{99m}Tc complex with N,N-bis(2-mercaptoethyl)-N',N'-diethylethylenediamine as ligand and 1-octanethiol as coligand is prepared, characterized and evaluated as potential brain perfusion radiopharmaceutical.

2.- Materials and Methods

2.1.- Synthesis of the ligand

The ligand was synthesized by reacting N,N-diethylethylenediamine with ethylene sulfide in toluene at 110°C, following a previously described method (9) . Purification was achieved by high vacuum distillation. Commercially available 1-octanethiol was used as coligand.

2.2.- Preparation of ^{99m}Tc complex

Preparation of the mixed ligand complex at tracer level was accomplished by using ^{99m}Tc -glucoheptonate as precursor. A vial containing a lyophilized mixture of 200 mg calcium glucoheptonate and 0.2 mg SnCl_2 was reconstituted with 5 mL water and 0.5 mL of this solution was mixed with 0.5-1 mL $^{99m}\text{TcO}_4^-$ (Elumatic III generator , Cis Biointernational) with an activity of 5 - 50 mCi (185 - 1850 MBq). Substitution was performed at different coligand/ligand molar ratios (1, 5 and 10) by adding the precursor (with radiochemical purity > 95%) to a centrifuge tube containing the ligand (0.02 moles) and the coligand . The mixture was agitated in a vortex mixer and left to react at room temperature for 10 minutes. The lipophilic species were extracted with CH_2Cl_2 and the organic layer dried with MgSO_4 , filtered and analyzed by paper chromatography (Whatman 1, ethyl acetate) and normal phase HPLC (Bondclone-Si column,

metanol:dicloromethane 50:50, 0.5 mL/min). Activity measurements were performed either in a Dose Calibrator (Carpintec CRC- 5R) or in a scintillation counter (3"x3" NaI (TI) crystal detector associated to an ORTEC monochanel analyzer). HPLC analysis was developed using a LC-10 AS Shimadzu Liquid Chromatograph coupled to a scintillation counter.

2.3.- Preparation of rhenium analog

Chemical characterization was developed at carrier level using the corresponding rhenium complex as structural model. The oxorhenium precursor trans-oxotrichlorobistriphenylphosphine rhenium (V) $[\text{ReOCl}_3(\text{PPh}_3)_2]$ was reacted with a mixture of ligand (0.2 mmoles) and coligand (molar ratio 1:1:5) in methanol under reflux, until green-yellow color of the precursor turned to green. After being cooled at room temperature the reaction mixture was extracted with CH_2Cl_2 and the organic layer dried and filtered. The solvent was removed under reduced pressure to aprox. 5 mL and 5 mL of isopropanol were added. Slow evaporation of the solvents afforded the product as green crystals. HPLC analysis of the reaction mixture and the crystals (using the same conditions as at tracer level) was also performed. Detection was accomplished with a photodiode array detector (SPD-M10A, Shimadzu) that recorded UV-vis spectra on flux.

IR spectrum was obtained from KBr pellets in the range 4000 to 400 cm^{-1} on a Bomen MB-102 FT-IR spectrophotometer. Elemental analysis was performed on a Carlo Erba EA 1108 analyzer.

2.4.- Crystallographic studies

Diffraction measurements were made on a Crystal Logic Dual Goniometer diffractometer using graphite monochromated Mo radiation. Unit cell dimensions were determined and refined using the angular settings of automatically centered reflections in the range $11 < 2\theta < 23$. Intensity data were recorded using a $\theta - 2\theta$ scan to 2θ (max) = 50 deg. with scan speed 1.5 deg/min and scan range 2.4 plus $\alpha_1\alpha_2$ separation. Three standard reflections monitored every 97 reflections showed less than 3% variation and no decay.

Table I.- Summary of Crystal,Intensity Collection and Refinement Data

	Complex ReOLC
Formula	C ₁₈ H ₃₉ N ₂ OS ₃
FW	581.92
Temp, K	298
Wavelength	Mo Ka 0.71073
Space group	P2 ₁ /n
a (Å)	16.506 (9)
b (Å)	9.997 (5)
c (Å)	15.339 (8)
β , deg	103.03 (1)
V (Å ³)	2466
Z	4
D _{calcd} (Mg m ⁻³)	1.567
Abs coeff, (μ), mm ⁻¹	5.19
Scan mode/speed (deg/min)	$\theta - 2\theta/1.5$
Scan range (deg)	2.4 + $\alpha_1\alpha_2$ separation
θ range (deg)	50
Reflections collected	4796
Independent reflections	4335 [R(int) = 0.0199]
Range of h, k, l	0 - 13, 0 - 13, 20 - 19
F (000)	1168
$[\Delta/\sigma]_{\max}$	0.802
$[\Delta\rho]_{\max}$ $[\Delta\rho]_{\min}$ (C/(Å ³))	1.24 and - 1.06
Refinement method	Full matrix least squares on F ²
Data/ parameters	4335/235
Goodness of fit on F ²	1.100
R indices [4335 refs I > 2 σ (I)] ⁽²⁾	R1 = 0.0530 wR2 = 0.1618
R indices (all data)	R1 = 0.0773 wR2 = 1618

(1) R1 based on F's, wR2 based on F²

Lorentz polarization correction and psi-scan absorption corrections were applied using Crystal Logic software. Symmetry equivalent data were averaged with $R=0.0199$ to give 4335 independent reflections from a total of 4796 collected. The structure was solved by direct methods using SHELXS-86 (10) and refined by full-matrix least squares techniques on F^2 with SHELXL-93 (11) using 4335 reflections and refining 235 parameters. All hydrogen atoms were introduced at calculated positions as riding on bonded atoms. All non-hydrogen atoms were refined anisotropically. The final values for R_1 , wR_2 and GOF for observed data are 0.0530, 0.1618 and 1.100 and for all data are 0.0773, 0.1618 and 1.097 respectively. The maximum and minimum residual peaks in the final difference map were 1.24 and - 1.06 e/ Å³. The largest shift/esd in the final cycle was 0.802. A summary of crystal, intensity collection and refinement data is presented in Table I.

2.5.- Preparation of ⁹⁹Tc complex

A solution of stannous chloride (11.3 mg) in HCl (1N, 1 mL) was added to an aqueous solution of K⁹⁹TcO₄ (Amersham International, 4 mg) and sodium gluconate (20 mg) to obtain ⁹⁹Tc-gluconate. The pH of the solution was adjusted to 7.5 with 0.1 N NaOH before adding it with stirring to a mixture of ligand (0.02 mmoles) and coligand (0.1 mmoles) in methanol. After 20 minutes stirring the solution was extracted with CH₂Cl₂. The organic phase was separated, dried over MgSO₄, filtered and analyzed by HPLC.

2.6.- Biodistribution studies

Normal mice (CD1, 25-30 g, 4 animals per group) were injected via tail vein with aprox. 100 µCi (3.7 MBq) of the HPLC purified ^{99m}Tc complex. At

different intervals after injection (1 to 60 minutes) the animals were sacrificed by neck dislocation. Whole organs, total urine volume and samples of blood and muscle were collected, weighted and assayed for radioactivity. Corrections by different sample geometry were applied when necessary.

3.- Results and Discussion

Simultaneous action of the tridentate ligand N,N-bis(2-mercaptoethyl)-N',N'-diethylethylenediamine and the monodentate coligand 1-octanethiol on the $^{99m}\text{Tc(V)}$ glucoheptonate at different coligand/ligand molar ratios, produced the formation of ^{99m}Tc lipophilic species, which were extracted by CH_2Cl_2 with high yield (> 80% in all cases). The organic layer was analyzed by paper chromatography and HPLC in order to study the different ^{99m}Tc species formed upon substitution. Paper chromatography demonstrated the presence of at least 2 species ($R_f=0$ and $R_f=1$ respectively) whose percentage correlated with the coligand/ligand ratio. HPLC analysis showed a major peak with a retention time of about 7 minutes and a secondary one of about 12. Besides part of the activity was not eluted from the column. The retained activity was approximately equal to the percentage of activity with $R_f = 0$ in paper chromatography. A molar ratio coligand/ligand of 5 yielded 80% activity elution from the HPLC column as well as 80% purity of the complex with 7 minutes retention time and was consequently selected to continue the study.

Chemical characterization was developed at carrier level using the corresponding rhenium complex as structural model. Rhenium, as technetium's third row congener, exhibits many of the chemical properties of technetium. ^{99}Tc complexes and Re complexes with identical ligands have essentially identical coordination parameters and Re has environmentally preferable non

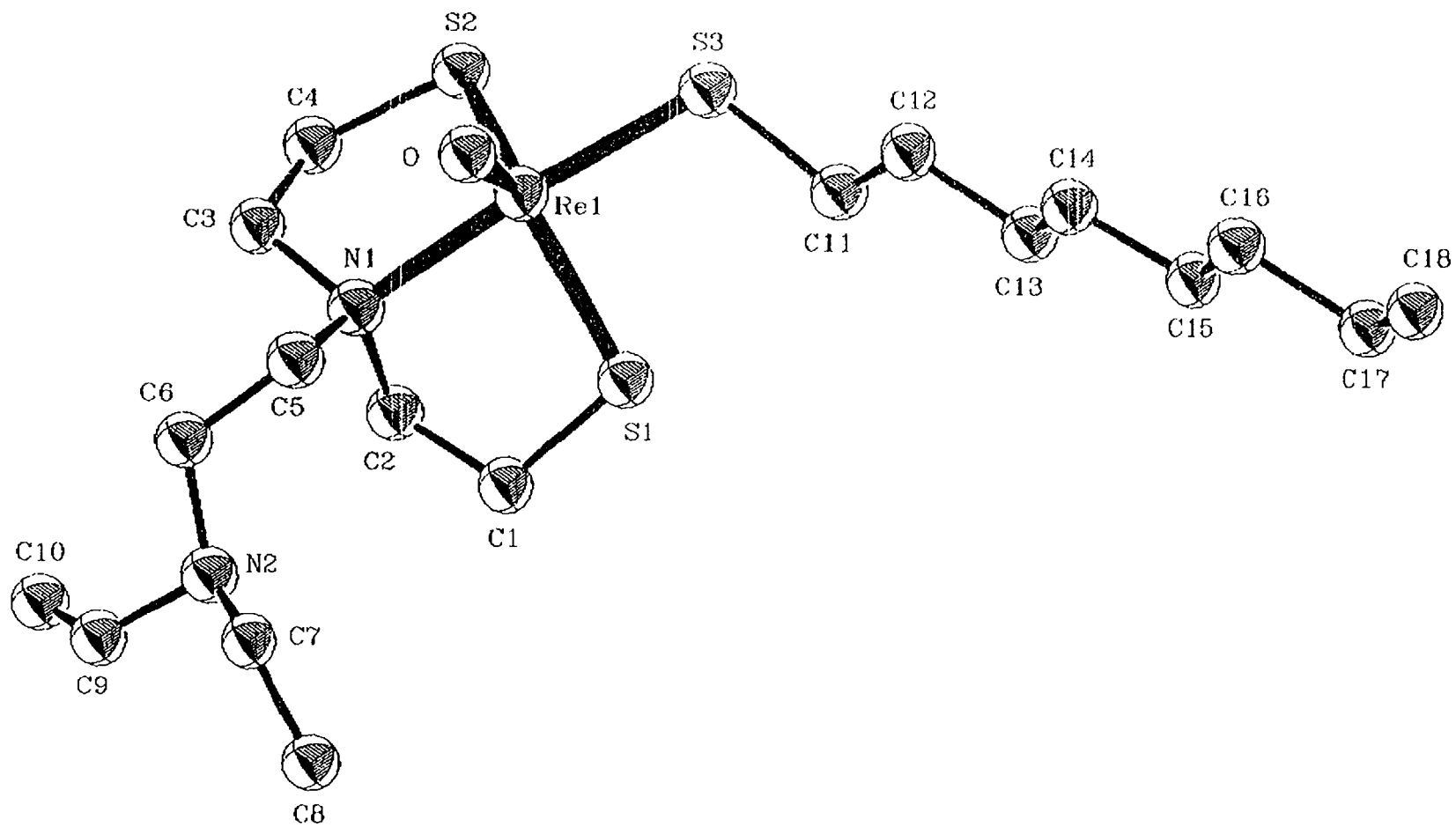


Figure 1.- ORTEP diagram of ReOLC with 50% thermal ellipsoid probability .

Table II.- Selected bond distances (Å) and angles (degrees) of ReOLC

	Complex ReOLC
Re - O1	1.682 (7)
Re - S1	2.277 (3)
Re - S2	2.271 (3)
Re - S3	2.277 (4)
Re - N1	2.188 (11)
O1 - Re - S1	119.97 (31)
O1 - Re - S2	117.55 (30)
O1 - Re - S3	105.70 (34)
O1 - Re - N1	95.38 (37)
N1 - Re - S1	82.48 (21)
N1 - Re - S2	83.94 (23)
N1 - Re - S3	158.77 (22)
S1 - Re - S2	121.74 (11)
S1 - Re - S3	88.96 (14)
S2 - Re - S3	84.14 (16)

radioactive isotopes. Substitution of ligand and coligand on the $[\text{Re}(\text{VO})]^{3+}$ precursor in a molar ratio 1:5 yielded only one complex according to HPLC analysis. The retention time was identical to the major $^{99\text{m}}\text{Tc}$ complex. Furthermore, when $^{99\text{m}}\text{Tc}$ and Re complexes were coinjected, both radioactivity (for tracer) and UV-vis detectors exhibited identical chromatographic profiles, suggesting the same chemical structure was formed under both chelating conditions. The rhenium complex was obtained as a crystalline product. It is neutral, stable and lipophilic, as indicated by its quantitative extraction from the

aqueous to the organic layer during isolation. ReO stretching vibration frequency (942.2 cm^{-1}) is consistent with other reported for monooxo rhenium(V) complexes and demonstrated the presence of the $[\text{Re}(\text{V})\text{O}]^{3+}$ core. UV-vis spectrum exhibited an intense band at approximately 398 nm, probably due to $\text{S} \rightarrow \text{Re}$ charge transfer transition. Additional absorptions at shorter wavelengths (235, 264 nm) correspond to ligand and coligand. Elemental analysis results were consistent with the presence of one molecule ligand and

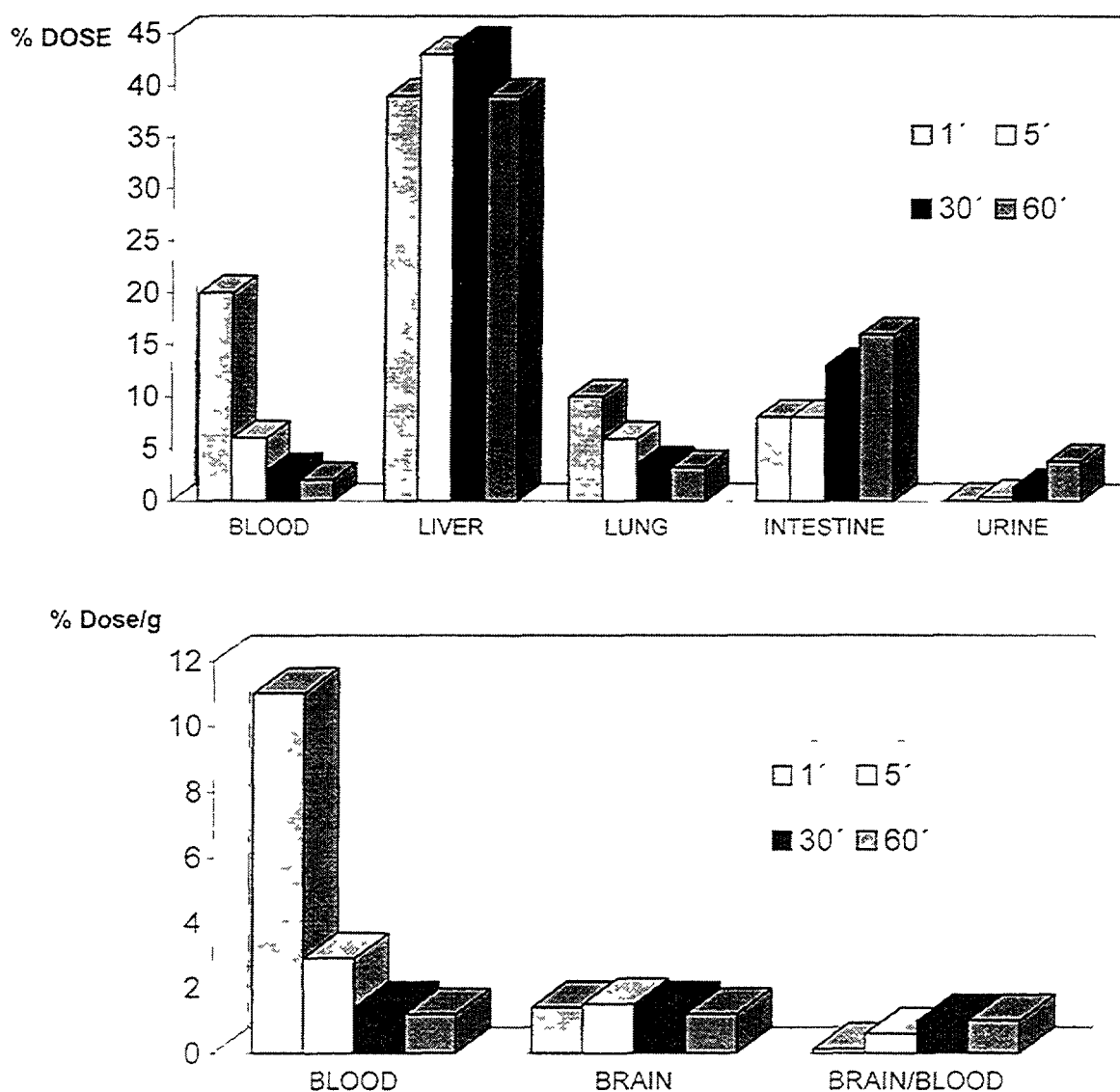


Figure 2.- Biodistribution results from $^{99\text{m}}\text{TcOLC}$ in mice

one of coligand per complex molecule. Figure 1 shows the ORTEP diagram for the complex. The coordination geometry is distorted trigonal bipyramidal, the calculated trigonality index, τ , being 0.62. The basal plane is defined by the SS atoms of the ligand and the oxo group, while the N of the ligand and the S of the coligand occupy the two apical positions. Table II shows selected bond distances and angles. All sulphur atoms underwent ionization leading to the formation of a neutral compound.

^{99}Tc complex was also prepared by exchange method using ^{99}Tc -gluconate as precursor. Although the mixed ligand complex could not be isolated due to the small amount of reagents employed (0.02 mmoles), HPLC profile was identical to the one observed for the rhenium complex suggesting the same chemical structure.

Biodistribution studies of the major $^{99\text{m}}\text{Tc}$ complex (eluted from HPLC) were also performed. Figure 2 shows activity (%D/organ) for selected organs and tissues, as well as brain and blood uptake and retention (% Dose/g of organ) as a function of time. The complex demonstrated high initial blood, lung and liver uptake ($20 \pm 5\%$, $10 \pm 2\%$ and $39 \pm 3\%$ respectively at 1 min. post injection), as expected from a lipophilic complex. Blood and lung clearance was rather fast while liver activity remained essentially constant during the period of study. Brain initial uptake was not notably high ($0.51 \pm 0.08\%$ at 1 min. post-administration) but remained constant with time (until 60 minutes). Excretion occurs mainly through hepatobiliary system as demonstrated by high intestinal activity ($16 \pm 1\%$ at 60 min. post-adm.), urinary excretion being very low ($3.8 \pm 0.8\%$ at 60 min.). Stomach and thyroid values were within acceptable levels ($0.12 \pm 0.01\%$ and $2.0 \pm 0.5\%$ respectively at 60 min.), indicating no "in vivo" decomposition. Prolonged brain retention together with fast blood clearance

determined a brain/ blood ratio that increased significantly with time (0.13 ± 0.01 % 1 min. post -administration and 1.0 ± 0.3 % at 60 min) .

4.- Conclusions

Neutral mixed-ligand oxotechnetium complexes with N,N-bis(2-mercaptoethyl)-N',N'-diethylethilenediamine as ligand and different aromatic thiols as coligands have already been successfully evaluated as potential brain radiopharmaceuticals. This study presents a novel compound in which the coligand is an aliphatic thiol.

Its coordination chemistry has been extensively studied through the rhenium model. The elucidated structure was similar to other mixed complexes with the same ligand but a molar ratio coligand/ligand of 5 was necessary to obtain the complex with high yield, suggesting that aliphatic thiols have less binding ability than aromatic thiols. Other aliphatic thiols should be studied to support this hypothesis. ^{99m}Tc complex was also formed in a reproducible way. Corroboration of the structure was achieved by comparing high performance liquid chromatographic profiles of rhenium/ $^{99}\text{Tc}/^{99m}\text{Tc}$ complexes. The results suggest that in this case rhenium complex is an adequate model to study the coordination chemistry at carrier level.

Biological evaluation demonstrated prolonged brain retention as well as high brain/blood ratios that make this complex promising for further development of brain perfusion imaging agents.

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UPTAKE OF ^{99m}Tc -TETROFOSMIN BY BREAST CANCER, SARCOMA AND MELANOMA CELL LINES

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XA9847986

Abstract

^{99m}Tc - tetrofosmin was developed for myocardial perfusion imaging. However, it has been suggested that this cationic tracer may be of value as a tumor-imaging agent. Very little is however known about the mechanism of uptake of ^{99m}Tc -tetrofosmin in heart tissue or tumor cells. We studied in-vitro the uptake of this radiopharmaceutical in breast cancer-, sarcoma- and melanoma cell lines. The influence of density of tumor cells, temperature- and time of incubation on the radioactivity uptake were analysed.

In these tumor cell lines the uptake of ^{99m}Tc -tetrofosmin was highest at a cellular density of 1.10^6 cell/ml and at a temperature of incubation of 37°C . This indicates that the uptake of ^{99m}Tc -tetrofosmin by these tumor cells occurs by a metabolism-dependent process, probably related to mitochondria, besides cation channel transport. ^{99m}Tc -tetrofosmin scintigraphy might thus be useful for detecting and imaging at a very early clinical stage these malignant tumors.

1. Introduction

The diagnosis of malignant disease consists of locoregional assessment and staging of disease throughout the body. Whereas local disease can often be diagnosed by simple techniques and treated surgically, metastatic spread often demands sophisticated diagnostic procedures and systemic therapy. Nuclear medicine imaging has greatly facilitated the staging process.

In 1995 Rambaldi et al (1) accidentally found a hot spot in a mammary gland during myocardial ^{99m}Tc -tetrofosmin (1,2-bis[bis(2-ethoxyethyl) phosphino]ethan) scintigraphy, a finding that has

encouraged further research into the clinical relevance of this new radiopharmaceutical. In this study the uptake characteristics of ^{99m}Tc -tetrofosmin was evaluated in tumor cell lines *in vitro*. The aim of the study was to investigate the potential relevance of ^{99m}Tc -tetrofosmin scintigraphy for tumor detection.

2. Material and Methods

Breast cancer- [MCF-7 (cloned carcinoma), ZR-75-1 (human adenocarcinoma) and SKBR-3 (human adenocarcinoma)], sarcoma- [A-204 (rhabdomyosarcoma), SW 684 (fibrosarcoma), SW

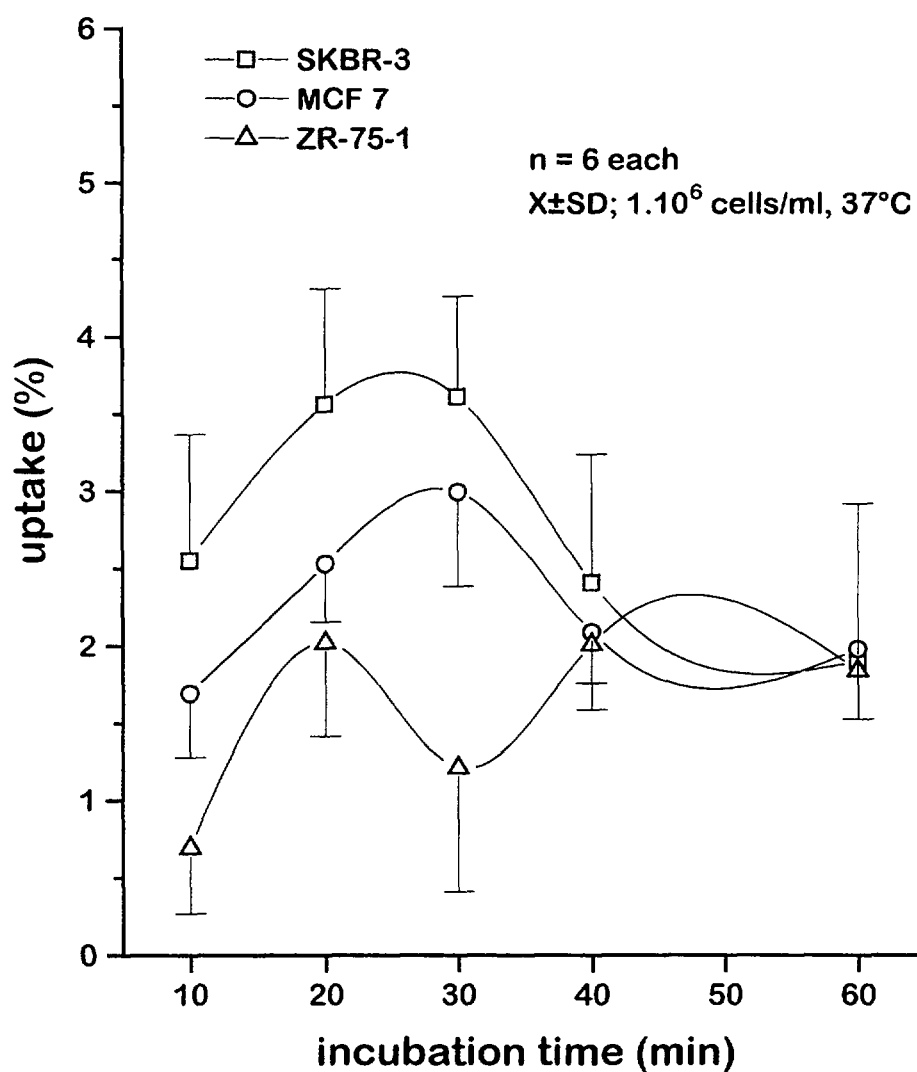


FIG. 1. Breast cancer cell lines uptake of ^{99m}Tc -tetrofosmin.

872 (liposarcoma), SW 982 (synovial sarcoma) and SW 1353 (chondrosarcoma)] and melanoma (SK-MEL 28 and 518 A-2) cell lines were investigated. The influence of density of tumor cells (1.10^5 - 1.10^6 cell/ml), temperature- (4°C , 22°C , 37°C) and time (10-60 minutes) of incubation on the radioactivity uptake ($100 \mu\text{Ci } ^{99\text{m}}\text{Tc}$) were analysed.

Statistical analysis was performed using Students *t* test. A value of $p < 0.01$ was considered as significant.

3. Results

Studies with breast cancer lines *in vitro* revealed a higher uptake (although not significantly different) of $^{99\text{m}}\text{Tc}$ -tetrofosmin for SKBR-3 ($3.61 \pm 0.65\%$), followed by MCF-7 ($2.99 \pm 0.33\%$) and

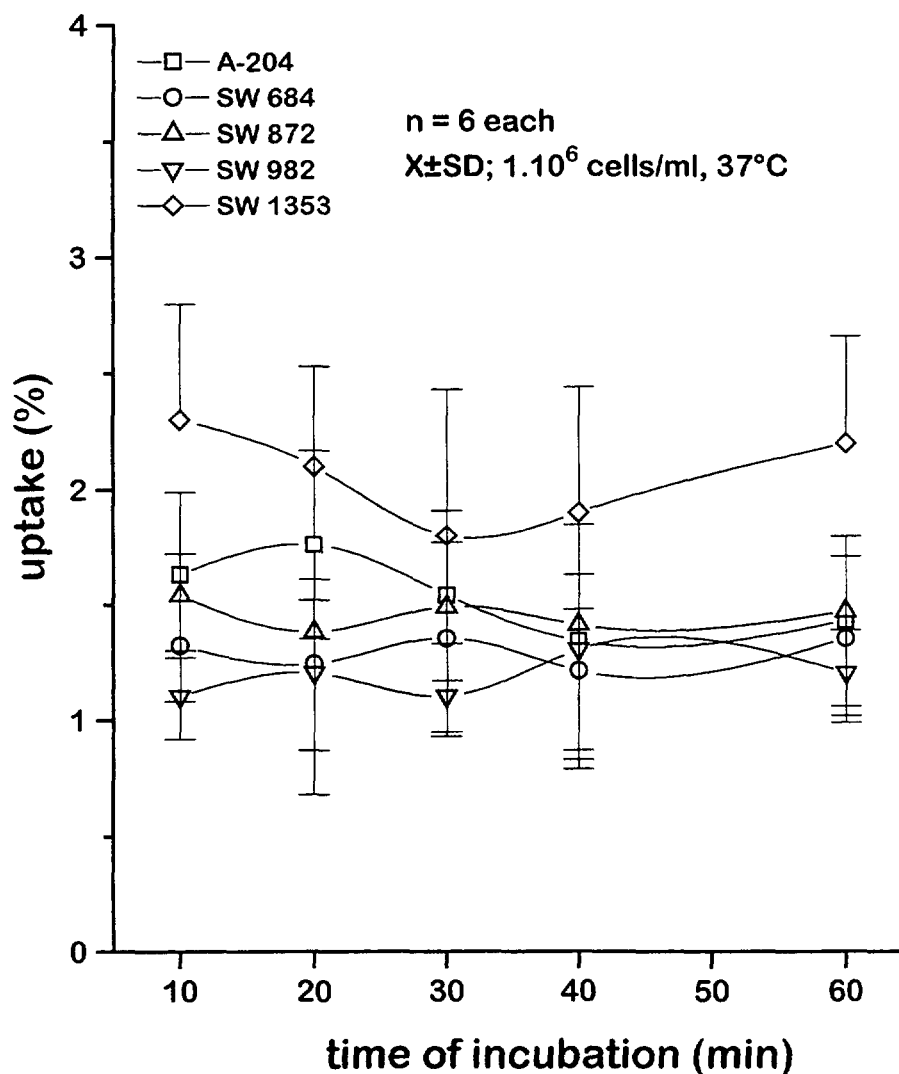


FIG. 2. Sarcoma cell lines uptake of $^{99\text{m}}\text{Tc}$ -tetrofosmin.

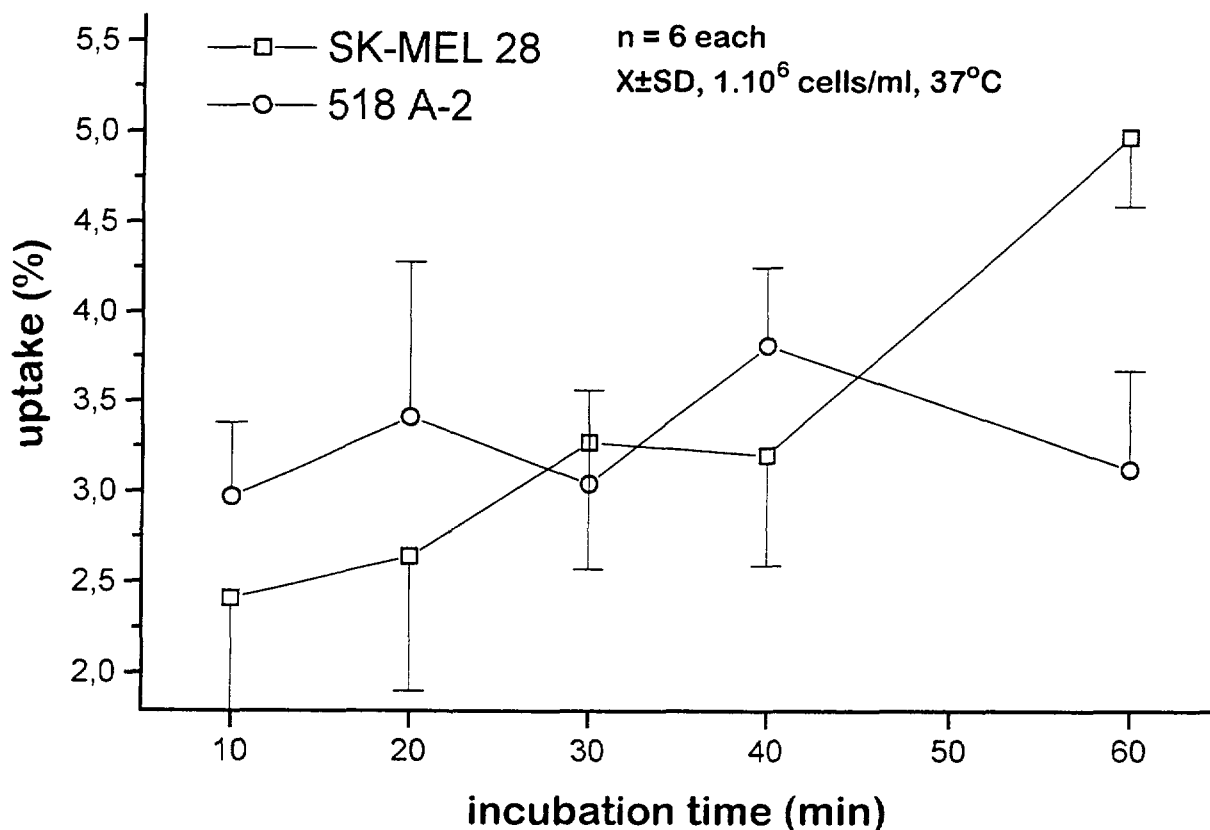


FIG. 3. Melanoma cell lines uptake of ^{99m}Tc-tetrofosmin.

ZR-75-1 (2.02 \pm 0.60%). In these tumor cell lines, the uptake was highest at a cellular density of 1.10⁶ cell/ml and at a temperature of incubation of 37°C and it reached a maximum at 30-40 minutes after incubation, starting to decline after about 60 minutes (Fig. 1). Significantly higher uptake of ^{99m}Tc-tetrofosmin by sarcoma cell line SW 1353 (2.30 \pm 0.40%) as compared to the other sarcoma cell lines studied (uptake in the range of 1-2%) was found (Fig. 2). Cell lines SW 684, SW 872 and SW 982 showed an uptake ^{99m}Tc-tetrofosmin obviously lower at 4°C and only slight incread at 37°C as compared to 22°C, whereas in the other cell lines, A-204 and SW 1353, the uptake was highest at 37°C. Sarcoma cells uptake of ^{99m}Tc-tetrofosmin was highest at a cellular density of 1.10⁶ cell/ml, reaching its maximum as early as after 10 minutes. In melanoma cell lines SK-MEL 28 and 518 A-2 the uptake of ^{99m}Tc-tetrofosmin was highest at 37°C and at a cellular density of 1.10⁶ cell/ml as well (Fig. 3).

4. Discussion

^{99m}Tc -tetrafosmin was introduced for myocardial perfusion imaging. However, this newly lipophilic cation has been recently examined in clinical studies as a radiopharmaceutical for tumor-imaging as well. It has recently been reported to be suitable for clinical evaluation of breast tumors (2) and for functional imaging of multidrug resistance (3), among others. Very little is however known about the mechanism of uptake of ^{99m}Tc -tetrafosmin in heart tissue or tumor cells. A possible role of cell membrane potential and of the Na^+/K^+ pump has been hypothesized for ^{99m}Tc -tetrafosmin cellular uptake (4). In this study we investigate various tumor cell lines for ^{99m}Tc -tetrafosmin uptake kinetics. In most cell lines studied, the uptake of ^{99m}Tc -tetrafosmin was highest at 37°C , which might indicate that it occurs by a metabolism-dependent process, probably related to mitochondria, besides cation channel transport as suggested. ^{99m}Tc -tetrafosmin was taken up by breast cancer-, sarcoma- and melanoma cells, thus strengthening the hypothesis that these malignant cells can be imaged with ^{99m}Tc -tetrafosmin scintigraphy at a very early clinical stage due to the high number of high activity of mitochondria in these cells. Present on-going investigations are being focused on the role of mitochondria in the cellular uptake of ^{99m}Tc -tetrafosmin. In vivo at present on-going studies are mandatory to evaluate the exact clinical value of ^{99m}Tc -tetrafosmin scintigraphy in malignant disease.

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EXCHANGEABLE PULMONARY WATER SPACE EVALUATION USING GIANT LIPOSOMES

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Abstract

The present work aims to study the potential use of liposomes for the evaluation of pulmonary exchangeable water space, important parameter in some pulmonary oedema situations. This study is based upon the delivery of a diffusible water radiotracer into pulmonary capillary network, which equilibrates with interstitial water space of the lung and returns to the blood circulation. The time constant of this phenomena depends on the magnitude of the water space under study. The release of the diffusible radiotracer in lung capillaries is performed using liposomes with specific formulation. The giant liposomes (15-30 μm \varnothing) used in this study are instable at 37°C. They are biocompatible, biodegradable, with low toxicity and showed no immunogenicity.

A water tracer labelled with $^{99\text{m}}\text{Tc}$, encapsulated in the aqueous phase of giant liposomes, has been used. Liposomes were prepared in sterile conditions and with apyrogenic materials. The lipid films composition is L- α -diestearoylphosphatidylcholine (DSPC), L- α -phosphatidyl-DL-glycerol (EPG) and cholesterol (CHOL) (60%/10%/30% mass ratio).

After iv injection at $\pm 20^\circ\text{C}$ in the femoral vein of Wistar rats (300g-600g) or albino rabbits (4.5-5Kg), the thermolabile liposomes will be entrapped in lung capillaries and release the radiotracer locally. When the radiodrug is diffusible we will evaluate the volume of the exchangeable pulmonary water analyzing the activity/time curves. These curves are slower for greater water spaces. When the radiotracer is non-diffusible, the disappearance curves are not influenced by the extravascular water space.

1. INTRODUCTION

Liposomes are artificial vesicles with a phospholipid-based constitution. They have been studied widely since the 70's as a system for the delivery or targeting of drugs to specific sites in the body. They are biocompatible, biodegradable, present low toxicity and lack of immunogenicity. Due to their structural versatility in terms of size, composition, surface charge and bilayer fluidity, liposomes offer the possibility of being the potential to be tailored in a variety of ways to ensure the production of

optimal formulations for clinical use [1]. This includes controlled retention of entrapped drugs in the presence of biological fluids, controlled vesicle residence in the blood circulation or other compartments in the body, and enhanced vesicle uptake by target cells. Furthermore, we can use almost any administration pathway, including intramuscular, oral, topical, intravenous, etc.[2, 3, 4, 5, 6].

We aim to study the potential use of giant liposomes for the evaluation of the exchangeable pulmonary water space, an important parameter in several pulmonary oedema situations.

Liposomes are used as carriers of a diffusible radiotracer to be delivered in lung capillaries and which equilibrate with interstitial water space. The tracer disappearance rate depends on the magnitude of the referred space.

2. MATERIAL AND METHODS

We produced thermolabile unilamellar giant vesicles (GUV's) with 15-30 μ m \varnothing from a mixture of L- α -diestearoylphosphatidylcholine (DSPC), L- α -phosphatidyl-DL-glycerol (EPG) and cholesterol (CHOL), in a 60%/10%/30% mass ratio, respectively. This lipidic composition has a transition temperature of $\pm 37^{\circ}\text{C}$.

We used *Wistar* rats, essentially males, 3 to 4 months old (300-600g), and albino rabbits (4-5.5Kg) (*Oryctolagus cuniculus hyplus*) as experimental model animals. The animals were previously anesthetized with Ketamine (50mg/ml) and chlorpromazine (2.5%), according to national and international standard procedures, in order to catheterize the femoral vein with an heparinized abocat for iv injection.

As radiotracers we used 2,3-dimercaptosuccinic acid (DMSA); mercaptoacetyltriglycine (MAG3); diethylenetriaminopentacetic acid, as a monocalcic trissodium salt (DTPA) and rhenium sulphide colloid (Re_2S_7), labelled with $^{99\text{m}}\text{Tc}$. The first two are hydrosoluble and diffusible tracers, whereas the last two are hydrosoluble but non-diffusible.

The liposome preparation followed the Reeves & Dowben protocol [7], modified by Needham & Evans [8] and ourselves. The labelling efficiency and radiochemical purity were always tested. The liposome dimensions and homogeneity were determined by Coulter Multisizer[®] and confocal microscope BioRad[®] 600MRC. All the material has been autoclaved and manipulations were done in aseptic conditions.

They were suspended in 0.2-0.4 ml of cooled saline. The animals were placed directly over the collimator in dorsal and GUV's were administered by iv injection in the previously catheterized femoral vein.

To perform the information acquisition we used a γ -camera (Maxi camera Digital GE) controlled by a computer, and a low-energy convergent collimator. We acquired 400-600 images, to 64*64 matrices, of 10 seconds each. To store, process and visualize the data a PC computer and homemade software have been used.

After information acquisition we determined the *in vitro* activity in several organs and we also obtained tissue samples for histologic studies.

On any image of the acquired file, 4 Regions of Interest (ROI's) were drawn: brain, chest, right kidney, liver+spleen area. The average brain activity per pixel has been considered the background activity. The values of mean activity per pixel of each ROI, after background deduction, were used to obtain regional activity/time curves. An exponential adjustment has been made for chest activity/time curves.

3. RESULTS AND DISCUSSION

Giant liposomes (>7-10 μ m) are mechanically retained by the first capillary network they reach. Our thermolabile vesicles, due to their special feature, burst in the lung capillaries and release locally the encapsulated radiotracer. Therefore their presence is transitory and they will be removed by disintegration and phagocytosis [3].

The specified lipidic formulation has already been approved for clinical use.

Either by confocal microscope or by Coulter[®] analysis, the liposomal population is homogeneous which proves that the technique enables the systematic production of giant liposomes (Figs. 1, 2).

When the radiotracer is diffusible, such as DMSA (Fig.3), evaluation of the exchangeable water volume will be possible analyzing the activity/time curves (Fig. 4). The tracer will equilibrate with interstitial water space of the lung and returns to the blood circulation. These curves will probably be slower for greater water spaces.

For a non-diffusible radiodrug (ex: DTPA)(Fig. 5), the disappearance curves are not influenced by the interstitial exchangeable water space (Fig. 6).

The lipidic formulation we prepared (DSPC/EPG/CHOL) (fully approved for clinical use) enables the use of a non-invasive technique to evaluate the exchangeable pulmonary water space, through encapsulated radiotracers delivered by giant liposomes.

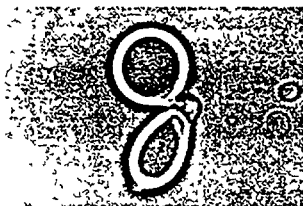


Fig. 1 - Giant unilamellar liposomes containing ^{99m}Tc -DTPA (15-30 μm , 100 \times).

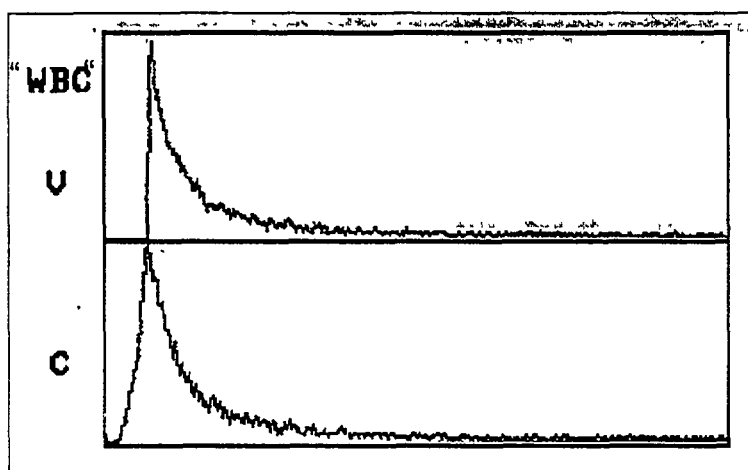


Fig. 2 - Coulter[®] graphic of our typical giant liposomal population: the system "recognizes" the liposomes as white blood cells (WBC) (dimension and volume): V=volume=85 to 90 fL; C=conductivity; $[] = \pm 3.3 \times 10^5/\text{ml}$; $\varnothing = \pm 15$ to 30 μm .

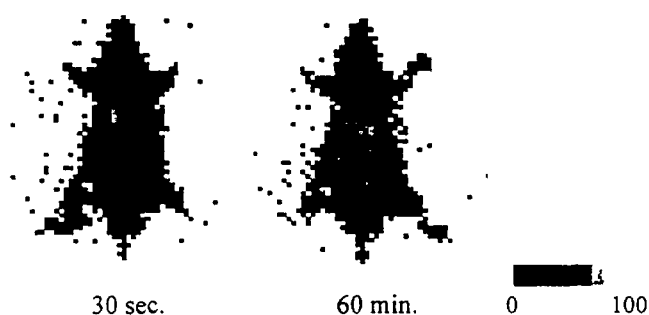


Fig. 3 - Scintigraphic images obtained after iv injection (femoral vein) of ^{99m}Tc -DMSA-GUV's in a rat. The gray colour scale shows higher activity values from black to white.

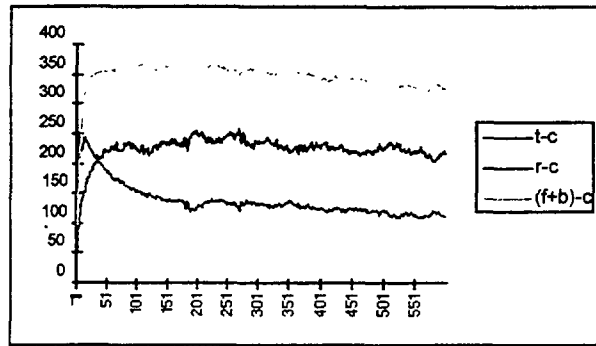


Fig. 4 - Activity/time curves of the rat, after background subtraction, for ^{99m}Tc -DMSA-GUV's: t-c = chest-brain; r-c = kidney-brain; (f+b)-c = (liver+spleen)-brain.

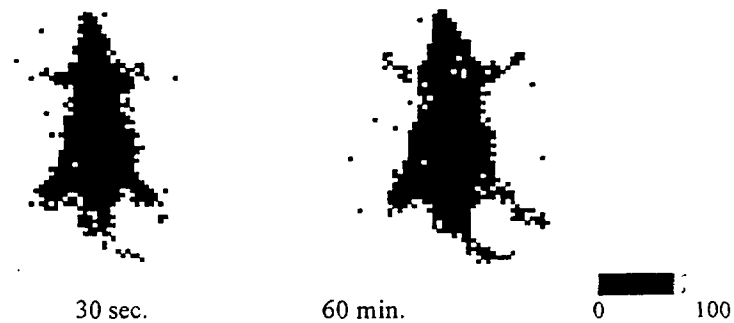


Fig. 5 - Scintigraphic images obtained after iv injection (femoral vein) of ^{99m}Tc -DTPA-GUV's in a rat. The gray colour scale shows higher activity values from black to white.

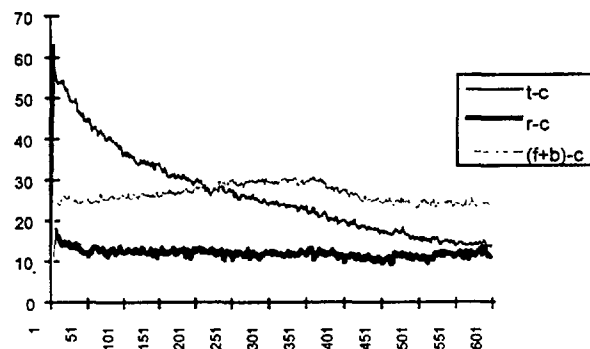


Fig. 6 - Activity/time curves, after background subtraction, for ^{99m}Tc -DTPA- t-c = chest-brain; r-c = kidney-brain; (f+b)-c = (liver+spleen)-brain.

We designed a formulation with phosphatidylglycerol (EPG) and cholesterol (CHOL) because:

- 1) the introduction of EPG, with a glycerol group in the polar head and negative surface charge, induces a more definite pulmonary liposomal affinity;
- 2) the CHOL enables us to control the release of entrapped hydrophilic substances in the aqueous phase, increases their *in vivo* and *in vitro* stability as well as the bilayer permeability at less than 41°C.

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Abstract

Ciprofloxacin (CIP) was labelled with $^{99}\text{Tc}^m$. The radiolabelled efficiency monitored by ITLC and HPLC was higher than 95%. The $^{99}\text{Tc}^m$ -CIP complex analyzed by those systems have shown that inactive and labelled CIP exhibit different chromatographic behavior. This finding together with octanol/saline partition coefficients determination indicated that CIP and $^{99}\text{Tc}^m$ -CIP correspond to different chemical structure. Biodistribution studies in inflamed mice shown that $^{99}\text{Tc}^m$ -CIP is rapidly distributed after i. v. administration with a predominant renal clearance. The radioactive preparation is able to localize bacterial and sterile inflammations induced by *Staphylococcus aureus*, *Escherichia coli* and turpentine, which suggest that its accumulation is due to increased blood flow together with enhanced vascular permeability as also postulated to other non-specific radiopharmaceuticals.

1. INTRODUCTION

Fluoroquinolones represent a new group of compounds that exhibit a very high activity against a broad spectrum of bacteria. ^{18}F -quinolones as Fleroxacin [1] and Trovafloxacin [2] which have three fluorine atoms in its native structure, have been labelled with ^{18}F by fluorine exchange and proposed as tracers for PET measurements in the study of the pharmacokinetics of these quinolones in normal and infected animals and humans. Due to the quantitative nature of PET measurements the absolute concentration of drug in all tissues of the body could be determined. The pattern of biodistribution obtained was consistent with the results of similar studies with ^{14}C [2]. Among the marketed quinolones ciprofloxacin (1-cyclopropyl-6-fluor-1,4-dihydro-4-oxo-7-(piperazinyl)-3-quinoline carbonic acid), a new quinoline with activity against both gram-negative and gram-positive organisms, has shown to be more potent than the structurally similar compounds [3]. Owing to its biological properties ciprofloxacin (CIP), has been described as a promising agent for the treatment of different kinds of infection. Vinjamuri et al. have recently developed a new technetium-99m radiopharmaceutical ($^{99}\text{Tc}^m$ -infecton) based on ciprofloxacin [4-8] as a bacterial infection specific imaging agent in differential diagnosis between infectious and sterile inflammations to overcome the limitation of some radiopharmaceuticals as leukocytes and human immunoglobuline. The aim of the present study was to investigate the radiochemical and biological properties of $^{99}\text{Tc}^m$ -ciprofloxacin, prepared according to the method proposed by Vinjamuri and evaluate its value in the imaging of infection *versus* sterile inflammation induced in lateral thigh of the mice by subcutaneous injection of *Staphylococcus aureus* and *Escherichia coli* or intramuscular turpentine oil administration.

2. MATERIALS AND METHODS

2.1. Chemicals

Ciprofloxacin (CIP) from Tecnimed (Sacavém, Portugal) was used. The chemical structure is presented in Fig.1. The purity of CIP was assayed using a HPLC (Perkin Elmer, Norwalk, USA) system with a Nucleosil C₁₈ column (8x250 mm) fitted with a fixed UV (280 nm Perkin Elmer, Norwalk, USA) detector. The mobile phase consisted of a mixture of acetonitrile and 0.05 M phosphate buffer adjusted to pH 3.0 with phosphoric acid (20:80, vol/vol), also adjusted to pH 3.0, to which a suitable amount of tetrabutylammonium bromide was added to obtain a 0.01 M solution. Chromatography was carried out at a flow rate of 0.5 mL/min. Pertechnetate was eluted from a ⁹⁹Mo-⁹⁹Tc^m generator (Amersham, UK). All the reagents were analytically pure.

2.2. Radiolabelling of ciprofloxacin

Ciprofloxacin was radiolabelled with ⁹⁹Tc^m according to Vinjamuri method [6]. To a suitable amount of ciprofloxacin an appropriate quantity of formamidine sulphonate acid (FSA) solution was added to a molar ratio CIP/FSA of 1.6:1. For labelling 0.5-1.5 mL (37-185 MBq) of ⁹⁹Tc^m-sodium pertechnetate solution were added and the reaction mixture was then boiled at 100° C for 10 min.

⁹⁹Tc^m-CIP labelling efficiency, radiochemical purity and stability of the ⁹⁹Tc^m-CIP were accomplished by the following chromatographic systems:

- (a) ITLC SG (Gelman Sciences Inc., Ann Arbor, MI) with saline eluent (labelled CIP and colloids remain at the origin while pertechnetate migrate with R_f=1).
- (b) HPLC analysis as above described (2.1.) using the HPLC system also equipped with a radioactive (Berthold, Wildbad, W. Germany) detector (⁹⁹Tc^m-CIP eluted at 7.07 min and ⁹⁹Tc^mO₄⁻ at 31.8 min).

The chromatographic behavior of non-labelled CIP in both systems ITLC SG and HPLC was also studied by UV detection.

2.3. Determination of octanol/saline partition coefficients

The lipophilic properties of ⁹⁹Tc^m-CIP were evaluated by determination of the partition coefficient between octanol and saline. In a test tube, 25 µL of an aqueous solution of ⁹⁹Tc^m-CIP was added to 2 mL octanol and 2 mL saline pH=3.0. The tubes were vortex-mixed for 1 min and centrifuged for 10 min at 2,500 g. The entire procedure was repeated at least three times. Aliquots of 100 µL of each phase were counted in a gamma counter. The lipophilicity of the non-labelled CIP was also determined using the same method except that the CIP distribution in octanol and saline fractions was measured by absorbance at 280 nm. The distribution of the samples between saline and octanol, expressed by the partition coefficient (P_{o/s}), was used to evaluate the lipophilic character of both labelled and non labelled CIP solutions. Results are the means of 6 determinations.

2.4. Total human serum protein binding

A sample of ⁹⁹Tc^m-CIP (100 µL) was incubated with 2 mL of human serum, at 37° C. Then 200 µL was analyzed on a Sephadex G-25 column (0.9 x 20.0 cm, Pharmacia, Uppsala, Sweden). Saline pH=3 was used as eluent. Elution fractions were collected from the column and counted in a gamma counter. A standard was prepared to determine the percentage of radiopharmaceutical protein bound.

2.5. “*In vitro*” biological studies

2.5.1. Biological activity of treated CIP

Following the procedure used for radiolabelling, but using saline instead of $^{99}\text{Tc}^{\text{m}}$ -sodium pertechnetate solution, an inactive reaction mixture of ciprofloxacin was prepared. The biological activity of this reaction mixture was evaluated in order to study the effect of FSA and heating, necessary for labelling purpose, on CIP. The biological activity of this inactive reaction mixture was evaluated through its inhibition capacity on *S. aureus* and *E. coli* cultures (Kirby-Bauer method). The medium used was tryptic soy agar. Samples of 100 μL of inactive reaction of ciprofloxacin solutions (corresponding from 20 to 2000 $\mu\text{g}/\text{mL}$ of ciprofloxacin) were loaded on *S. aureus* and *E. coli* plates and then incubated at 37° C for 24h. Non treated CIP was used as standard. The biological activity of each sample was evaluated by measurement of the diameter of the inhibition circle.

2.5.2. $^{99}\text{Tc}^{\text{m}}$ -CIP uptake by bacteria

The uptake of ciprofloxacin by bacteria was determined by *in vitro* incubation of $^{99}\text{Tc}^{\text{m}}$ -CIP with 2 mL of bacterial broths (tryptic soy agar) containing *S. aureus* and *E. coli* ($\sim 10^7$ organisms) at 37° C with continued stirring up to 24 h. The incubated suspension was then centrifuged and then the tubes were aspirated and the pellets washed with 0.5 mL of saline and then counted. The results were expressed as percent of total radioactivity. $^{99}\text{Tc}^{\text{m}}\text{O}_4^-$ bacterial uptake was also determined as control.

3. Animal experiments

3.1. Infection models

A single clinical isolate of *S. aureus* and *E. coli* from biological samples were used to produce focal infection. Individual colonies were diluted in order to obtain turbid suspensions containing approximately 2×10^8 organisms/mL. The optimization of these infection conditions was previously established [9]. Groups of 6 female CD-1 mice, weighting approximately 25 g each, were injected with 100 μL of each suspension in the right lateral thigh muscle. Twenty-four hours later, when gross swelling was apparent in the infected thigh the $^{99}\text{Tc}^{\text{m}}$ -CIP (100 μL) was i.v. administered to each animal. At 4 and 24 h following injection the animals were sacrificed. Both thighs (target and non-target) were dissected and counted and target/non-target thigh radioactivity ratio was then determined.

3.2. Sterile abscess/inflammation

Sterile abscess/inflammation was induced by injecting 100 μL of turpentine oil intramuscularly (i. m.) in the right lateral thigh muscle. At 1 to 8 days after the turpentine oil administration $^{99}\text{Tc}^{\text{m}}$ -CIP (100 μL) was i.v. administered to each animal. At 4 h following injection the animals were sacrificed. Both thighs (target and non target) were dissected and counted and the target/non target thigh radioactivity ratio was then determined. Based on data obtained, for uptake study in sterile abscess, the $^{99}\text{Tc}^{\text{m}}$ -CIP was administered 24 h after turpentine administration.

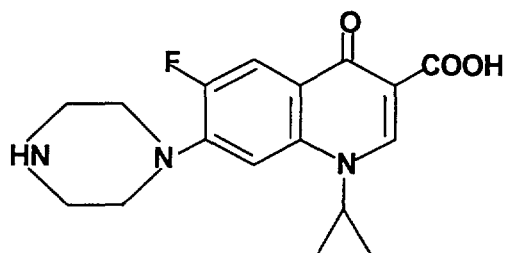


FIG.1. Chemical structure of ciprofloxacin.

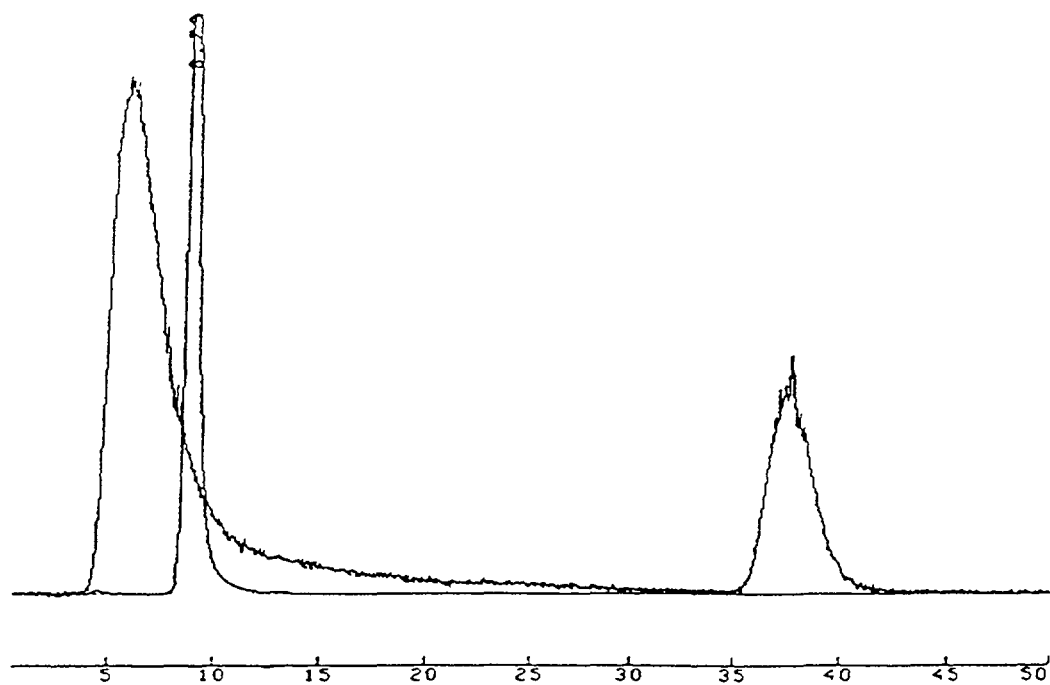


FIG.2. UV (top row) and radioactive (bottom row) HPLC profiles of $^{99}\text{Tc}^{\text{m}}$ -CIP. Retention times: CIP = 6.1; $^{99}\text{Tc}^{\text{m}}$ -CIP = 8.8; $^{99}\text{Tc}^{\text{m}}\text{O}_4^-$ (co-injected) = 37.1. Experimental conditions: Nucleosil C_{18} column. Mobile phase: mixture of acetonitrile and 0.05 M phosphate buffer(20:80), 0.01 M tetrabutylammonium bromide, pH=3.

3.3. Biodistribution patterns

The *in vivo* behavior of the labelled CIP preparation was evaluated in groups of 6 inflamed female mice CD-1 (randomly-bred Charles River mice, Wilmington, MA), weighing approximately 25 g each. Animals were intravenously injected with 100 μl (5-10 MBq) via the tail vein. The radioactive dosage administered was determined by counting the animal immediately after injection in a dose calibrator (Aloka, Curiometer IGC-3, Aloka Co Ltd, Tokyo, Japan). The mice were maintained on normal diet *ad libitum*. At 4 h and 24 h post-injection the animals were killed by cervical dislocation and their activity measured again. The difference between the radioactivity in the injected and sacrificed animal was assumed as the urinary excretion. Tissue samples were then removed for counting in a gamma counter (Innotron, Hydragamma, Innotron Ltd, Oxford, England). The results were expressed as percent of injected dose per organ (% ID/ organ). Mean values are given with the standard deviation. For blood, the activity was calculated assuming that this organ constitutes 7% of the total body weight.

3.4. Imaging Studies

A separate set of mice with the same bacterial and sterile inflammations induced as described were intravenously injected with 100 μ l (5-10 MBq) of the radiolabelled CIP. Scintigraphic images were obtained with a GE gamma-camera equipped with a LEAP collimator and connected to a Starcam 4,000I computer. All the images were acquired in a 128x128 matrix at 4 and 24 h after administration of the radiolabelled CIP.

4. Statistical methods

The results of the $^{99}\text{Tc}^{\text{m}}$ -CIP bacterial uptake were analyzed by an analysis of variance. The level of significance was set at 0.05.

3. RESULTS AND DISCUSSION

Chemical purity of CIP determined by HPLC was higher than 98 %. Radiolabelled efficiency of $^{99}\text{Tc}^{\text{m}}$ -CIP monitored by ITLC and by HPLC analysis was higher than 95%. Therefore no post-labelling purification was required. Recovery activity from the HPLC column, expressed as a percentage of the initial injected activity, was higher than 85% indicating low presence of insoluble $^{99}\text{Tc}^{\text{m}}$ -labelled species as well as a low adsorption to the stationary phase. The $^{99}\text{Tc}^{\text{m}}$ -CIP complex, analyzed by the systems above referred, has shown to be stable at least during 4h. U.V. and radioactive detection made evident that inactive and labelled CIP exhibit different chromatographic behavior in both systems ITLC and HPLC: by ITLC inactive and labelled species migrate with R_f 0.7 and 1.0 (not shown) and by HPLC were eluted at 8.8 and 6.1 min respectively. In Fig.2 the UV (top row) and radioactive (bottom row) profiles of $^{99}\text{Tc}^{\text{m}}$ -CIP are presented. Thus, this different chromatographic behavior suggests that CIP and radiolabelled CIP correspond to different chemical structures. In fact as the CIP group can only occupy one or two sites in the technetium coordination sphere the formation of a complex involving more than one molecule of CIP could occur. In addition taking into account that the role of FSA was not completely established, we could speculate that the FSA could act not only as reductant agent but also as a coligand to get a stable $^{99}\text{Tc}^{\text{m}}$ -CIP complex.

The octanol-saline partition of $^{99}\text{Tc}^{\text{m}}$ -CIP was 0.087 ± 0.016 ($n=6$). The octanol-saline partition of CIP was 0.52 ± 0.02 ($n=3$). Thus CIP presents higher lipophilic character when compared with that of $^{99}\text{Tc}^{\text{m}}$ -CIP. This finding together with the difference assigned in their chromatographic profiles support the hypothesis that CIP and the final $^{99}\text{Tc}^{\text{m}}$ -complex are different chemical compounds.

The total plasma protein binding of $^{99}\text{Tc}^{\text{m}}$ -CIP amounted to $56.8 \pm 7.4\%$ ($n=5$). Since only non-protein bound fraction of a substance can be filtered glomerularly the high protein binding indicates that the renal clearance of $^{99}\text{Tc}^{\text{m}}$ -CIP probably is also accomplished by tubular extraction.

Biological activity studies of treated CIP (in presence of FSA and heated at 100° C) when compared with CIP (starting material) made evidence that the CIP did not lose bacterial activity, under the labelling chemical conditions, since for both identical inhibition of *S. aureus* and *E. coli* cultures were observed.

The uptake of $^{99}\text{Tc}^{\text{m}}$ -CIP by *S. aureus* and *E. coli* was 2.2 and 1.4% ($n=2$) and the $^{99}\text{Tc}^{\text{m}}\text{O}_4^-$ uptake 7.4 and 9.2% ($n=2$) respectively. Higher $^{99}\text{Tc}^{\text{m}}\text{O}_4^-$ uptake values were found for both *S. aureus* and *E. coli* suggesting that other than specific binding can be involved, probably bacterial membrane permeability. According the biological activity studies

performed, the low $^{99}\text{Tc}^{\text{m}}$ -CIP bacterial uptake observed can not be attributed to a lost of CIP biological activity during the labelling procedure. Therefore it should be assume as a characteristic of $^{99}\text{Tc}^{\text{m}}$ -CIP complex formed.

The ratio target/ non target thigh radioactivity obtained, for optimization of experimental conditions to induce sterile abscess, were: 7.5, 4.5, 4.4, 3.2, 3.5, 3.2, 2.5 at 1, 2, 3, 5, 6, 7 and 9 days after turpentine administration. As observed the highest value was obtained 24 h after turpentine administration. Therefore this interval of time was chosen for $^{99}\text{Tc}^{\text{m}}$ -CIP administration. Previous studies [8] have also shown that this was the optimized interval to induce established infections with *S. aureus* and *C. coli*.

The tissue distribution of labelled CIP preparation, expressed as percentage of injected dose per organ (% ID/organ) in mice, with bacterial and sterile inflammations induced, 4 h and 24 h after intravenous administration is presented in table I. The $^{99}\text{Tc}^{\text{m}}$ -CIP is rapidly distributed after i.v. administration as shown by the value of the renal elimination and serum level. The high hydrophilic character of $^{99}\text{Tc}^{\text{m}}$ -CIP is in accordance with its predominant renal clearance.

TABLE I. BIODISTRIBUTION DATA IN PERCENT INJECTED DOSE PER TOTAL ORGAN FOR $^{99}\text{Tc}^{\text{m}}$ -CIP 4 AND 24 H AFTER I.V. ADMINISTRATION IN INFECTED / INFLAMED MODEL (*S. aureus*, *E. coli* and Turpentine).

Organ	<i>S. aureus</i>		<i>E.coli</i>		Turpentine	
	4 h	24 h	4 h	24 h	4 h	24 h
Blood	4.7 \pm 1.1	0.6 \pm 0.2	6.2 \pm 0.4	0.4 \pm 0.0	3.8 \pm 0.6	0.8 \pm 0.1
Liver	13.4 \pm 0.8	6.9 \pm 1.7	12.3 \pm 3.2	8.7 \pm 1.2	12.5 \pm 0.6	5.6 \pm 0.3
Spleen	0.4 \pm 0.0	0.3 \pm 0.1	0.8 \pm 0.2	0.8 \pm 0.3	0.3 \pm 0.1	0.2 \pm 0.1
Heart	0.1 \pm 0.0	0.0	0.2 \pm 0.0	0.0	0.1 \pm 0.0	0.0
Lung	0.3 \pm 0.0	0.1 \pm 0.0	0.5 \pm 0.0	0.2 \pm 0.0	0.4 \pm 0.0	0.1 \pm 0.0
Kidney	8.9 \pm 0.4	1.9 \pm 0.5	9.0 \pm 0.1	1.8 \pm 0.2	4.9 \pm 0.2	1.8 \pm 0.1
Stom.	0.6 \pm 0.1	0.1 \pm 0.0	0.4 \pm 0.0	0.2 \pm 0.0	0.8 \pm 0.2	1.2 \pm 0.4
Norm.thigh	0.7 \pm 0.2	0.2 \pm 0.0	0.8 \pm 0.1	0.2 \pm 0.0	0.7 \pm 0.1	0.2 \pm 0.0
Inf. Thigh	1.4 \pm 0.3	0.4 \pm 0.1	1.6 \pm 0.4	0.4 \pm 0.0	2.9 \pm 0.3	0.7 \pm 0.1
Urine	50.4 \pm 4.2	74.5 \pm 6.1	45.7 \pm 3.3	67.2 \pm 1.0	41.0 \pm 2.2	65.2 \pm 2.0

TABLE II. RADIOACTIVITY RATIO BETWEEN THE TARGET THIGH AND NON TARGET THIGH OBTAINED AT 4, 6 AND 24 H AFTER I.V. ADMINISTRATION OF $^{99}\text{Tc}^{\text{m}}$ -CIP.

Infected/ Inflamed model	Target / non target (n=3)		
	4 h	6 h	24 h
<i>S. aureus</i>	2.2 \pm 0.5	2.2 \pm 0.4	2.9 \pm 0.9
<i>E. coli</i>	2.0 \pm 0.4	1.7 \pm 0.3	2.2 \pm 0.7
Turpentine	4.5 \pm 0.9	4.1 \pm 0.6	3.3 \pm 0.6

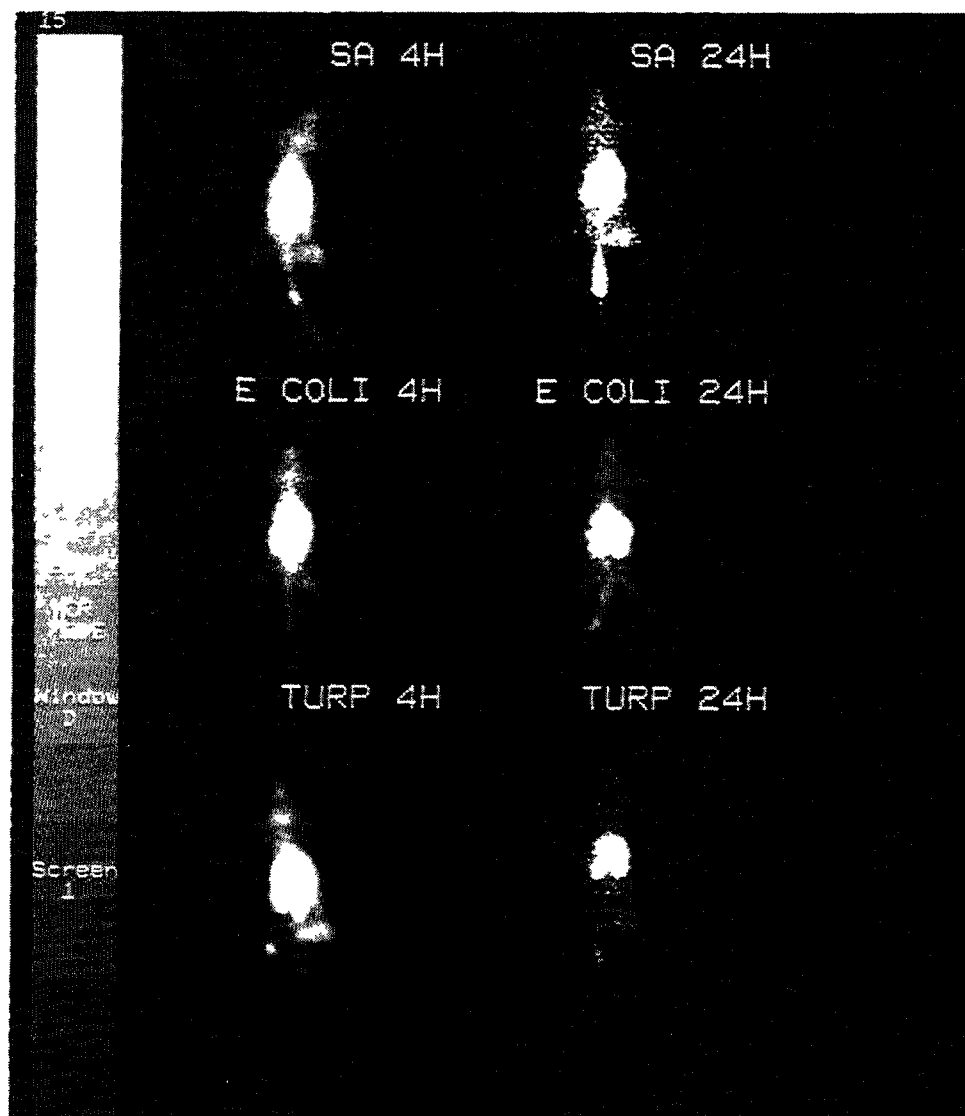


FIG.3. Planar whole body gamma camera images of mice (inflamed in the right thigh) injected with $^{99}\text{Tc}^{\text{m}}$ -CIP at 4 and 24 h post administration.

In Table II are presented the radioactivity ratios between the target thigh and non target thigh obtained at 4, 6 and 24 h after administration of $^{99}\text{Tc}^{\text{m}}$ -CIP. At 4 and 6 h the target thigh/ normal thigh radioactivity ratios indicated that higher binding affinity to the inflammation induced with turpentine was observed. However at 24 h no significant difference ($p < 0.05$) was found in its ability to localize in bacterial or sterile inflammations.

Whole body images of inflamed mice at 4 and 24 h after $^{99}\text{Tc}^{\text{m}}$ -CIP administration are presented in Fig.3 at 4 and 24 h post administration. At 4 and 24 h both *S. aureus* and *E. coli* focal infections are visualized. The sterile inflammation is clearly visible at 4 h post administration as well as at 24 h. Based on biological data we observed that $^{99}\text{Tc}^{\text{m}}$ -CIP was taken up both by bacterial and sterile inflammations. This finding led us to admit that the accumulation detected could be related to increased blood flow to the area together with enhanced vascular permeability, which facilitate the passage of the tracer to the extravascular space, as also postulated to other non specific radiopharmaceuticals.

4. CONCLUSIONS

No particular difficulty was experienced in radiolabelling CIP. Radiolabelled efficiency of $^{99}\text{Tc}^{\text{m}}$ -CIP monitored by ITLC and HPLC was higher than 95%. Therefore no post-labelling purification was required. Chromatographic analysis by ITLC and HPLC as well as the determination of octanol / saline partition coefficients indicated that CIP and $^{99}\text{Tc}^{\text{m}}$ -CIP correspond to different chemical structure. According the biological activity studies performed, the low $^{99}\text{Tc}^{\text{m}}$ -CIP bacterial uptake observed can not be attributed to a loss of CIP biological activity during the labelling procedure. Therefore it should be assume as a characteristic of $^{99}\text{Tc}^{\text{m}}$ -CIP complex formed. The biological properties of CIP, base to use the CIP molecule as starting material to develop specific infection imaging agent could not be met. Biodistribution studies in inflamed mice have shown that $^{99}\text{Tc}^{\text{m}}$ -CIP is rapidly distributed after i.v. administration with a predominant renal clearance. The radioactive preparation is able to localize both bacterial and sterile inflammations induced by *S. aureus*, *E. coli* and turpentine however its accumulation may be due only to increased blood flow together with enhanced vascular permeability as also postulated to other non specific radiopharmaceuticals.

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Abstract

Lately, the pineal body has been the subject of a large variety of studies. Only recently it has been understood the role played by this endocrine gland to maintain the balance of the human body and also in animal models. Although small in dimensions, the pineal body is a very active organ, able to transmit precise temporal information. It probably participates in the synchronization of several organic functions. The present work aims to study a possible use of ^{99m}Tc -glucose-6-P as a tracer for the pineal gland.

Histautoradiographic studies have been performed in *Wistar* rats. Tomoscintigraphic studies were acquired in patients and in albino rabbits (*Oryctolagus cuniculus hyplus*). The labelling efficiency and the radiochemical purity of the labelled products have always been tested. Animal and human SPECT exams, show an activity focus projected over the area corresponding to the pineal body localization. Autoradiographic studies using $[1-^{14}\text{C}]$ -glucose-6-P did not reveal a more relevant activity at the pineal level, probably due to its hepatic conversion to ^{14}C -glucose.

1. INTRODUCTION

Lately, the pineal body has been subject of a large variety of studies. Only recently has it been understood the role played by this endocrine gland to maintain the balance of the human body and also in animal models. Although small in dimensions, the pineal body is a very active organ, able to transmit precise temporal information. It participates in synchronisation of several organic functions.

Due to its reduced size and anatomical location, the methods for *in vivo* studies were almost non-existent. Research was done based upon indirect determinations such as *in vitro* dosages in plasma, urine and, eventually, in cerebrospinal fluid.

The present work aims to study a possible use of ^{99m}Tc -glucose-6-phosphate (^{99m}Tc -glucose-6-P) as a tracer for the pineal gland.

2. MATERIAL AND METHODS

2.1. HISTOLOGIC AND AUTORADIOGRAPHIC STUDIES

4 Wistar rats (weighting 350-450g) were injected in the dorsal tail vein with 3.7×10^3 Bq (10 μ Ci) of D-[6- 14 C]-Glucose (*D*-[6- 14 C]-Glucose, Amersham Life Science[®], aqueous solution with 3% ethanol and specific activity 2.07GBq/mmol). To another group of 4 animals, with the same characteristics, approximately 10 μ Ci of D-[1- 14 C]-Glucose-6-phosphate dissodium salt (*D*-[1- 14 C]-Glucose-6-phosphate dissodium salt, DuPont[®], aqueous solution with 0.5 ml ethanol; specific activity 2.1GBq/mmol and concentration 3.7 MBq/ml), corresponding to 1.7mmol/ml (0.53mg/ml) were administered, also in the dorsal tail vein.

Two hours after injection the animals were sacrificed by decapitation. After careful opening of the cranium, brain+pineal body+cerebelum were removed as a block. Immediately after the organs were frozen with isopentane (Merck) at -30° to -50°C. OCT (Tissue-Tek, Miles Inc.) was used as an embedding medium and the histological sections ($\pm 15\mu$ m) were performed with a SHANDON Cryotome and removable Feather knives (Type C35). The sections were mounted on pre-prepared coverslides with poli-L-lysine and stored at -25° to -30°C.

In order to do autoradiographic studies, coverslides (with respective sections) were glued on a propylene sheet with identical dimensions to the film used (*Hyper-film β max*, Amersham Life Science[®] - one sided emulsion, 8mm thick and silver halogenate granules of 1.6 \times 0.45mm), to ensure its motionless throughout the process.

Autographic procedure followed usual rules concerning the sample contact with the film. 4 to 5 weeks after films were processed (*D19 Kodak*[®] developer diluted to 1/7 and *AP44 Agfa* universal fixative at $\pm 20^\circ\text{C}$ for 4 to 5 min. each). After drying at room temperature image acquisition of the films was performed using a video system coupled to an optic microscope. The microscope was connected to a computer system enabling posterior granule density determination (granule/mm¹)(Nikon Optophot - 2)(20 \times enlargement). The image system used was composed of: CCD video+CUE camera+Sony Trinitron Super Fine Pitch monitor, IBM 466 DX2/V computer+14L10 IBM monitor+Cue.2 - Olympus software.

¹ We used the D-glucose-6-phosphate from Sigma Chemical, CO[®] (aqueous solution with a 255mg/ml concentration)

In each field, 3 identical areas ($2136.87\mu\text{m}^2$) in each ROI (Region of Interest) were chosen, as well as in the background (RF) to measure the granule density ($\text{granule}/\text{mm}^2$). This relationship has been determined using the average of three values obtained for each ROI. At the end the signal/background (S/R) for each tissue sample was determined.

The coverslides used for autoradiography were stained for optic observation using two different staining procedures: hematoxyline/eosin and silver impregnation (Bielschowsky technique modified by Glees-Marsland).

2.2. SCINTIGRAPHIC IMAGE ACQUISITION (DYNAMIC AND SPECT ACQUISITIONS)

2.2.1. GLUCOSE-6-P LABELLING PROTOCOL AND LABELLING QUALITY CONTROL

The labelling technique of glucose-6-P with $^{99\text{m}}\text{Tc}^1$ was adapted from the Upstate Medical Center, Syracuse, New York Nuclear Medicine Division $^{99\text{m}}\text{Tc}$ labelling protocol for renal scintigraphic imaging, using stannous chloride. 1 to 2mg of $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ (diluted in 1 ml of hydrochloric acid 1N, freshly prepared) were mixed up with 2 to 4ml of pertechnetate. The mixture was continuously agitated at $\pm 30\text{Hz}$ with a vortex for 5 min.. Afterwards $\pm 0.5\text{ml}$ of the D-glucose-6-P solution was added and shaken up for some minutes. The pH adjustment to 6-7.5 has been done with a sodium hydroxide solution 3M. The final mixture was filtered throughout a column ($\pm 2\text{cm}^2$) of anionic exchanging resin² in order to remove most of the free pertechnetate.

The D-glucose-6-P labelling efficiency was tested with microchromatographies to quantify the presence of free and reduced pertechnetate. 31 ET paper strips ($8 \times 1\text{cm}$) as stationary phase and acetone and butanone as solvents, were used. In both cases the determined labelling efficiency values were 95-99%.

2.2.2. DYNAMIC SCINTIGRAPHIC IMAGE ACQUISITION

A gamma camera (Maxicamera II General Electric 400AC) controlled by a Starport Computer was used for acquisition of the dynamic images.

² The resin was previously processed with deionized water.

After quality control, the ^{99m}Tc -D-glucose-6-P was injected iv. into 4 Wistar rats (average dose of 74MBq in 0.3-0.5ml, in femoral vein, previously catheterized with a 24-26G abocat) and 6 albino rabbits (*Oryctolagus cuniculus hyplus*) (average dose of 200MBq, injected also in the catheterized femoral vein; 22 or 24G abocat) for *in vivo* studies of glucose-6-P biodistribution. The animals, previously anesthetized im. with internationally recommended dosage³ of ketamine 50mg and chlorpromazine at 2%, were placed directly over the collimator in dorsal *decubitus*.

The image acquisition was started immediately before the radiotracer injection to ensure that the initial image, corresponding to the arrival of the product into the organism, was properly recorded. Sequences of 700 images, 5 sec. each, were acquired to 64*64 matrices, corresponding to a total of ± 60 min.. Acquisition was performed using a low energy and convergent collimator.

5 ROI's were drawn over the acquired image⁴, respectively projected over the chest, right kidney, muscle and two cranial regions: the exterior one corresponding to the animal head and an inner one representing with more accuracy its brain. The mean activity values were then obtained for each ROI. The mean value for muscle (background activity) was deduced from the mean values of the other 4 ROI's. 4 activity/time curves were obtained, being each one previously smoothed⁵ and normalized for the maximum of each curve.

The rabbit's SPECT (single photon emission computerized tomography) exams were done with the referred gamma camera (GE 400AC) and with a 3 head camera (Neurocam GE).

For the 12 SPECT patient's exams a Maxicamera GE 400T controlled by a computer system was used. Acquisition was performed to 128*128 matrices [1].

According to the acquisition type, two collimators were selected: low-energy high-resolution and convergent for dynamic and tomographic animal exams, or a low-energy high-resolution and parallel hole for human tomographies.

Rabbit's tomographic acquisition was started after the dynamic one, approximately 90 min. post radiotracer injection. The exam performed with the one head camera had a total of 64 views of 64*64 matrices, of 40 sec each, according to a circular orbit of 360°. For the Neurocam exams 128 views of 128*128 matrices, of 40 sec each, were acquired. In this case two SPECT studies were performed: the first one after ^{99m}Tc -glucose-6-P injection into the femoral vein, and the second one, 10 min. after, with an iv injection of ^{99m}Tc -HMPAO.

³ For rabbits 3-5ml of the 3:1 mixture was used; for rats the im dose was of 0.6-0.8ml of the 10:3 solution.

⁴ The processing and visualization program has been developed in the Biophysics Dept., Medical School of the University of Coimbra.

⁵ The curves were smoothed applying a mobile average of 3 points to the curves; the activity value in the *i* interval was chosen to be the average in the three adjacent time intervals, centered in *i*.

The tomographic reconstruction was done by filtered retroprojection with a commercial GE software. The selected filters were Rampa and Hanning, with a slice frequency of 0.8.

For the patients' exams, after iv injection of 370-555MBq (10-15mCi) of ^{99m}Tc -glucose-6-P, 128 views of 128*128 matrices, 30 sec each, were acquired in a 360° orbit.

3. RESULTS

3.1. HISTOLOGIC AND AUTORADIOGRAPHIC STUDIES

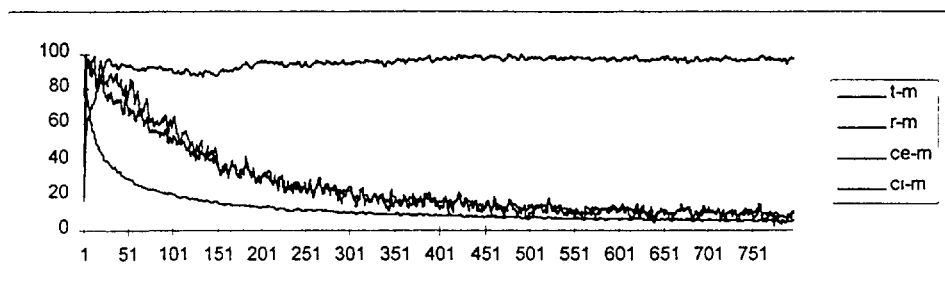
The autoradiographic sections, either with D-[6- ^{14}C]-glucose or with D-[1- ^{14}C]-glucose-6-P, show that both radiotracers are fixed almost uniformly and homogeneously over all the brain area, being impossible to differentiate hyper or hypoactivity zones (Fig.1). Nevertheless, the relationship signal/background obtained from the same images, observed with the optic microscope, showed that this value is higher when D-[1- ^{14}C]-glucose-6-P is used (Table I). The histological stainings performed



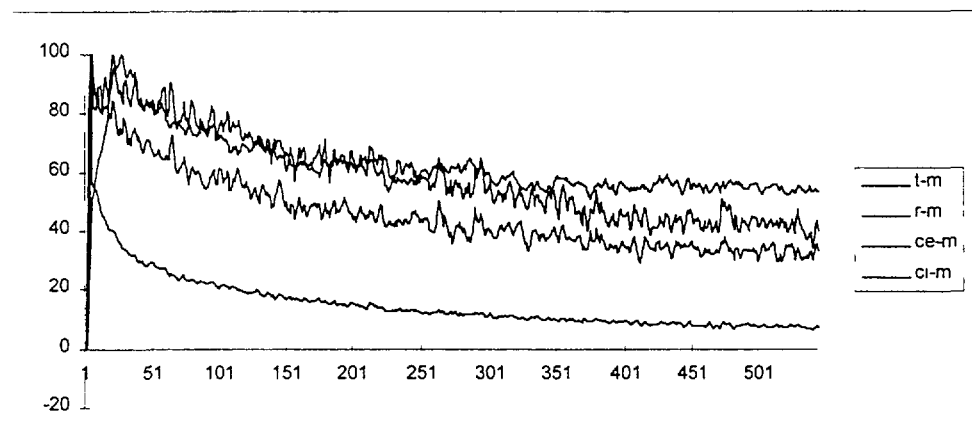
Fig. 1 - Autoradiographic image (optical microscope), post computer processing of brain+epiphysis of a rat, with [1- ^{14}C]-glucose-6-P (200 ×).

Table I. Mean granule density values obtained for autoradiographic sections (tissue and background) and respectively signal/background relationships for D-[6- ^{14}C]-glucose and D-[1- ^{14}C]-glucose-6-P.

Pineal body	D-[6- ^{14}C]-glucose	D-[1- ^{14}C]-glucose-6-P
mean density	11553.33	809.00
mean RF	666.66	304.00
S/R	1.73	2.66



a)



b)

Fig. 2 - a) Activity/time curves of the selected ROI's in a rat: t-m=chest-muscle, r-m=kidney-muscle, ce-m=craneal area-muscle, ci-m=cerebral area-muscle; b) Activity/time curves of the selected ROI's in a rabbit: t-m=chest-muscle, r-m=kidney-muscle, ce-m=craneal area-muscle, ci-m=cerebral area-muscle;



Fig. 3 - Sagittal slices of a rabbit head: exam performed with ^{99m}Tc -glucose-6-P on the left and with ^{99m}Tc -HMPAO on the right. Grey colour scale showing activity increase from black to white.

over the same sections, after autoradiographic film development, enabled us to correctly identify the pineal body.

3.2. DYNAMIC SCINTIGRAPHIC IMAGES

The initial image, both in rats and rabbits, shows that the injected product is totally present in the chest and venous way leading to it. At 10 sec we can observe that the product reached all the body, but is predominantly present at the cardiac, pulmonary and renal levels, traducing mainly the vascular phase of the study. 30 min. after, the radiotracer activity concentrates at renal and vesical levels. We can observe in the 3 images an activity focus corresponding to the abocat used for injection. On the other side, through the activity/time curve analysis (Fig. 2) we may say that the predominant activity is located at renal level (r-m curve), in both species. At chest level (t-m curve) a fast mean activity decrease during the first 6 min. of each acquisition was observed, being almost constant afterwards. Both curves at cranial level show that the activity in the two ROI's (ce-m and ci-m curves, respectively) have a slow decrease during the first 15 min., being constant until the end of the exam. These curves are almost parallel throughout the acquisition.

3.3. CEREBRAL SPECT

Although the reduced dimensions of the used animals heads, the reconstructed images of the SPECT exams performed after ^{99m}Tc -D-glucose-6-P show a predominant radiotracer fixation at the ocular, thyroid and salivary glands areas and a very diffuse activity over the facial zone. The injected ^{99m}Tc -D-glucose-6-P is not fixed at cerebral level, not crossing the blood-brain barrier. Nevertheless, at the base of the cranium, particularly in the sagital images, it can be observed a relatively important activity focus, projected over the location of the pineal body. On the other hand, the SPECT slices obtained after administration of ^{99m}Tc -HMPAO show, as predicted, that this radiotracer cross the blood-brain barrier. In these slices it can not be identified the area corresponding to the epiphysis, as this radiotracer is fixed all over the brain (Fig.3).

In the transaxial tomographic slices performed in the patients, the ^{99m}Tc -D-glucose-6-P do not cross the blood-brain barrier either (Fig.4). In these slices it is also possible to identify a small activity focus projected over the pineal body location.

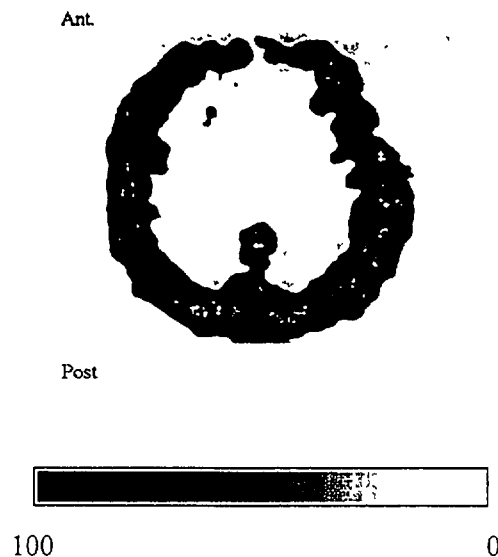


Fig. 4 - Transverse slice of a human head: exam performed with ^{99m}Tc -glucose-6-P. Gray colour scale showing activity increase from black to white.

4. DISCUSSION AND CONCLUSION

In rats, the histologic staining done in autoradiographic sections enabled the correct identification of the epiphysis. Nevertheless, the autoradiographic images of the same sections did not show significant differences between the two radiotracers, although, macro and microscopically, it is observed a relative increase of the intensity with the D-[1- ^{14}C]-glucose-6-P.

The glucose-6-P does not cross the cellular membrane, however, we can observe in all the sections of every tissue sample a relatively homogeneous activity in the autoradiographic images for the D-[1- ^{14}C]-glucose-6-P. The time interval (± 2 hours) between the radiotracer administration and the animals decapitation would enable the conversion of an high amount of the injected D-[1- ^{14}C]-glucose-6-P, due to glucose-6-phosphatase at the hepatic level, into D-[1- ^{14}C]-glucose ($\text{glucose-6-P}^{2-} + \text{H}_2\text{O} \rightarrow \text{glucose} + \text{Pi}^2$, this reaction is thermodynamically favourable). This enzyme is very important, it produces glucose from glucose-6-P in the last step of glucogenesis and glucogenolysis, but it does not participate in the glycolytic way [2].

The activity/time curves show that distribution of ^{99m}Tc -glucose-6-P is predominant at renal level, while at cranial and brain levels, after a fast decrease during the first 15 min., it stays stable throughout the acquisition (60 min.).

The SPECT studies performed both in animals and humans, show an activity focus over the zone corresponding to the pineal body location.

The results in rats using D-[1-¹⁴C]-glucose-6-P and ^{99m}Tc-glucose-6-P, are not identical to the ones obtained in humans and other species. In fact, the contrast between the epiphysis and the surrounding tissue in man, after ^{99m}Tc-glucose-6-P injection, enabled to identify the gland systematically (Fig.5). These results were obtained with a group of 12 patients.

The fact that autoradiographic exams with D-[1-¹⁴C]-glucose-6-P did not show a predominant activity at the epiphysis level could be due to its hepatic conversion into [1-¹⁴C]-glucose, as previously pointed out. In the future we intend to do new autoradiographic studies using [1-¹⁴C]-glucose-6-P and to sacrifice the animals earlier. Unfortunately, at the moment, this product is not available in the market.

In previous research done in dogs, sacrificed after ^{99m}Tc-glucose-6-P administration, there was an high fixation at pineal body location. In two of four cases, the relationship signal/background corresponded to 8 [3]. The apparent disagreement between the results in rats and rabbits and the ones for humans and dogs, may point for another choice of animal model. Unfortunately this last specie was not available for medical research due to difficulties inherent to the national and international legislation.

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**SYNTHESIS AND CHARACTERIZATION OF TWO NOVEL
TcO AND ReO MIXED LIGAND COMPLEXES
(3+1 COMBINATION, SNS/S) FOR HYPOXIA IMAGING**

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Abstract

As part of our project to develop small-size, neutral, mixed ligand oxotechnetium and oxorhenium complexes of the general formula MOL_1L_2 , we have synthesized and characterized two novel complexes of the general formula $MO[EtN(CH_2CH_2S)_2][p-O_2NC_6H_4S]$ where $M=Re$ (complex **1**) or $M=Tc$ (complex **2**) as a new approach for tissue hypoxia imaging. Complex **1** has been synthesized by exchange reaction in equimolar quantities of the tridentate and the monodentate ligand on $ReOCl_3(PPh_3)_2$. Only the *syn* isomer has been isolated and characterized by elemental analysis, IR, UV-vis, and 1H NMR. Crystal data reveal a trigonal bipyramidal geometry around the metal. The basal plane is defined by the sulfur atoms of the tridentate ligand and the oxygen, while the two apical positions are occupied by the nitrogen of the tridentate ligand and the sulfur atom of the monodentate thiol. Complex **2** is prepared by exchange reaction using Tc-99m-glucosheptonate as precursor and equimolar quantities of the two ligands. Only one complex, the *syn* isomer, is formed. Its identity is confirmed by comparative HPLC studies with complex **1**.

1. INTRODUCTION

In recent years, considerable efforts have been invested in labelling a number of nitroimidazole derivatives with Iodine-123, Carbon-11 and Fluorine-18 for application in the non-invasive imaging of hypoxic tissues [1]. Technetium complexes with the nitroimidazole-BATO derivatives or the PnAO-1-(2-nitroimidazole) ligand system have also been developed [2,3]. They bind to ischemic tissue of cerebral infarction in rats and in hypoxic myocardium in rabbits.

Recently we have reported the synthesis of technetium ($[^{99}\text{Tc}]$ and $[^{99\text{m}}\text{Tc}]$) neutral mixed ligand complexes of the general formula $\text{TcO}\{[\text{X}-\text{CH}_2\text{CH}_2\text{N}(\text{CH}_2\text{CH}_2\text{S})_2](\text{S}-\text{R})\}$ [4,5]. It was found that usually only the *syn* isomer is formed at both carrier and tracer level, although the formation of two isomers (*syn* and *anti*) is possible. The $^{99\text{m}}\text{Tc}$ complexes were evaluated in mice and rats as potential brain perfusion imaging agents and were found capable of penetrating the blood brain barrier. Most of them resulted in significant retention in the brain. It was also found that the biodistribution was grandly influenced by both X and R substituents [5].

As part of our project to develop small-size, neutral, mixed ligand oxotechnetium and oxorhenium complexes of the general formula MOL_1L_2 , we now present the synthesis and characterization of two novel complexes, $\text{MO}[\text{EtN}(\text{CH}_2\text{CH}_2\text{S})_2][p\text{-O}_2\text{NC}_6\text{H}_4\text{S}]$ where $\text{M}=\text{Re}$ (complex 1) or $\text{M}=\text{Tc}$ (complex 2) as a new approach for tissue hypoxia imaging. This system combines the radiosensitizer properties of aromatic nitro-compounds (3) on the one hand, and the advantages of technetium mixed ligand complexes (3+1 combination, SNS/S donor atom set) on the other.

2. EXPERIMENTAL SESSION

2.1. Materials and methods.

IR spectra were recorded as KBr pellets in the range 4000-500 cm^{-1} on a Perkin-Elmer 1600 FT-IR spectrophotometer and were referenced to polystyrene. The NMR spectra were recorded in deuteriochloroform on a Bruker AC 250E spectrometer. Melting points were determined using an Electrothermal 9100 capillary melting point apparatus and are uncorrected. Elemental analyses were performed on a Perkin-Elmer 2400/II automatic analyzer. High performance liquid chromatography (HPLC) analysis was performed on a Waters chromatograph equipped with 600E delivery system and a μ -Bondapak C-18 RP (10 μm , 3.9 mm x 300 mm) column using 60 % methanol as the mobile phase at a 1.0 ml/min flow rate. The rhenium complex was detected by a photodiode array detector, Waters 991 PDA, recording the UV-visible spectrum of the eluting complexes. Detection of the technetium ($^{99\text{m}}\text{Tc}$) complex was accomplished by a Beckman 171 radioisotope detector.

All chemicals were reagent grade. Solvents used in chromatographic analysis were HPLC grade. The tridentate ligand, 2,2'-dimercaptotriethylamine, was synthesized according to the literature [6,7]. The 4-nitrothiophenol, used as coligand, was purchased from Fluka. $\text{ReOCl}_3(\text{PPh}_3)_2$ was prepared according to literature [8].

$[\text{}^{99\text{m}}\text{Tc}]\text{NaTcO}_4$ was obtained in physiological saline as commercial $^{99}\text{Mo}/^{99\text{m}}\text{Tc}$ generator eluate (Cis International). Commercial glucoheptonate kits containing a lyophilized mixture of 200 mg calcium glucoheptonate and 0.2 mg SnCl_2 (Gluco/Demoscan, NCSR "Demokritos") were used.

2.2. Synthesis of $\text{ReO}[\text{C}_2\text{H}_5\text{N}(\text{CH}_2\text{CH}_2\text{S})_2][p\text{-SC}_6\text{H}_4\text{NO}_2]$

To a stirred suspension of trichlorobis(triphenylphosphine)rhenium(V) oxide (333 mg, 0.4 mmol) in methanol (10 ml) 1 N CH_3COONa in methanol (4 ml) was added. A

mixture of 66 mg (0.4 mmol) 2,2'-dimercaptotriethylamine (L_1H_2), and 62 mg (0.4 mmol) of 4-nitrothiophenol (L_2H) was added under stirring. The solution was refluxed until the green-yellow color of the precursor turned to dark-green. After being cooled to room temperature, the reaction mixture was diluted with CH_2Cl_2 (30 ml) and then washed with water. The organic layer was separated from the mixture and dried over $MgSO_4$. The volume of the solution was reduced to 5 ml and then 5 ml of methanol were added.

Analysis of the solution by HPLC (C-18 RP column using methanol/water 85/15 as mobile phase and a flow rate of 1 ml/min) demonstrated the formation of two complexes. The major product was isolated as green crystals (66 mg, 32% yield) FTIR (cm^{-1} , KBr pellet) : 2978, 2934, 1590, 1560, 1503, 1333, 1083, 1025, 957 ($Re=O$). 1H NMR (250 MHz, $CDCl_3$) δ : 1.42 (t, 3H, $J=7.5$ Hz, $NCH_2CH_3^*$), 2.65, 3.50 (m, 4H, $SCH_2^*CH_2N$), 3.29, 3.60 (m, 4H, $SCH_2CH_2^*N$), 3.92 (q, 2H, $J=7.5$ Hz, $NCH_2^*CH_3$), 7.78 (d, 2H, $J=10$ Hz, aromatic H) and 8.20 (d, 2H, $J=10$ Hz, aromatic H). Anal % Calcd for $C_{12}H_{17}N_2O_3S_3Re$: C: 27.69; H: 3.29; N: 5.39. % Found: C: 28.18; H: 3.15; N: 5.71.

The minor complex was detected by HPLC in a very small amount (yield <1%). Our attempts to isolate this complex have failed.

2.3. X-ray crystal structural determination of **1**

Diffraction measurements were made on a P2₁ Nicolet diffractometer with Ni-filtered Cu K α radiation. Unit cell dimensions were determined by using the angular settings of 25 automatically centered reflections in the range $24^\circ < 2\theta < 54^\circ$. Intensity data were recorded using a θ - 2θ scan ($2\theta_{max}=130^\circ$). Lorentz, polarization and psi-scan absorption corrections were applied using Crystal Logic software. All non-hydrogen atoms were refined anisotropically. Complex **1**: reflections collected/unique/used 2914/2745 [$R_{int}=0.0119$]/2523. The structures were solved with direct methods using

SHELXS-86 [9] and refined by full-matrix least-squares techniques on F^2 with SHELXL-93 [10].

2.4. Synthesis of $^{99m}\text{TcO}[\text{C}_2\text{H}_5\text{N}(\text{CH}_2\text{CH}_2\text{S})_2][p\text{-SC}_6\text{H}_4\text{NO}_2]$

A Gluco/ Demoscan kit was reconstituted with 10 mL of water and then, a 1.0 mL aliquot was mixed with 0.5-1.0 mL of (^{99m}Tc) pertechnetate solution (5-10 mCi). The $^{99m}\text{Tc(V)O}$ -glucoheptonate solution was added to a centrifuge tube containing equimolar quantities (0.02 mmol) of the tridentate ligand: 2,2'-dimercaptotriethylamine (L_1H_2 : 3.3 mg) and the monodentate ligand: 4-nitrothiophenol (L_2H : 3.1 mg). The mixture was agitated in a vortex mixer and left to react at room temperature for 10 min. This time was sufficient for a quantitative exchange between $^{99m}\text{Tc(V)O}$ -glucoheptonate and the ligands (ITLC-SG; physiological saline $R_f = 1$ for $^{99m}\text{Tc(V)O}$ -glucoheptonate and $R_f = 0$ for ^{99m}Tc mixed-ligand complex). The complex was extracted with CH_2Cl_2 (3x1.5 mL) and the combined organic extracts were dried over MgSO_4 , and filtered. The extraction was nearly quantitative for all preparations (85-99%).

Samples of the organic extracts (50 μL , 50-100 μCi) analysed by HPLC showed one major radioactive peak (>95%). The ^{99m}Tc complex (major radioactive peak) had the same retention time with the analogous Re complex thus suggesting the same chemical structure for both complexes. The ^{99m}Tc complex was isolated and thus purified from excess of ligands and small amounts of radiochemical impurities by manual collection of HPLC eluents.

3. RESULTS AND DISCUSSION

The rhenium mixed ligand complex **1**, *syn*- $\text{ReO}[\text{C}_2\text{H}_5\text{N}(\text{CH}_2\text{CH}_2\text{S})_2][p\text{-SC}_6\text{H}_4\text{NO}_2]$, was synthesized by reacting equimolar quantities of the tridentate

$\text{C}_2\text{H}_5\text{N}(\text{CH}_2\text{CH}_2\text{SH})_2$ and the monodentate *p*-HSC₆H₄NO₂ ligands with $\text{ReOCl}_3(\text{PPh}_3)_2$ precursor in refluxing sodium acetate and methanol. The complex was extracted in dichloromethane and isolated by recrystallization from $\text{CH}_2\text{Cl}_2/\text{MeOH}$. The yield of the reaction was relatively low (32%).

Formation of two isomers, *syn* and *anti* during coordination of the tridentate and the monodentate thiol, is expected according to the orientation of N-ethyl substituent of the tridentate ligand in respect to the ReO^{3+} core [11]. However, these isomers are not always both formed [4,5,12]. Indeed, in this study only the *syn* isomer (complex **1**) has been isolated. The HPLC analysis showed that a very small amount of a second complex was present in the filtrates after isolation of complex **1**. The second complex which was not isolated, might be the *anti* isomer. Complex **1**, gave correct elemental analysis and was characterized by IR, UV-vis and NMR spectroscopy and x-ray crystallography. It is soluble in acetone, dichloromethane and chloroform, slightly soluble in methanol or ethanol and insoluble in ether, pentane and water. It is stable in the solid state as well as in organic solutions as shown by HPLC. Its stability is not affected by the presence of air or moisture.

The infrared spectra of **1** exhibits a strong $\text{Re}=\text{O}$ stretching band at 957 cm^{-1} . This value is consistent with those reported for several other well characterized monooxo complexes of rhenium [11, 13]. Strong bands, arising from the NO_2 group, were observed at 1503 and 1333 cm^{-1} . Moreover, the IR spectra shows that both the tridentate and monodentate ligands were combined to form the oxorhenium complex. The absence of bands associated with SH stretching modes is a sign of the deprotonation of this group upon complexation with oxorhenium.

The electronic absorption spectra of complex **1** was determined during HPLC analysis using the photodiode array detector. The UV-vis spectra is characterized by an intense band in 406 nm which is probably due to $\text{S}\rightarrow\text{Re}$ charge transfer transition

where S is the sulfur donor atom of the monodentate thiol. The results are consistent with those of other *syn* isomers with this ligand system reported previously [11].

The ^1H NMR data confirm the presence of both types of ligands in complex 1. The integration of the peaks (data not shown) demonstrates that in each case one tridentate and one monodentate ligand were combined to form the oxorhenium(V) mixed ligand complex. The signals of the tridentate ligand fall in the region 1.42-3.92 ppm. The signals in the region 2.65-3.60 ppm are assigned to the methylene proton of the two chelated $\text{NCH}_2\text{CH}_2\text{S}$ moieties. The four protons of each moiety are diastereotopic, each one resulting in a multiplet. The methylene protons of the ethyl-side chain (C-5) are found in a deshielded environment at 3.92, indicating that the complex has the *syn* configuration [11]. The two doublets at 7.78-8.20 (4H, $J=10.0$ Hz) are characteristic of the protons of a *para* substituted benzene ring.

Crystals suitable for X-ray analysis are obtained by slow evaporation from $\text{CH}_2\text{Cl}_2/\text{EtOH}$. The X-ray crystallography for complex 1 established the *syn* configuration of the ethyl side chain. An ORTEP diagram is shown in Fig. 1, and selected bond distances and angles are given in Table 1. The metal-oxygen bond distance is 1.67 (1) Å. The metal sulfur bond distances are in the range of 2.273(3)-2.303(3) Å, while the Tc-N1 bond distance is 2.24 (1) Å. All of these parameters are in general agreement with the structural parameters for a large number of complexes containing the same donor atoms [11, 13]. The bond angles between the diametrically opposite coordinated atoms N1-Tc-S3 and S1-Tc-S2 are 158.2 (1) and 123.9 (6) Å respectively. Analysis of the shape-determining angles using the approach of Addison [14], yields a trigonality index, τ , of 0.57 ($\tau=0$ for square pyramidal geometry and $\tau=1$ for trigonal bipyramidal geometry). Thus, the coordination geometry around the metal can be described as distorted trigonal bipyramid. The basal plane is defined by the sulfur atoms of the tridentate ligand and the oxygen, while the two

apical positions are occupied by the nitrogen of the tridentate ligand and the sulfur atom of the monodentate thiol. During complexation all sulfur atoms undergo ionization so that the overall charge of the complex is zero.

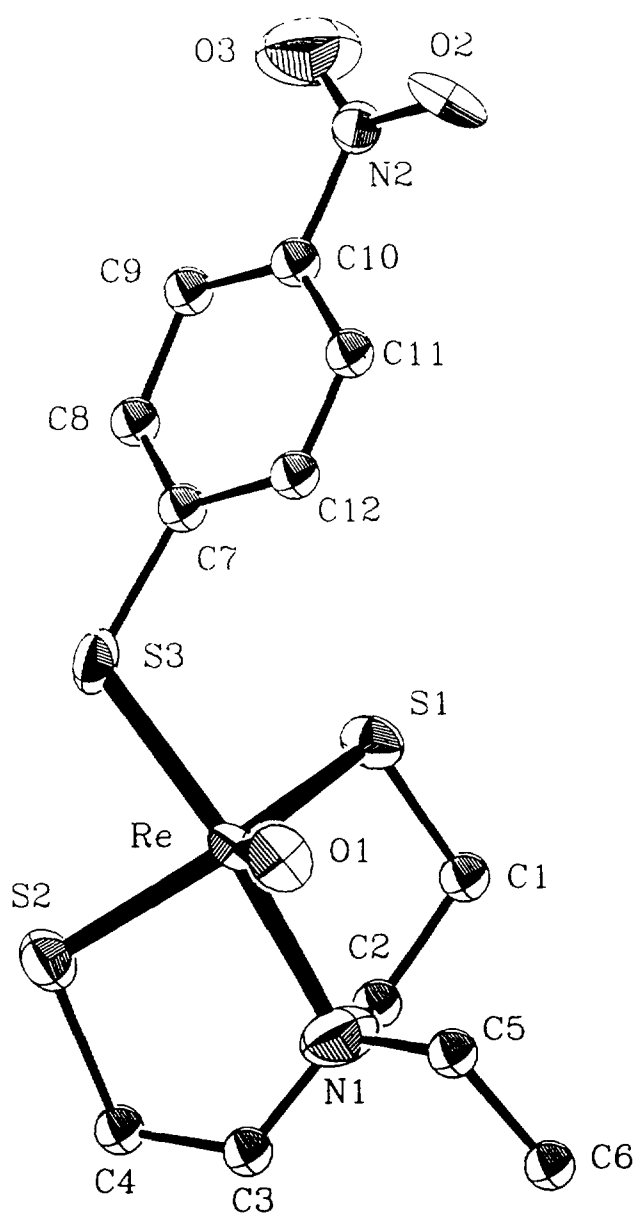


Fig.1 ORTEP diagram of *syn* - $\text{ReO}[\text{EtN}(\text{CH}_2\text{CH}_2\text{S})_2][p\text{-O}_2\text{NC}_6\text{H}_4\text{S}]$, complex 1.

Table 1. Selected bond distances (Å) and angles (degrees).

Complex 1			
Re	-O1		1.67 (1)
Re	-S1		2.287 (3)
Re	-S2		2.273 (3)
Re	-S3		2.303 (3)
Re	-N1		2.24 (1)
O1	-Re	-S1	118.9 (1)
O1	-Re	-S2	116.7 (4)
O1	-Re	-S3	104.9 (4)
O1	-Re	-N1	96.6 (1)
N1	-Re	-S1	83.6 (3)
N1	-Re	-S2	83.7 (3)
N1	-Re	-S3	158.2 (4)
S1	-Re	-S2	123.9 (1)
S2	-Re	-S3	83.8 (1)
S3	-Re	-S1	88.7 (1)

Complex 2, $^{99m}\text{TcO}[\text{C}_2\text{H}_5\text{N}(\text{CH}_2\text{CH}_2\text{S})_2][p\text{-SC}_6\text{H}_4\text{NO}_2]$, was prepared by exchange reaction using Tc-99m-glucoheptonate as precursor and equimolar quantities of the two ligands. The reaction mixture was extracted with dichloromethane and the radiochemical purity of the extracts was checked by HPLC (C-18 μ -Bondapak, MeOH:H₂O, 85:15, 1.0 ml/min). Under these conditions a major peak was monitored in the radiochromatogram and was isolated by manual collection of the eluents. The radioactivity of the peak was more than 95% of the radiochromatogram. When the Re complex (**1**), which is a surrogate of technetium and the isolated ^{99m}Tc complex were coinjected, both radioactivity (for ^{99m}Tc) and UV (for Re) detectors exhibited identical

chromatographic profiles, demonstrating that the same chemical species (*syn* isomer) were formed under both chelating conditions.

Further studies are in progress in order to evaluate the behavior of the complex in experimental animals.

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DEVELOPMENT OF A NOVEL ^{99m}Tc -LABELLED BRAIN PERFUSION AGENT



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Abstract

A method has been described for radiolabeling of salbutamol with technetium-99m (^{99m}Tc). To a 1 ml solution, containing 5 mg salbutamol and 2 mg of ascorbic acid, was added a clear solution (10 μl) of $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ (100 μg) in distilled water. The pH of the solution was adjusted to 8.5, mixed gently with 10 mCi (370 MBq) of ^{99m}Tc elute and incubated at room temperature for 15 minutes. The resulting solution was passed through 0.22 μ filter and radiolabeled fraction was quantified using paper chromatography. The radiochemical analysis, employing the use of above-mentioned radioanalytical technique, revealed that greater than 97% of the radioactivity was bound to salbutamol and rest of the activity was in the form of free pertechnetate ($^{99m}\text{TcO}_4$).

Biological data, obtained after i.v. injection of ^{99m}Tc -salbutamol to female albino rabbits, revealed a fair uptake in the brain at 30 min and 60 min post injection time intervals. On the basis of animal biodistribution data it is suggested that ^{99m}Tc -salbutamol can be successfully applied as a brain perfusion agent.

Introduction

The clinical value of measuring regional cerebral blood flow (rCBF) has long been recognized [1]. Nuclear medicine is rapidly establishing itself as an important tool in the in vivo study of the brain neurochemistry. Several neuroligands have been developed which offer valuable new insights in various neural disorders from brain injury to vascular diseases [2-4]. ^{99m}Tc and iodine-123 (^{123}I) are well suited radionuclides for single photon emission computed tomography (SPECT) studies [5]. Compared to currently used ^{123}I -labeled brain perfusion agents [6-14], the ready availability, low cost, instant kit formulation at the clinical site and ideal nuclear emission are the advantages of ^{99m}Tc -labeled rCBF agents [5].

Several neutral, lipophilic ^{99m}Tc chelates have been known to cross the blood-brain barrier (BBB) but only a few are efficiently extracted by the brain and retained there for long enough to be useful as brain perfusion agents with SPECT. Many derivatives of these chelates have been evaluated. Among these, hexamethylpropyleneamine oxime (^{99m}Tc -HMPAO), the first Food and Drug Administration (FDA) approved ^{99m}Tc -labeled rCBF agent, has considerable potential for routine clinical studies using SPECT [15]. HMPAO has two stereoisomers due to two asymmetric carbon atoms (at 3 and 9 positions) and these two isomers have dramatic differences in their

cerebral retention characteristics [16]. Synthesis of d,l-HMPAO requires prolonged purification steps and ^{99m}Tc -HMPAO is unstable in vitro [17]. ^{99m}Tc -l,l-ethylcysteinate dimer (^{99m}Tc -ECD) have been shown to be quite suitable for human SPECT brain imaging studies [18] but its synthesis involving the use of liquid ammonia is complex.

Salbutamol, 2-tert-butylamino-1-(4-hydroxy-3-(3-hydroxymethylphenyl)-ethyl)-ethanol, is a direct acting sympathomimetic agent with predominantly beta-adrenergic activity and has a selective action on β_2 receptors. Salbutamol, a bronchodilator, may be inhaled, given by mouth or injected [19]. A molecule of salbutamol contains three oxygen and one nitrogen as donor atoms and thus was expected to form complex with reduced ^{99m}Tc .

Keeping in view the need for development of a safe, convenient, stable and particularly cheap radiopharmaceutical, salbutamol, closely resembling the structure of dopamine, has been labeled with ^{99m}Tc for rCBF SPECT studies. The aim of the present study is to develop a sensitive and stable nuclear neural diagnostic agent with optimum imaging ability which would help to monitor the cerebral flow disorders. This article describes the details of a recently developed ^{99m}Tc -salbutamol brain perfusion agent.

Experimental

1 Material and Methods

Salbutamol was a gift from Glaxo Laboratories. All the chemicals used in this study were of analytical grade and were procured from commercial sources. ^{99m}Tc generator was imported from Amersham International Ltd. UK. The equipment used was composed of gamma camera GCA 40A interfaced to a nuclear medicine computer (Toshiba Japan) for imaging studies; freeze-dryer, consol 12, The VirTis Co. NY for preparation of lyophilized kits; dose calibrator, Nuclear Associates, UK for assessment of radioactivity and Albino rabbits (female) for biodistribution studies. Chromatographic separations were carried out on Whatman No. 1 paper. Round bottom glass developing chambers were employed to develop radiochromatograms. Binding efficacy of ^{99m}Tc -salbutamol complex was determined by ascending chromatography using appropriate solvents. Well-type gamma counter by Thorn EMI UK was used for radioactivity counting.

2 Radiolabeling

To a 1 ml solution, containing 5 mg salbutamol and 2 mg of ascorbic acid, was added a clear solution (10 μl) of $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ (100 μg) in distilled water. The pH of the solution was adjusted to 8.5, mixed gently with 10 mCi (370 MBq) of ^{99m}Tc elute and incubated at room temperature for 15 minutes. The resulting solution was passed through 0.22 μ filter and radiolabeled fraction was quantified using paper chromatography.

3 Quality Control

Following preparation of ^{99m}Tc -labeled salbutamol, the radiochemical purity of the final product (^{99m}Tc -labeled salbutamol) was determined using paper chromatography. The radiochromatograms were developed in a circular development chamber for a period of 10-15 minutes using chloroform and acetone as eluents. The paper was removed from the development tank and 2 cm segments were cut and radioactivity was measured using gamma counter. All kits used had radiochemical purity greater than 97%. The results of radiochromatography, obtained from kits up to 4 hr post reconstitution, were identical to those obtained at injection time and confirmed the stable and pure labeling of salbutamol with ^{99m}Tc .

4 Biodistribution studies

The distribution of ^{99m}Tc -salbutamol was evaluated in four 1-2 kg female rabbits that were allowed food and water. One mCi (37 MBq) of ^{99m}Tc -salbutamol complex was injected in the marginal ear vein of the rabbit. Immediately after i.v. injection, gamma camera was started for

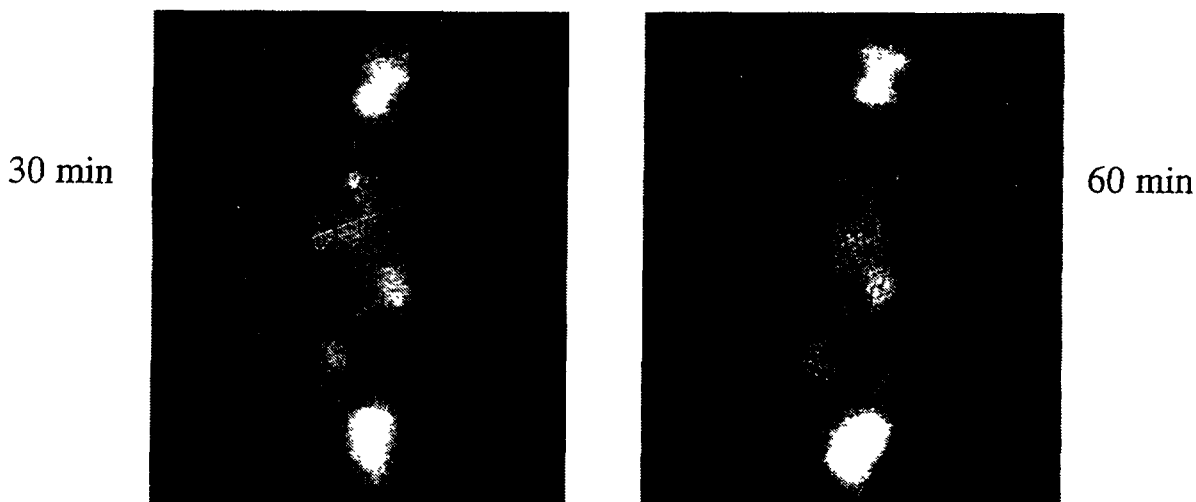


FIG. 1. Posterior views of Rabbit, after intravenous injection of ^{99m}Tc -salbutamol at 30 min and 60 min post injection time intervals, showing uptake in brain.

dynamic study and the static images were taken after 15 min, 30 min and 90 min using computerized gamma camera. Organ uptake at different time intervals was calculated using computer interfaced to gamma camera and detailed biodistribution studies are in progress. Preliminary results of biodistribution studies, indicating a fair uptake in the brain, are depicted in Fig. 1.

Discussion

^{99m}Tc -labeled propyleneamineoxime (^{99m}Tc -PnAO), initially developed at the University of Missouri [20, 21], was found to be a stable neutral lipophilic chelate. ^{99m}Tc -PnAO freely diffuses across the BBB with a high first-pass extraction efficiency that is similar to ^{123}I -IMP. However, it was not retained in the brain long enough for brain SPECT studies.

It was recognized that simple N_4 ligand chemistry could be utilized to prepare a number of small neutral, lipophilic compounds that could potentially be more useful as rCBF agents. A series of this type of agents was synthesized and screened at Amersham International, UK [22]. One of these agents, HMPAO (4,8-diaza-3,6,6,9-tetramethyl-undecane-2,10, dione-bisoxime) has been found to have high cerebral extraction and long retention in the brain. These properties make it quite suitable for brain SPECT studies [15]. Initial clinical brain imaging studies with these neutral lipophilic chelates have been reported [23]. HMPAO has two stereoisomers due to two asymmetric carbon atoms (at the 3 and 9 positions), and these two isomers have dramatic differences in their cerebral retention characteristics [16]. The meso form is cleared rapidly from the brain; however, d, l-HMPAO demonstrated excellent cortical retention. ^{99m}Tc -d, l-HMPAO is somewhat unstable in aqueous solution [17]. Thus, it is recommended that the product be used within 30 min of preparation. Causes of this instability and ways to correct it are being explored.

Diamine dithiol (N_2S_2) ligands are known to form single, stable, neutral lipophilic complexes with ^{99m}Tc . Several derivatives have been synthesized and evaluated. Among them, ^{99m}Tc -N-piperidinyethyl-diaminodithiol (NEP-DADT) and methyl-NEP-DADT have given promising results in experimental studies [24]. In humans, the most promising results have been obtained with ^{99m}Tc -ethyl cysteinate dimer (ECD; DuPont, Billerica, Mass.). Only the l, l-ECD distereoisomer is retained in the brain whereas ^{99m}Tc d, d-ECD is not. ^{99m}Tc l, l-ECD crosses the BBB and rapid conversion of the lipophilic chelate to a hydrophilic form takes place inside the cells, it cannot diffuse back and is retained in the brain for a long period of time ($T_{1/2} > 24\text{h}$) [25].

QNB(R)-3quinuclidinyl-4-iodo-benzilate has been labeled with ^{123}I with high specific activity [26] Radioiodinated QNB has been used to image the receptor distribution in normal subjects and in patients with Alzheimer's disease [27] The work on ^{123}I -epidepride, presented by Komhuber et al 1995 [28], is an important effort to improve SPECT imaging of neuroreceptors Recently, Leslie [2] has compared ^{123}I -IBZM and ^{123}I -epidepride for SPECT imaging of dopamine D_2 receptors and showed that latter provided higher quality images of striatum with enhanced target-to-background ratios Further evaluation of the clinical utility of these agents is awaited Radioiodinated (^{123}I -IMP, ^{123}I -IBZM and ^{123}I -epidepride) as well as $^{99\text{m}}\text{Tc}$ -labeled ($^{99\text{m}}\text{Tc}$ -HMPAO, $^{99\text{m}}\text{Tc}$ -ECD) agents, that are excellent for brain perfusion studies, are now available

It is important to note that the use of ^{123}I -labeled radiopharmaceuticals is limited to the countries where the cyclotrons are part and parcel of the nuclear medicine establishments because ^{123}I is cyclotron produced and due to short half life ($T_{1/2} = 13 \text{ hr}$) it is not feasible to import this radionuclide Several countries such as Pakistan don't have even single cyclotron facility in the country As cyclotron is an expensive technology, the use of ^{123}I -labeled radiotracers for brain perfusion studies is not expected in the near future in developing countries Furthermore, the cold kits ready to be labeled with $^{99\text{m}}\text{Tc}$ are quite expensive and it is not feasible for nuclear medicine departments in Pakistan to use these brain perfusion agents on regular basis for brain SPECT studies Salbutamol is a cheap drug and animal studies have revealed encouraging results In case it works well in human studies, it would be possible to use $^{99\text{m}}\text{Tc}$ -salbutamol as a brain perfusion agent in developing countries

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Abstract

^{99m}Tc -isonitriles have been shown to be a very promising substitute for Thallium-201 (^{201}Tl) for myocardial perfusion imaging. In this study, the lyophilized kit of Methoxyisobutylisonitrile (MIBI) was prepared and labeled with ^{99m}Tc . Several factors affecting the labeling yield such as the kit's stannous content, boiling time during labeling, and the volume of ^{99m}Tc used during reconstitution were also investigated. The radiochemical purity (RCP) determination of the labeled product was analyzed by HPLC, solvent-extraction, TLC, and ITLC-SG chromatographic methods in various systems. Animal biodistribution study performed in rats indicated the ^{99m}Tc -MIBI accumulation in the myocardial is up to 3 hours with little or no redistribution. Toxicity studies performed indicate no clinical signs of abnormality in mice at injected dose equivalent in amount of 100 times the human dose in proportion to body weight. Stability studies of the labeled complex performed at room temperature showed no change in radiochemical purity (> 95%) 6 hours post-preparation. Compatibility and comparative studies were done using both MINT and commercially available MIBI kits and ^{99m}Tc generator eluates. From the results obtained the MINT produced MIBI kits were found to be comparable in quality to that of commercials.

INTRODUCTION

Over the years, Thallium-201 (^{201}Tl) has been the prime radionuclide for the assessment of the myocardial perfusion and the diagnosis of coronary artery diseases. However, due to several drawbacks such as its unsatisfactory nuclidic properties, limited availability, relatively high cost and biological redistribution, the replacement of ^{201}Tl by an agent based on a more suitable isotope has been a longstanding goal of diagnostic nuclear medicine. ^{99m}Tc is the radionuclide chosen not only because it is readily available and cheap but also its 140 keV gamma photon is much better suited for gamma camera imaging. Thus, several ^{99m}Tc -cationic isonitrile compounds have recently been developed to address these problems viz, Tetrabutylisonitrile, TBI [1], Carbomethylisopropylisonitrile,

CPI [2] and MIBI [3]. Of these agents ^{99m}Tc -MIBI was found the better myocardial agent. It gives relatively low lung and liver activity with sufficient myocardial retention to give a good target to background ratio. This ^{99m}Tc -MIBI agent has been administered safely to patients and good initial myocardial uptake has been demonstrated.

Accordingly, the objective of this study was to develop MIBI kit for myocardial perfusion studies. This study includes the determination of the kit's (a) optimum SnCl_2 contents (b) boiling time during labeling with ^{99m}Tc (c) optimum volume of ^{99m}Tc activity needed for reconstitution (d) radiochemical purity determination using various systems (e) compatibility with various ^{99m}Tc generator sources (f) biological distribution in rats (g) toxicity and (h) comparative study using commercially available kits.

MATERIAL AND METHODS

1. ^{99m}Tc -MIBI Preparation

a) *MIBI kit preparation* The MIBI compound used in the preparation of lyophilized MIBI kits was provided by Greece and Thailand. The MIBI kits were prepared using almost the same composition as that of Cardiolite (DuPont) kit. 19.5 mg of anhydrous SnCl_2 was dissolved in 3.0 ml concentrated (37%) hydrochloric acid and the solution was then labeled as Solution A. 10 mg of $[\text{Cu}(\text{MIBI})_4]\text{BF}_4$ was added into 8 ml of water and stirred until it is completely dissolved. Then 10 mg of L-cysteine hydrochloride monohydrate, 25 mg sodium citrate and 220 mg mannitol were added and stirred respectively until dissolution. After purging with oxygen-free nitrogen gas for 15 minutes, 0.1 ml of solution A was added. The final solution was adjusted to pH 5.4 – 5.9 with 1M NaOH. Final volume was adjusted to 10 ml with sterile water for injection (SWFI). One ml of the final solution was each dispensed through 0.22 μm millipore filter in a 10 ml borosilicate vial. The content of the vials were then lyophilized at 0 °C for 30 hours and stored under vacuum.

b) *Labeling with ^{99m}Tc* Labeling was performed by reconstitution of the lyophilized kit with 1.0 – 3.0 ml ^{99m}Tc generator eluate (500 – 6000 MBq). The vial was swirled for a few seconds and placed in a boiling waterbath for a minimum period of 10 minutes.

2. Determination of Radiochemical Purity (RCP) of ^{99m}Tc -MIBI

The RCP of ^{99m}Tc -MIBI was determined using 4 different radiochemical methods

a) *Thin Layer Chromatography (TLC) Method* 2 drops of ^{99m}Tc -MIBI solution were applied side by side on top of a drop of pre-applied ethanol wet spot on TLC plate (7.5 x 2.5 cm). The TLC plate was developed with ethanol in chromatographic tank. Only the ^{99m}Tc -MIBI migrated with ethanol to the solvent front.

b) *Instant Thin Layer Chromatography Silica Gel (ITLC-SG) Method* The ITLC-SG strip (10 x 90 cm) was spotted 2 cm from the bottom, placed in a solvent tank (solvent: acetone/0.9% saline). Each strip was cut into 3 portions which were assayed separately in a gamma well-counter. ^{99m}Tc -reduced hydrolyzed remains at the origin in the acetone system and free $^{99m}\text{TcO}_4$ migrate to the solvent front in the saline system.

c) *Solvent Extraction Method* A few drops of ^{99m}Tc -MIBI were dropped into a tube containing a mixture of water:chloroform (1:1). The tube was then vortexed for 1 minute, allowed to stand for phase separation. Transfer the top phase into a separate tube. The activity in each phase was measured in a dose calibrator. The percent bound was calculated as the activity in the organic phase divided by the total activity in both phases.

d) *High Performance Liquid Chromatography (HPLC) Method* The radiochemical HPLC analysis was done on a VARIAN 9010 using Micropak (MCH15) C18 reverse-phase column. MeOH-0.5(NH₄)₂SO₄ (95:5) was used as mobile phase with a flow rate of 1 ml/min. Isocratic mode and NaI(Tl) crystal as the detection system.

3. Optimum Condition for Kit Formulation and Labeling

a) *Effect of stannous chloride on kit labeling* Lyophilized kits were prepared as prescribed previously with stannous chloride contents varies from 30 to 150 µg per kit. Labeling with ^{99m}Tc were done accordingly and their radiochemical purity were determined by ITLC-SG method.

b) *Effect of heating time on kit labeling* Reconstitute each of MIBI kits with 2 ml of ^{99m}Tc generator eluate (about 50 mCi). Separately, placed the reconstituted kits in boiling water bath for 2, 5, 10, 15, 20 and 30 minutes, respectively. Controls were left at room temperature. Percent labeling were then determined by TLC method.

c) *Effect of ^{99m}Tc Volume on Labeling* Reconstitute each of MIBI kits with 35 - 37 mCi of ^{99m}Tc in volume varies from 1, 2, 3, 4, 5, 6, 7, 8 and 10 ml per vial. The kits were then placed in boiling water for 10 minutes. Percent labeling were then determined by TLC method.

4. Compatibility Studies: Radiochemical purity determination and animal biodistribution studies of MIBI kits labeled with Methyl Ethyl Ketone (MEK) extracted ^{99m}Tc were done according to the procedures described earlier. Results obtained were compared to those obtained using ^{99m}Tc eluate from both the Amersham and MINT produced chromatographic generators.

5. Biodistribution Studies: 40 to 50 μCi in 30 to 40 μL ^{99m}Tc -MIBI was injected into the tail vein of Sprague-Dawley rat. The rats were then sacrificed and dissected at specific time. Tissue samples were collected, weighed, and counted in gamma scintillation counter. Organ distribution and uptake were determined in percent injected dose per organ (% ID/organ) and also as % ID per gram of body weight.

6. Stability Studies: Radiochemical purity determination and its animal biodistribution studies of ^{99m}Tc -MIBI labeled compound were done. The labeled ^{99m}Tc -MIBI was kept in the lead pot at room temperature for 6 hours before its RCP determination and animal studies were done.

7. Toxicity Studies: The toxicity study was performed by intravenous injection in male mice of MIBI kit content in equivalent to 100 times the human dose in proportion to body weight. The animals were kept under observation over a period of 1 month.

8. Comparative Studies: RCP determination and animal biodistribution studies of labeled ^{99m}Tc -MIBI compound were repeated using commercial kits (Cardiolite). Results obtained were compared with those obtained using MINT produced kits.

RESULTS AND DISCUSSION

1. ^{99m}Tc -MIBI Preparation: Each MIBI kit contains a freeze-dried powder of 1.0 mg $[\text{Cu}(\text{MIBI})]\text{BF}_4$, 1.0 mg L-cysteine hydrochloride monohydrate, 2.5 mg sodium citrate, 22.0 mg mannitol and 65 μg SnCl_2 , anhydrous. Labeling with ^{99m}Tc generator eluates gives a labeling efficiency of 95%. A minimum of 90% labeling is required for any clinical application.

2. Radiochemical purity: All four methods studied was found to give equivalent results (Table 1). The average time for drying and developing the reference Al_2O_3 TLC plate was about 23 minutes, whereas the average time taken for developing the solvent-extraction and ITLC-SG was 5 minutes. The combination of acetone and saline with ITLC-SG produced results equivalent to the recommended alumina-ethanol system with TLC system. However, the behavior of $^{99\text{m}}\text{Tc}$ -MIBI in this ITLC-SG system was the opposite to those obtained with the routine polar $^{99\text{m}}\text{Tc}$ -radiopharmaceuticals. With this ITLC-SG system, acetone determines $^{99\text{m}}\text{Tc}$ -colloid and saline determines free pertechnetate ($^{99\text{m}}\text{TcO}_2$), % $^{99\text{m}}\text{Tc}$ -MIBI was determined by difference from 100%. This sort of behavior also explained the lipophilicity of the radiopharmaceutical as has been previously reported [4].

3a. Effect of SnCl_2 on Labeling: The optimum quantity of SnCl_2 needed for the kit formulation was found to be about 65 μg per kit. SnCl_2 kit content lower than 50 μg or higher than 80 μg per kit would result in labeling efficiency lower than 90%. Human studies required at least 90% labeling.

3b. Effect of Boiling Time: The labeling of MIBI kit required a minimum of 10 minutes heating in boiling water. Labeling the kit with $^{99\text{m}}\text{Tc}$ at room temperature or with shorter heating period would result in very poor labeling efficiency (about 55-85%).

3c. Effect of $^{99\text{m}}\text{Tc}$ volume on Labeling: The labeling yield was found to be greatly dependent on the volume of the $^{99\text{m}}\text{Tc}$ eluates used for kit reconstitution. Labeling of kit with $^{99\text{m}}\text{Tc}$ was best done using activity in the range of 1.0 to 3.0 mCi.

4. Compatibility Studies: It has been often reported that kits designed for use with $^{99\text{m}}\text{Tc}$ from alumina generator are not compatible with $^{99\text{m}}\text{Tc}$ obtained by solvent (MEK) extraction. Thus, it is essential to check the performance of this kit using $^{99\text{m}}\text{Tc}$ pertechnetate ($^{99\text{m}}\text{TcO}_4$) from different specifications in pH, MEK content, Molybdenum content etc. This compatibility study would enable us to determine whether subtle alterations in kit composition are required in order to achieve high labeling efficiency. From the results obtained, the MINT produced kits were found to be compatible with all three $^{99\text{m}}\text{Tc}$ generators eluates giving a minimum labeling of 97.0% (Table 2).

5. Animal Studies: The studies performed in rats indicated the ^{99m}Tc -MIBI myocardial accumulation is up to 3 hours. The lack of significant myocardial washout or redistribution could mean that this agent possibly functions as a chemical microsphere and bound tightly within myocytes. It also gives relatively low lung and liver activity with sufficient myocardial retention to give a good heart to background ratio. Tissue distribution data in rats also showed that ^{99m}Tc -MIBI is excreted primarily in the urine and to a lesser extent in the faeces. Approximately 20% of the activity is excreted within the first 3 hours post-injection. Its rapid blood clearance, rapid and high myocardial uptake, no or very limited myocardial redistribution, its favorable target/non-target ratios (heart/lung, heart/liver, heart/spleen, heart/blood) has made it an excellent ^{99m}Tc labeled compound for myocardial perfusion studies.

6. Stability Studies: The labeled ^{99m}Tc -MIBI complex showed no change in radiochemical purity (>95%) 6 hours post-preparation (Table 2). The ITLC-SG and TLC methods used for RCP determination indicated practically no formation of undesirable $^{99m}\text{TcO}_2$ and $^{99m}\text{TcO}_4^-$. The animal studies done using these labeled compounds indicated similar biological distribution as to those of the freshly prepared ^{99m}Tc -MIBI. Its 6 hours stability post-preparation at room temperature would ensure its continuous supply for daily routine clinical use.

Table 1 Radiochemical purity determination

Methods	Solvent	% Labeling (MINT kit)		% Labeling (Cardiolite kit)		Time (min)
		10 min.	6 hours	10 min.	6 hours	
TLC	Ethanol	98.9 \pm 0.21	99.0 \pm 0.32	99.5 \pm 0.4	99.3 \pm 0.62	23
ITLC-SG	Saline/ Acetone	98.4 \pm 0.31	98.8 \pm 0.12	98.9 \pm 0.57	99.3 \pm 0.26	5
Solvent Extraction	$\text{CHCl}_3/\text{H}_2\text{O}$ (1:1)	98.2 \pm 0.6	98.1 \pm 0.54	98.8 \pm 0.7	98.3 \pm 0.65	5
HPLC-RP C18 (5)	$\text{MeOH}/(\text{NH}_4)_2\text{SO}_4$ (95:5)	99.5 \pm 0.2	99.3 \pm 0.13	99.7 \pm 0.22	99.5 \pm 0.19	16

Each value is the mean \pm SD of 5 results

Table 2 Compatibility study of ^{99m}Tc -MIBI

^{99m}Tc Sources	% Labeling	
	10 min.	6 hours
Chromatographic generator (Amersham)	98.7 \pm 0.7	99.1 \pm 0.6
Chromatographic generator (MINT)	98.2 \pm 0.31	98.9 \pm 0.44
MEK Extracted ^{99m}Tc (MINT)	97.9 \pm 0.13	98.9 \pm 0.44

Each value is the mean \pm SD of 5 results

7. Toxicity Studies: No clinical signs of abnormality was observed over a period of 1 month in mice injected with a dose equivalent in amount of 100 times the human dose in proportion to body weight. Similar results were also observed [5] using higher dosage of 900 times the human dose.

8. Comparative Studies: From the results obtained it was found that both MINT and Cardiolite kits gave equivalent biological distribution and organs uptake in rats. This study indicates that both kits are comparable in quality.

CONCLUSION

Finding in this study indicated that the MINT produced MIBI kit for myocardial perfusion imaging is comparable in quality to that of commercials. However, special precaution should be taken during the kit preparation process in order to achieve satisfactory labeling (>90%) for it to have any clinical application.

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BIOEVALUATION

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**CANCER-AFFINE RADIOPHARMACEUTICALS FOR THE STUDY OF
BIOCHEMICAL NATURE OF CANCER AND IN THE EARLY DIAGNOSIS
AND FOLLOW-UP OF CANCER AND ITS SYSTEMIC THERAPY**

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Abstract

Cancer patient needs less diagnosis but an effective therapy. The systemic nature of cancer, often right from its inception, requires systemic therapy with cancer-affine radiopharmaceuticals which contain radionuclide species recognizing both the primary and secondary cancers which have generally different biochemical properties. Cancers may be classified into two groups: I. CATIONIC COMPLEX-AFFINE TUMOURS: Lung cancer, thyroid cancer, primary breast cancer, renal cell carcinoma, bone metastases from anionic complex-affine cancers,; II. ANIONIC COMPLEX-AFFINE TUMOURS: Primary prostate cancer, melanoma, hepatocellular carcinoma, osteosarcoma, Ewing's sarcoma, bone metastases from cationic complex-affine cancer. With cancer-affine citratogallate-67 complexes we have diagnosed and followed up, and with citratoyttrate-90 complexes we have treated advanced breast, prostate, renal cell cancer patients. The patient preparation by advising to avoid cancer risk factors and to take cancer preventing and radiopharmaceutical stabilizing diets during diagnosis and therapy have given better



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results. Friendliness, caring, visits and telephone calls from the therapist group help to obtain better outcomes of the diagnosis, and mainly of the therapy. The complexes of these radionuclides with other chelating agents EDTA and DPTA are expected to give better images and cure of advanced cancer patients. Cancer-affine formulations of Tc-99m(V), Re-186(V) and Re-188(V)-DMSA are being studied for their future use in early diagnosis and follow-up, and for the systemic therapy of cancer which will show affinity for them.

INTRODUCTION

Despite enormous efforts devoted to identify cancer-causing environmental and dietary risk factors [1-5], and find diets rich in those foods and drinks that protect against or have curative action [4-7], cancer still remains the major cause of morbidity and mortality [1,8,9]. Several decades of basic research and trials of promising new therapies could not so far lead to an effective therapy of cancer [8-11]. Since cancer has been found to be a systemic disease right from its inception [12,13], nonsystemic modes of therapy (surgery and teletherapy) cannot cure the patient [8-11], and are responsible for serious side effects because they affect both healthy and diseased tissue and are unable to reach all cancer lesions.

Although systemic in nature, also the chemotherapy administered today, due to its empiricity, is rarely effective and causes serious toxicity [8,10,14].

Nuclear medicine has been defined [15-18] as "the application of radioactive materials (called radiopharmaceuticals) to the diagnosis and treatment of patients and the study of human disease". In spite of several radionuclides with very attractive physical properties to be applied in the systemic detection and therapy of cancer and very refined instrumentation (γ - and positron-cameras available today), the results of cancer imaging and cancer therapy after systemic administration of the radiopharmaceuticals,

commercially available, are not always satisfactory. Thus, computed tomography (CT) and magnetic resonance imaging (MRI), for example, have replaced radionuclidic liver and brain scans [19,20] and the fate of other radionuclide imagings may be the same, if the quality of radiopharmaceuticals is not improved. Also radionuclidic cancer therapy with many β - and α -emitting radiopharmaceuticals used is not effective and often causes serious myelosuppression [21-24]. Even the use of different monoclonal antibodies to target the radionuclides to the cancer for radioimmunodetection (RID) and for radioimmunotherapy (RIT) has not led to the tumour-specific concentration of the radionuclides [25-29]. The object of our research has been, therefore, to obtain the radionuclides for cancer diagnosis and cancer therapy in the chemical form in solution which when injected to cancer-bearing subjects seek out cancer sites and leave the healthy organs untouched [30]. Such radionuclide species in solution we call cancer-affine radiopharmaceuticals. Due to the chemical similarities and the possibility to extend the conditions of cancer-affine γ -emitting radiopharmaceuticals to the synthesis of the β -emitting homologue radionuclide, we chose the diagnosis and therapy radionuclide couples: Ga-67---Y-90 and Tc-99m---Re-186 or Re-188.

MATERIALS AND METHODS

Gallium-67 and yttrium-90 citrate solutions are obtained from different suppliers. Unfortunately, the radiopharmaceutical formulation in the commercial Ga-67 is not the same [30].

The nature and stability of the radionuclide species in the radiopharmaceutical solution is examined chromatographically and electrophoretically.

The conditions under which the radiopharmaceutical will be stable both in vitro and in vivo is also found by chromatography and electrophoresis.

The effect of drinks and nutrients on the biochemical properties of the radiopharmaceuticals were studied chromatographically and electrophoretically in order to get better diagnostic and therapeutic results.

The affinity of the radionuclide for cancer was examined by total-body scintigram of the cancer patient.

For yttrium-90, we developed [31] a procedure to study its distribution in patients with a γ -camera fitted with a low energy and ultra-high-sensitivity collimator and with the window set at the Bremsstrahlung peak (72.2 KeV).

The cancer-affine species of the radionuclides in the radiopharmaceutical formulation was recognized by the selective uptake of the radionuclide in the total-body image for both diagnostic and therapeutic radiopharmaceuticals.

Only cancer-affine radiopharmaceuticals were administered in our Institutes to cancer patients who came either for diagnosis or for radionuclidic therapy. Before the radiopharmaceutical administration the patients were prepared for the examination or treatment by :1) Friendly atmosphere reserved for them, 2) Advice to eliminate all risk factors which possibly were responsible for their cancer, and 3) the recommendation of nutrients which prevent or have a curative action on their disease. This was very important for patients admitted for the radionuclidic therapy.

Patients with advanced cancer are treated with I-131, Y-90 citrate and with Sr-89 chloride solutions made cancer-affine. For simplicity, this paper will deal with only metal radionuclide complex species of Y-90 and Sr-89 only.

RESULTS

1 COMMERCIAL RADIOPHARMACEUTICALS FOR CANCER DIAGNOSIS

Despite very refined radionuclide distribution study instrumentation (γ -camera for the detection of γ - and β -emitting radionuclides, and positron emission tomography (PET)

camera) available today for high quality imaging of even small cancerous lesion seated deep in the body [32,33], the radionuclide image of the cancer patients and the outcome of the therapy have been giving unsatisfactory results due to the very empirical nature of the radiopharmaceuticals employed for these purposes.

Gallium-67 citrate solutions available commercially, since 1969 when its first use for tumour imaging was found [34], has still been being supplied by radiopharmaceutical suppliers in the formulations where the sodium citrate concentration, for the same concentration of the radionuclide, varies from 1.67 mg/ml to 50 mg/ml [30,35]. We have shown [30] that the number, nature, and stability of Ga-67 species in the commercial Ga-67 varies greatly and depends mainly on the concentration of the chelating ligand, citrate, in the radiopharmaceutical solution. We further showed that the Ga-67 radiopharmaceuticals from one supplier may be suitable for some type of cancer and their metastases, while that from the other supplier may give better results for other types of cancer, which have affinity for the Ga-67 species present in these solutions. The radionuclide is mainly present as easily hydrolysable cation in the low sodium citrate concentration Ga-67 radiopharmaceuticals, while more stable anionic citratogallate-67 is main constituent of the radiopharmaceutical solutions with higher ligand concentration. Gallium-67, present even in 50 mg/ml sodium citrate containing solution, is not purely citratogallate-67 but contains low concentration of the cationic Ga-67 as well which hydrolyses. Without giving any importance to the fact that how this difference in the chemical nature and stability of Ga-67 can affect the uptake of the radionuclide in the tumour and in the healthy organs of the cancer patient, the nuclear physician injects Ga-67 citrate from different suppliers and expects high quality tumour imaging. From the results obtained from different Ga-67 radiopharmaceuticals, without giving any importance to which Ga-67 species one is dealing with, "the mechanism of Ga-67 localization in malignant disease" [36] is often proposed. We have shown [30] that for each type of cancer there is a specific sodium citrate formulation which gives the maximum localization of the radionuclide in the cancer sites and minimum in the healthy organs. Such

radiopharmaceutical formulations we call “cancer-affine” radiopharmaceuticals. We further observed that the radionuclide, both from lower and from higher ligand concentrations, concentrates mainly in healthy organs, like liver, although by different mechanisms [30].

Similarly, more recent tumour imaging radiopharmaceutical, pentavalent technetium-99m-dimercaptosuccinate (Tc-99m-DMSA) is being used in different formulations and the results of this difference are being studied.

2 BIOCHEMICAL NATURE OF CANCER

The selective uptake of gallium-67 from radiopharmaceuticals, the composition of which had been previously studied by chromatography and electrophoresis, before being injected to the cancer patient for total body scan for cancer sites, permitted the classification of cancer into two groups [37]:

- (I) CATIONIC Ga-67-AFFINE TUMOURS, and
- (II) ANIONIC Ga-67-AFFINE TUMOURS.

Later observations with radiopharmaceuticals containing other radionuclides (In-111, In-114m, Tl-201, and I-131) of known chemical composition have led to the present classification of tumours [38,39]:

- (I) CATIONIC COMPLEX-AFFINE TUMOURS: Lung cancer, thyroid cancer, primary breast cancer, renal cell carcinoma, bone metastases from anionic complex-affine cancers,
- (II) ANIONIC COMPLEX-AFFINE TUMOURS: Primary prostate cancer, melanoma, hepato-cellular carcinoma, osteosarcoma, Ewing's sarcoma, bone metastases from cationic complex-affine tumours,

3 IDEAL RADIONUCLIDES FOR CANCER DIAGNOSIS AND THERAPY

Table 1 shows the elements the radionuclides of which have been used for cancer diagnosis and therapy. Considering the biochemical nature of cancer and the biochemical

Table I. Elements whose radionuclides are used for γ -imaging, pure β therapy, $\gamma + \beta$ therapy, and α -therapy.





H -0.65																	He 2				
Li 1.47	Be 1.57															B 1.30	C 1.54	N 1.87	O 1.51	F 0.75	Ne 0.89
Na 1.03	Mg 1.03	In=Charge Density														Al 1.58	Si 1.52	P 1.43	S 1.09	Cl 0.55	Ar 0.64
K 0.75	Ca 2.02	Sc 3.70	Ti 5.88	V 8.47	Cr 11.54	Mn 16.06	Fe 14.68	Co 14.76	Ni 2.30	Cu 2.78	Zn 2.70	Ga 4.84	Ge 7.54	As 10.9	Se 14.3	Br 0.51	Kr 36				
Rb 0.68	Sr 1.79	Y 3.26	Zr 5.06	Nb 7.25	Mo 9.68	Tc 5.56	Ru 5.97	Rh 4.41	Pd 6.15	Ag 0.79	Cd 2.06	In 3.70	Sn 2.15	Sb 3.95	Te 10.7	I 0.45	Xe 54				
Cs 0.60	Ba 1.49	La 2.63	Hf 5.13	Ta 7.35	W 9.68	Re 5.56	Os 4.55	Ir 4.88	Pt 2.50	Au 0.73	Hg 1.82	Tl 3.16	Pb 1.67	Bi 3.13	Po 8.95	At 11.3	Rn 86				
Fr 1.05	Ra 1.40	Ac 2.54	Rf 1.04	Ha 1.05	106	107	108	109	110	111	112	113	114	115	116	117	118				
119	120	121	154	155	156	157	158	159	160	161	162	163	164	165	166	167	168				

Ce	Pr	Nd	Pm	Sm	Eu	Gd	Tb	Dy	Ho	Er	Tm	Yb	Lu
2.80	2.83	2.88	2.83	3.00	3.06	4.84	3.23	3.26	3.30	3.37	3.45	3.49	3.53

Th	Pa	U	Np	Pu	Am	Cm	Bk	Cf	Es	Fm	Md	No	Lr
3.92	4.08	4.12	4.21	4.30	4.35	96	97	98	99	100	101	102	103

122	123	124	125	126									151
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Periodic Table of Elements (— so far unknown elements)

 V-Imaging,
  Pure β -Therapy,
  γ + β -Therapy,
  α -Therapy

properties of different elements of the Periodic Table, we have shown [40] that the Third Group element radionuclides will be most suitable for the preparation of radiopharmaceuticals for cancer diagnosis and therapy. The middle members of the group

Ga-67 ($t_{1/2}=78$ h; γ emission: 90, 180, 300 KeV), and

Y-90 ($t_{1/2}=64$ h, pure β emission. 2.27 MeV),

are ideal for the synthesis of radiopharmaceuticals with suitable complexing agents respectively for the systemic diagnosis and therapy of cancer.

The radionuclides of the elements of lower and higher Groups of the Periodic Table have greater tendency to form respectively cationic and anionic species in aqueous

solution and therefore cannot easily diagnose or cure advanced cancer because the primary and secondary tumours have affinity for different ionic forms of the radiopharmaceutical or pharmaceutical.

The rich chemistry and the radionuclidic properties of the radionuclide homologues:

Tc-99m ($t_{1/2}$ =6 h; pure γ emission: 140 KeV), and

Re-186 ($t_{1/2}$ =3.8 d; β -emissions: 1.07, 0.93 MeV; γ emission: 1.37 KeV)

Re-188 ($t_{1/2}$ =16.7 h; β -emissions: 2.12, 1.96 MeV; γ emission: 155 KeV)

are, however, very attractive for synthesizing cancer affine radiopharmaceuticals for the diagnosis and therapy cancer. We are working on the biomedical properties of the DMSA complexes of this radionuclide couple.

4 IDEAL LIGANDS FOR CANCER-AFFINE RADIOPHARMACEUTICAL SYNTHESIS

The radionuclide stability and the nature of its complexes for the diagnosis and therapy of cancer demands the use of stronger chelating agents: EDTA and DTPA, being studied in our laboratories.

Due to the rarity of monoclonal antibodies [29], much work on the labelling with different radionuclides could not be done. Our work [39] with antihepatoma antibody (hepama-1) showed that the antibody labelled with I-131, Ga-67, or Y-90, has little affinity for the cancer and concentrates mainly in the healthy liver. Intra-arterial administration of I-131-hepama-1 reduced the hepatocellular mass to permit its surgical removal [41]. Further studies on the labelling of hepama-1 with other radionuclides are in progress both in Rome and Shanghai in order to make this antibody clinically useful in the diagnosis and therapy of hepatocellular carcinoma (HCC) patients.

5 CANCER-AFFINE Ga-67 AND Y-90 RADIOPHARMACEUTICALS FOR EARLY DIAGNOSIS AND SYSTEMIC THERAPY

Gallium-67 and yttrium-90, in sodium citrate solutions of concentration higher than 80 mg/ml, are stable and are present mainly as stable anionic citratogallate-67 or citratoyttrate-90. They can be administered in the diagnosis and therapy of advanced anionic complex-affine cancers, like advanced prostate cancer patients [42-44] or in the diagnosis and pain palliation of operated advanced breast cancer [45] or renal cell carcinoma [46] patients with diffused bone metastases refractory to other therapies. All patients were pain free within 3 to 4 h p.i. but needed second injection first at weekly, then at longer intervals.

Gallium-67 and more so yttrium-90, due to their high charge density (Table I), are easily hydrolysed in the solution of lower sodium citrate concentrations, and the hydrolysed Ga-67 or Y-90 concentrates easily in the liver, spleen, healthy bone, and the intestine as shown in the Ga-67 scans of a normal patient injected with commercial Ga-67 solution containing 1.67 mg/ml sodium citrate (Fig.1).

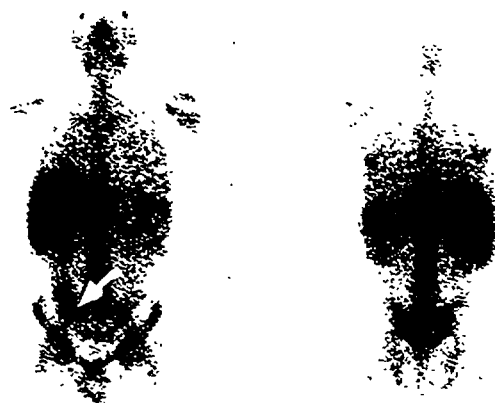


Fig. 1. Total-body distribution of Ga-67 in a healthy man injected with Ga-67 solution containing 1.76 mg/ml sodium citrate.



Fig 2 A Segmentary bone scan with Tc-99m-MDP of a prostate cancer patient

B Anterior (a) and posterior (p) total body scintigrams with cancer-affine Ga-67 of the same patient 40 days later shows many additional bone metastases

Freshly diluted Ga-67 solution, from the Ga-67 radiopharmaceuticals with higher sodium citrate concentration, does not show high uptake in healthy organs and localizes mainly in bone metastases as shown in Fig.2 B of a prostate cancer operated patient with diffused bone metastases. The patient had developed additional bone metastases during the period of 40 days between Tc-99m bone scan (Fig. 2 A) and the Ga-67 imaging.

Bremsstrahlung scans of the patients showed the concentration of the pure β -emitting radionuclides in all bone metastases seen previously on Ga-67 or bone scintigraphy of the patients [44-47].

5. IN VIVO STABILIZATION OF RADIOPHARMACEUTICALS FOR CANCER DIAGNOSIS AND THERAPY

Several attempts [23, 48, 49] have been made to obtain better results for radionuclide cancer therapy or pain palliation by administering another therapy radiopharmaceutical or pharmaceutical which has similar therapeutic action. Unfortunately, the combined therapy results were not better than those obtained with a single agent, and the toxicity of each component to the patient was additive [49].

We have been examining the effect of several drinks which will have beneficial effects on the patient's health and will stabilize also the radiopharmaceutical in vivo and thus could improve its therapeutic action.

The stability of the radiopharmaceutical was examined chromatographically and electrophoretically in several daily drinks. The results showed:

- 1) Coffee, tea and both red and white wine made insoluble species of the radionuclide (Ga-67, Y-90, Sr-89), and therefore had to be avoided.
- 2) Orange and tomato fresh juice stabilized the anionic complex radiopharmaceuticals, while the lemon juice has stabilizing action on the cationic complex radiopharmaceuticals.
- 3) Solid food before the administration of the radiopharmaceuticals favoured the intestinal uptake of the radiopharmaceuticals and therefore had to be avoided.

The patients are, therefore, advised to avoid solid food, coffee, tea, and alcohol at least for ten hours before and 3 to 4 hours after the injection of the radiopharmaceutical.

We advise the patients coming for radionuclide therapy to avoid the cancer risk factors (tobacco, alcohol, and saturated fat) for ever.

Patients were advised to drink much orange juice during Y-90 and lemon juice during Sr-89 therapy both before and for long time after the administration of the radiopharmaceuticals [45, 50].

Better results are obtained when the patients followed our advice regarding their daily habits and food.

6. THE ADVANCED CANCER PATIENT IN OUR NUCLEAR MEDICINE DEPARTMENT

Like Dr. Henkin [22], we also believe that: "Where the therapist is caring, spends time with the patient and offers reassurance and support, outcomes tend to be better than when those conditions are not met". We try to smile and telephone to the patients and their relative often, and they are glad to see and hear us.

7. CONCLUSIONS

Cancer-affine γ - and β -emitting radiopharmaceuticals can respectively detect and cure cancer patients.

Better results of diagnosis and therapy of cancer with radiopharmaceuticals are obtained if the patients are kept on food and drinks which stabilize the radiopharmaceutical in vivo and if the nuclear medicine laboratory provides a friendly and caring atmosphere to the patient.

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Abstract

¹⁶⁶Ho-Chitosan is a complex of ¹⁶⁶Ho and N-glucosamine with 400 to 500 kD MW, which chelates metal ions and degrades slowly in vivo. In mice, ¹⁶⁶Ho-Chitosan administered intraperitoneally was uniformly bound to the peritoneal wall(94%), and the surface dose calculated by Monte Carlo simulation (EGS 4 code) was 81 Gy/uCi/cm². ¹⁶⁶Ho-Chitosan was administered intraperitoneally as an adjunct in the treatment of ovarian cancer with diffuse spread of malignant cells in the peritoneal surfaces including the diaphragm. 97-99% of ¹⁶⁶Ho-chitosan was localized within the peritoneal cavity, and more than 90% of ¹⁶⁶Ho-chitosan was attached to the peritoneal wall. Partial response were observed in 4 among 5 patients with ovarian cancer without severe toxicity. Intracavitary radiation therapy with ¹⁶⁶Ho-chitosan in the cystic brain tumor, 5 of 8 cysts were shrunken in size with thinning of the wall, 2 out of 8 showed growth retardation. For large or multiple primary liver cancers, which were inoperable and resistant to chemotherapy. ¹⁶⁶Ho-chitosan was used for intraarterial injection, because this solution became gel with neutral pH. In the primary liver cancer, radioactivity was distributed in the territory of selected hepatic arterial branch, and partial responses were observed in 2 cases. For the large solitary liver tumor, which was not operable due to its location, direct intratumoral injection of the radioisotope had a limited response. ¹⁸FDG PET was a useful tool to follow up those radionuclide therapy, and guide to plan the next therapy. In one case of the large single metastatic stomach cancer in the liver, ¹⁸FDG PET was done two weeks after intratumoral injection of

^{166}Ho -chitosan, which showed cold defects matched with distribution of ^{166}Ho -chitosan, and second injection was guided by PET image. Various methods of the administration of ^{166}Ho -chitosan could be used for the treatment of the cancers.

Introduction

Many therapeutic radiopharmaceuticals have been used in various ways over 50 years, and due to their physical characteristics many of them disappeared. New radionuclides were tried with the progress of the technologies in radiopharmaceutical chemistry[1,2]. ^{166}Ho is a good therapeutic radionuclide because of its suitable half-life(26.8 hours), high beta energy(1.78, 1.86MeV) and 6% gamma ray(80.6keV) for imaging. Chitosan is a kind of N-glucosamine with 400 to 500 kD MW, which chelates metal ions and degrades slowly in vivo[3]. ^{166}Ho -chitosan could be suitable for intracavitary, intratumoral and intraarterial injection, because this solution become gel with neutral pH. High radiation doses can be delivered to the peritoneal serosa by administering radioisotope intraperitoneally, as an adjunct in the treatment of ovarian cancer with diffuse spread of malignant cells in the peritoneal surfaces including the diaphragm[4-8]. Intracavitary radiation therapy is an effective method to treat the brain cystic tumor[1,9]. Intraarterial infusion of the radioisotope to the large or multiple primary liver cancers, which are inoperable and resistant to chemotherapy, were tried with various results[10-13]. For the large solitary liver tumor, which is not operable due to its location, direct intratumoral injection of the radioisotope may have a role[14]. In this paper, animal experiments for the dosimetry of the intracavitary therapy, and intracavitary, intraarterial and intratumoral administration for various cancers were tried and reported. For the selected case, ^{18}F FDG PET was used to follow up the effect of the radionuclide therapy, and to plan the next therapy.

Material and Method

1. Beta dosimetry in peritoneal cavity of rat.

To evaluated the absorbed dose by the beta particles emitted from ^{166}Ho to the cavitary wall, ^{166}Ho -chitosan was injected into the peritoneal cavity of rat, and whole body autoradiographs were taken at 6 hrs and 24 hrs later to determine the biodistribution and dosimetry. To see the attached fraction to the peritoneal surface, the whole body radioactivity was counted, ascites was removed and both whole body and ascitic fluid were counted. Simulation of energy transfer from the beta particles to the cavity wall using the Monte Carlo code EGS4 was done, and used as a standard for the planning therapy. The radiation absorbed dose to the cyst wall is assessed in same way for different sizes of the tumoral cyst and a varying amount of ^{166}Ho -Chitosan complex, or ^{166}Ho -CHICO, administered into the cyst.

Table I. Patient Profiles with Intraperitoneal ^{166}Ho -Chitosan Administration

	Case 1	Case 2	Case 3	Case 4	Case 5
Age	31	51	40	65	52
Initial FIGO stage	IIIc	IIIc	IIIc	IIIc	IIIc
Pathologic type	Immature teratoma	Mucinous cancer	Malignant sex cord tumor	Mucinous cancer	Clear cell carcinoma
Previous treatment	Surgery X 2 ChemoTx1 RadioTx2 ChemoTx3	Surgery X 2 ChemoTx4	Surgery X 2 ChemoTx5	Surgery X 3 ChemoTx6	Surgery X 3 ChemoTx7
Disease status	Recurrent	Persistent	Persistent	Persistent	Recurrent
Residual mass	> 4 cm	> 4 cm	> 4 cm	> 4 cm	> 4 cm

1 BEP(bleomycin, VP-16, Cisplatin) 7 cycle, 2 Open field technique, 3 VAC(vincristine, adriamycin cyclophosphamide) 1 cycle, 4 CAC(cyclophosphamide, adriamycin, carboplatin) 3 cycle, Et-P(etoposide, cisplatin) 3 cycle, Et-I(etoposide, ifosfamide) 3 cycle, 5 CAP 6 cycle, Carboplatin 3 cycle, CC(cyclophosphamide, carboplatin) 1 cycle, 6 CAP 6 cycle, TP(taxol, cisplatin) 2 cycle, 7 Melphalan 6 cycle, CP(cyclophosphamide cisplatin) 8 cycle, TP 1 cycle

Table II. Absorbed dose (Gy) to a 10 μ m-thick peritoneal wall at a varying depth for 1.3 μ Ci/cm² of ¹⁶⁶Ho surface source and 0.1 μ Ci/ml of ¹⁶⁶Ho volume source.

target depth (mm)	Surface source (Gy)	volume source (Gy)	total absorbed dose (Gy)
0.0	105.	17.8	123.
1.0	5.08	3.50	8.59
2.0	1.42	1.58	3.00
3.0	.428	.605	1.03
4.0	.122	.205	.327

2. Intraperitoneal ¹⁶⁶Ho-Chitosan therapy

Since October 1996, intracavitary ¹⁶⁶Ho-chitosan therapy were tried to see the biodistribution, dosimetry, toxicity and responses in 5 ovarian cancer patients with peritoneal metastasis, who did not responded to chemotherapy or recurred after surgery and chemotherapy (Table 2). Clinical protocol was reviewed by the ethical committee of Korea Cancer Center Hospital, and patients signed on the informed consent before therapy. Patients were received cytoreductive surgery, and preitoneal catheter (Bard Titanium implanted Port, Bard Access System, Salt Lake City, Utah) was implanted. Seven to fourteen days after the surgery, ^{99m}Tc-sulfur colloid was injected via the port, and abdominal scintigraphy was obtained to see the distribution of the radioisotope. When the ascites was small, one to two liter of normal saline was infused via the port before the injection of the radioisotope. 25 to 40 mCi of ¹⁶⁶Ho-Chitosan with 50 to 100 ml normal saline was injected via the port, and abdominal massage was done to distribute the radioisotope evenly. Toxicity were evaluated according to the WHO classification. For three patients, cisplatin-based systemic chemotherapy was combined.

3. Intracavitary ^{166}Ho -Chitosan therapy

Since October 1996, intracavitary ^{166}Ho -chitosan therapy were tried in the unresectable seven cystic brain tumors (2 cases of metastatic brain tumors from lung cancer, 1 case of recurrent trigeminal neurinoma, 3 cases of recurrent low grade cystic astrocytomas, and 1 case of craniopharyngioma).(Table 2). Clinical protocol was reviewed by the ethical committee of Korea Cancer Center Hospital, and patients signed on the informed consent before therapy. The cyst volume and wall thickness were measured by MRI before ^{166}Ho -Chitosan injection. The Ommaya reservoir was installed stereotactically into the cyst. ^{166}Ho -Chitosan is injected into the Ommaya reservoir. After ^{166}Ho -Chitosan injection, the distribution of isotope was monitored by gamma camera. Two injections were administered in two cases, and one injection in all the others. The response was evaluated with MRI.

4. Intraarterial and intratumoral injection of ^{166}Ho -Chitosan in the primary and metastatic hepatic tumors.

Intraarterial injection in inoperable primary liver cancer just before chemoembolization was also tried in 58 year old man with 3 cm mass in the right dome of the cirrhotic liver in July 1996. After the chemoembolization, scintigraphy was done to see the distribution of ^{166}Ho -Chitosan. Four weeks after the therapy, liver CT and serum alpha fetoprotein was followed up.

Ultrasound-guided intratumoral injection of the ^{166}Ho -Chitosan was done to the 50 year old male, who had subtotal gastrectomy due to stomach cancer in August 1996, with single large liver mass by abdominal CT metastasized from the stomach cancer in August, 1997. Three weeks after the injection of ^{166}Ho -Chitosan, ^{18}F FDG whole body

PET scan was performed 40 minutes after the injection of 10 mCi of ^{18}F FDG with GE Advance PET scanner to see the effect of the therapy, to find other metastatic lesion and to plan the second therapy with ^{166}Ho -Chitosan.

Results and Discussion

1. Beta dosimetry in peritoneal cavity of rat.

The whole body autoradiography of rat with intraperitoneal injection of ^{166}Ho -Chitosan showed confined radioactivity within the preitoneal cavity(Figure 1). 97-99% of ^{166}Ho -chitosan was localized within the peritoneal cavity, and the radioactivity attached to the peritoneal surface was more than 90% of injected dose. The distribution of the radioactivity on the peritoneal surface observed by the autoradiography was quite even. Dose to the peritoneal surface was 80.8 Gy/uCi/cm² (85%)from the radioactivity on the surface itself, and 7.40 Gy/uCi/ml (15%) from the peritoneal fluid (Table 2). The volume of the ascites could be estimated by the $^{99\text{m}}\text{Tc}$ -sulfur colloid injection and counting the total injected radioactivity and the radioactivity of the ascites drawn. With the standard data of the peritoneal surface area, it could be possible to calculate the peritoneal surface absorbed dose directly.

2. Intraperitoneal ^{166}Ho -Chitosan therapy

The distribution of the intraperitoneally administered ^{166}Ho -Chitosan was confined to the preitoneal cavity up to 77 hours after the injection(Figure 2). Four patients showed decreased ascites volume, or or decreased viscosity of the ascites. Three patients died in three months after the therapy. One patient showed grade 4 diarrhea, and grade 3 anemia



Figure 1. Whole body autoradiography of the rat with intraperitoneal injection of ^{166}Ho -Chitosan, showing the radioactivity coating the peritoneal surface.

Table III. Clinical Effects after ^{166}Ho -Chitosan Application

	Case 1	Case 2	Case 3	Case 4	Case 5
Ascites tapping before Ho	3/weeks serotic fluid	ascites(+) but not done due to viscosity	2/weeks serotic fluid	Ascites(+) but not done due to viscosity	small localized cyst in gastro-colic ligament
No. of injection	1 cycle	4 cycles	2 cycles	6 cycles	1 cycle
Dosage (mCi/Total)	40	40, 40, 35, 30	35, 35	25, 30, 30, 35, 35, 30	7
Combined chemotherapy	None	Cisplatin + Cytosin	Cisplatin	Carboplatin	None
Disease status after last ^{166}Ho -Chitosan	No change Death after 2 months later	Marked decreased ascites, Death 3 months later	Marked decreased ascites, Death 2 months later	Liquefied & decreased ascites, Stable disease status	Decreased speed in collecting fluid, Stable disease status

Table IV. Toxicity Evaluation(WHO Classification; total 14 cycles)

	Grade 1	Grade 2	Grade 3	Grade 4
Abdominal pain	4	4	-	-
Nausea/Vomiting	6	-	-	-
Diarrhea	3	3	-	1
Fever	-	2	-	-
Anemia	1	5	1	-
Leucopenia	1	3	1	-
Dyspnea	-	1	-	-
Allergic reaction	1	-	-	-
Proteinuria	1	-	-	-
Hematuria	3	-	-	-
Peripheral neuropathy	-	-	3	-

Table V. Clinical Effects of Cystic brain Tumors after ^{166}Ho -Chitosan Therapy

Patient Number	Sex/ Age	Diagnosis	Injected Dose(mCi)	Volume of the Cyst (ml)		Thickness of the WaLL(mm)	
				Before/After Therapy	Before/After Therapy	Before/After Therapy	Before/After Therapy
1	M/66	Metastatic Lung Cancer	15, 15, 15	6.3	2.7	3	2
2	F/39	Neurilemmoma	15, 15	24	24	3	3
3	M/63	Metastatic Lung Cancer	15, 20	16.8	6.2	1	2
4	F/34	Astrocytoma	10	60	2	1	1
5	F/26	Astrocytoma	10	47.4	10.6	1	2
6	M/14	Craniopharyngioma	20, 15	10.7	16.1	3	3
7	M/26	Astrocytoma	20	47	53.5	3	1
8	F/49	Metastatic Breast Cancer	20, 5	14.3	0	2	1

and leukopenia was noted each. Partial responses were observed in 4 among 5 patients with ovarian cancer without severe toxicity (Table 3,4). There was no response in patient without combined chemotherapy, who died two months later, suggested synergism between the radioactivity and the chemotherapeutic agents, or higher radiation dose to be effective alone. ^{166}Ho -Chitosan was distributed rather uniformly and not affected by the gravity, if there was no adhesion between the peritoneal surfaces, which could be an advantage over the radioactive particles. Toxicity from the damage of the intestinal mucosa limits the administered dose, and chitosan complex with radioisotope of less beta energy should be considered for higher radiation dose.

3. Intracavitary ^{166}Ho -Chitosan therapy

Five of 8 cases were shrunk in size with thinning of the cyst wall, 2 of 8 cases showed growth arrest, and one case showed progression (Figure 3, Table 5). Using the Monte Carlo simulation, when 10 mCi of ^{166}Ho -Chitosan was uniformly bound to the cyst wall surface of a 3 cm-diameter cyst, the absorbed dose was 40.06 Gy, 14.96 Gy, 5.315



Figure 2. Scintigram of a patient 77 hour after intraperitoneal injection of ^{166}Ho -Chitosan, showing radioactivity confined to the peritoneal cavity.

Gy, and 1.660 Gy at 1 mm, 2 mm, 3mm, and 4 mm in depth, respectively. When 10 mCi of ^{166}Ho -Chitosan was uniformly diffused in the cystic fluid of a 3 cm-diameter cyst, the absorbed dose was 601.7 Gy, 188.7 Gy, 73.87 Gy, and 20.78 Gy at 1 mm, 2 mm, 3mm, and 4 mm in depth, respectively. Estimated surface dose of cyst wall was between 79 and 2566 Gy. No one showed systemic absorption of ^{166}Ho -Chitosan, and specific complication associated with isotope injection. ^{166}Ho -Chitosan intracavitary radiation therapy for cystic brain tumor may be safe, and reliable method and deserves further evaluation. Accurate dosimetry was difficult, because some radioactivity remained in the reservoir, and the fraction of the radioactivity in the cyst could not be measured accurately. When the wall of the cyst located just beside the ventricle, there could be a danger of the rupture and communication between cyst and ventricle, which spread the radioactivity in the CSF space.

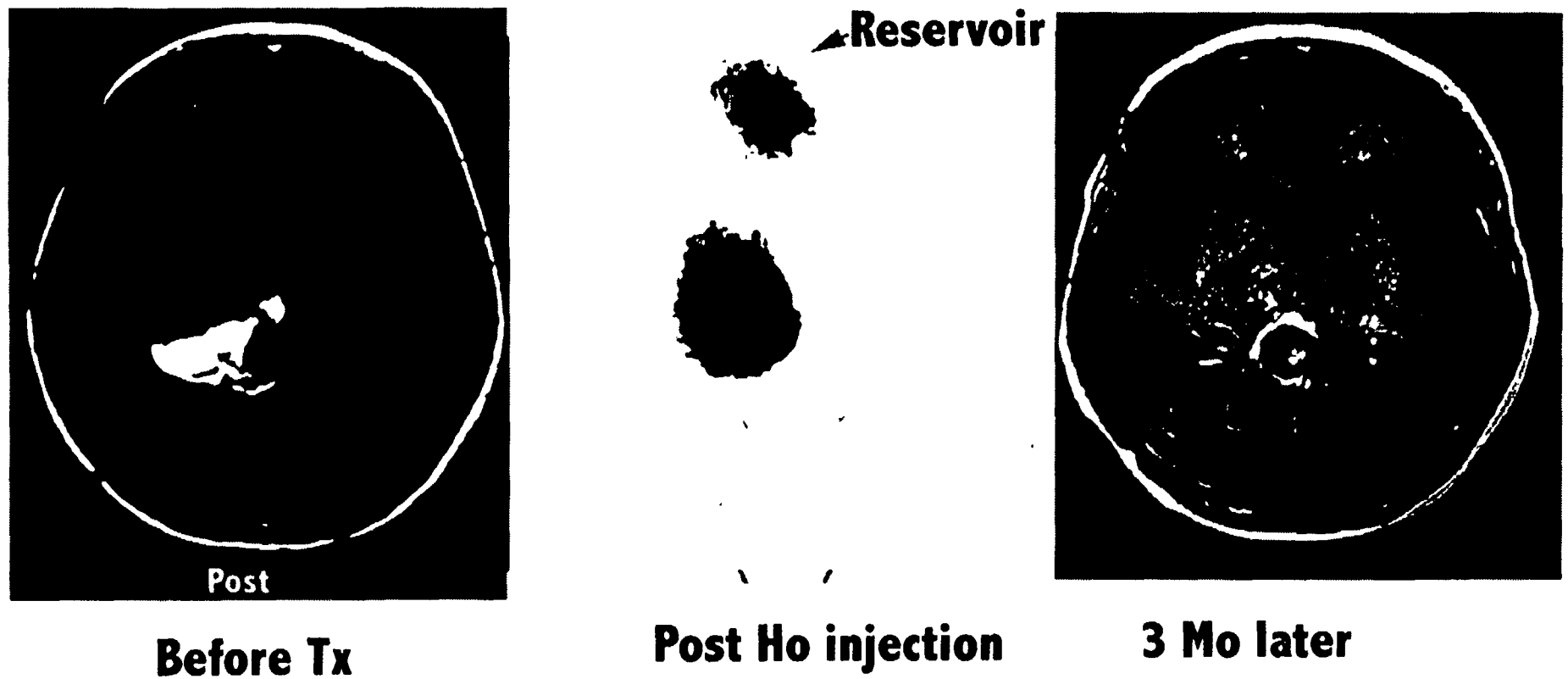
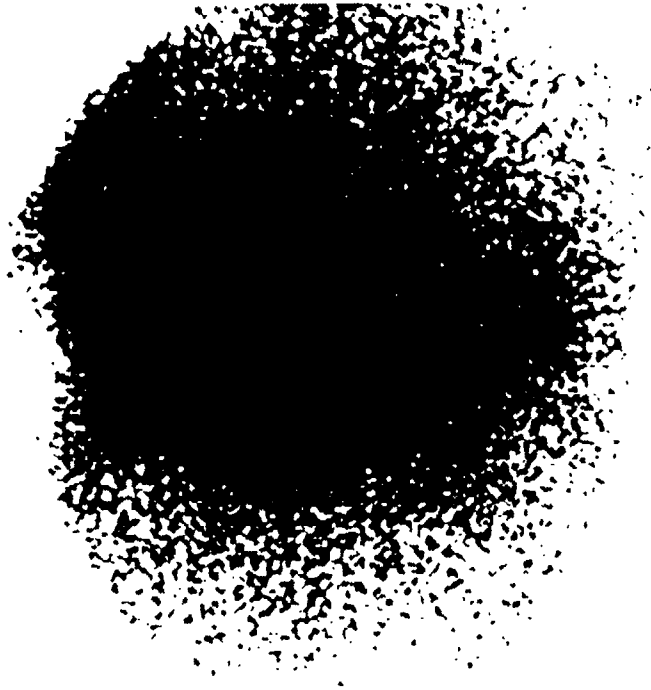


Figure 3. MRI and scintigram of a patient with cystic astrocytoma before and after ^{166}Ho -chitosan therapy. MRI before therapy(left), scintigram after injection of ^{166}Ho -chitosan(middle), MRI three months after therapy(right).



After Ho injection



After TAE

Figure 4. Distribution of the radioactivity after the intraarterial injection of ¹⁶⁶Ho-chitosan followed by chemoembolization. Scintigram(left), angiogram(right).



Figure 5. Intratumoral injection of ^{166}Ho -chitosan in the single large liver mass metastasized from the stomach cancer. Ct before therapy(left), scintigram after injection of ^{166}Ho -chitosan(middle), ^{18}F FDG-PET three weeks after therapy(right).

4. Intraarterial and intratumoral injection of ^{166}Ho -Chitosan in the primary and metastatic hepatic tumors.

In the primary liver cancer, after the intraarterial injection of ^{166}Ho -Chitosan, radioactivity was distributed in the territory of selected hepatic arterial branch, and partial responses were observed in 2 cases(Figure 4). ^{166}Ho -chitosan could be suitable for intraarterial injection, because this solution become gel with neutral pH. Many clinical trial were reported with radioiodinated lipiodol, but the gamma energy of the ^{131}I is too strong for good gamma imaging and need isolation of the patients. Besides, some radioactive oil leaked from the liver to the lung[10], which was not seen with ^{166}Ho -Chitosan. For the tumor with less arterial blood supply, combination of the intraarterial and intratumoral injection could be effective.

Intratumoral injection of ^{166}Ho -Chitosan showed persistent radioactivity in the injected site, and cold area in the mass by ^{18}F FDG-PET matched with the distribution of injected ^{166}Ho -Chitosan(Figure 5). In this case, the second injection of the ^{166}Ho -chitosan was guided by that PET image. Since ^{166}Ho -Chitosan did not diffuse far from the injected site(less than one cm in the tissue), which acted as a point source, multiple injection for the large mass is necessary. For the accurate dosimetry, microscopic autoradiography after the injection of ^{166}Ho -Chitosan into tissue should be obtained to see the distribution of the ^{166}Ho -Chitosan in three dimension, and Monte Carlo simulation should be done. Because of its localized distribution after intratumoral injection, it would be easy to escalate the dose to get higher radiation dose.

The viscosity of ^{166}Ho -chitosan can be changed for each method of administration, so that different in vivo distribution or diffusion in the tissue could be obtained. Further studies with different composition of ^{166}Ho -chitosan or complex with other radioisotope such as ^{153}Sm are expected.

In conclusion, ^{166}Ho -chitosan seemed to be safe and effective agent for the treatment of various cancers in various way, and many clinical trials and good responses were expected

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**NOVEL APPROACHES TO ANIMAL AND HUMAN PHARMACOLOGY:
PHARMACOKINETIC IMAGING WITH $^{195\text{m}}\text{Pt}$ -CISPLATIN AND
 $^{195\text{m}}\text{Pt}$ -CARBOPLATIN AND CORRELATIVE FUNCTIONAL IMAGING
OF THE PATHOPHYSIOLOGICAL STATUS OF TUMORS**

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Abstract

Pharmacokinetic imaging (the measurement of the time course of a drug at a target site), when combined with functional imaging (the measurement of the functional properties of the host's target site), provide the key elements needed to understand whether a drug can reach its desired target site (tumor) and whether it will do so at rates and in amounts that will make that drug effective in that individual.

We have now developed a method that allows for the concurrent measurement of all the three spaces of tumors: the cellular space can be studied by using the $^{195\text{m}}\text{Pt}$ -labeled drugs, the vascular space by using $^{99\text{m}}\text{Tc}$ -RBC's and the sum of all extracellular spaces by using ^{111}In -DTPA. Performing such imaging studies in such an order allows for concurrent measurements, inasmuch as the energy window of the gamma camera can be moved from $^{195\text{m}}\text{Pt}$ (mostly 66-77 keV) to $^{99\text{m}}\text{Tc}$ (centered on 141 keV) to ^{111}In (energies above 180 keV).

Studies in an animal tumor model have shown that the parameters of cisplatin or of carboplatin can be estimated from the imaging data following administration of the $^{195\text{m}}\text{Pt}$ -labeled drugs. As a next step, the functional status of tumors is of particular importance, given that changes in the tumor's pathophysiology will modify greatly the ability of drugs to be delivered to that target site - and hence, to be effective as antitumour agents. This is especially critical for drugs whose tumor targeting is governed by first passage phenomena, such as cisplatin and 5-FU, but very different for drugs whose targeting is governed by slow diffusion processes. These functional studies allow the direct measurement of the relative contributions that the well vascularized regions, the poorly vascularized regions, and the non-vascularized regions will make in allowing drugs to be targeted to tumor cells, as well as of the relative proportion of the vascular and the IF spaces in such regions.

A new approach to chemotherapeutic planning is proposed: the use of radiolabeled drug, in trace (radiopharmaceutical) doses, may allow an estimate of which fraction of that drug will target the desired effector sites. Such knowledge can then be used to select the methods of drug administration that is likely to be the most effective for optimizing drug delivery, and, thereby, enhancing the therapeutic effectiveness of that drug. This use of radiopharmaceuticals for drug monitoring (Pharmacokinetic Imaging) complements the current uses of radiopharmaceuticals for diagnostic studies and for delivery of therapeutic uses.

Introduction:

The ability of a drug to be effective in acting on any given target site requires that this drug be delivered to this organ or tissue at the required rate and in the required amounts to achieve the desired pharmacodynamic outcome. Yet in most clinical studies, whether in patient treatment or in drug development, we have not had, until now, good methods that would be able to measure the fate of the drug *in vivo*, especially its biodistribution, metabolism and excretion, and thereby, its ability to target the effector site for that agent. This is because the methods of drug analysis that are commonly used today in human studies require a sample to be analyzed *ex-vivo*, with the consequence that sampling has been primarily limited to blood, excreta and other accessible body tissues. Tissue sampling, through biopsies or surgical methods, can only be used under very special circumstances, and then only for a single time point. These limitations in human sampling clearly restrict our ability to gain accurate information on the level and the rate of drug targeting.

On the other hand, in animal studies, where destructive tissue sampling is possible, one can readily analyze the concentration of drugs in organs and tissues. However, both because each animal will provide only data for a single time point and because of interanimal variations, the pharmacokinetic analysis of such data will fail to detect small changes. Such changes can however be measured using noninvasive methods, as they will allow the sequential collection of data from a single individual. The uniqueness of noninvasive studies, using ^{195m}Pt, had been reported at the 2nd. IAEA conference on Radiopharmaceuticals, in 1984. (1,2). What is more, it is possible, in the same individual, to measure the effect of various factors that may modify the biodistribution of a drug. Thus, noninvasive studies of labeled drugs constitute a unique source for measuring pharmacokinetic /pharmacodynamic parameters.

Until recently, most Nuclear Medicine studies have been aimed at achieving a diagnostic outcome; the radiopharmaceuticals that are used for such diagnostic studies are optimized for obtaining the best diagnostic result. We will see later in the presentations at this symposium that there is now an emerging interest in using the ability of radiopharmaceuticals to achieve therapeutic effects. Also, the use of drugs radiolabeled with PET emitters for the study of drug biodistribution and targeting has become a well-established approach (3,4), especially now that almost any drug can be radiolabeled intrinsically. Such intrinsic radiolabeling is required in order to follow the fate, *in vivo*, of the chemical entity whose (except, perhaps, proteins, where extrinsic radiolabeling is possible).

However, and while radiolabeling allows for the highest degree of sensitivity of detection of any method for measuring drugs and their metabolites in living systems, the information generated is that of the sum of all radiolabeled products present in the region-of-interest (ROI) from which data have been collected. It is, however, possible to extract further information from nuclear imaging data, when the raw kinetics of the radiolabeled compound can be analyzed using a pharmacokinetic approach, a method we have designated as "Pharmacokinetic Imaging" (5).

One of the most important problems in clinical pharmacology is to assess the association between the amount of drug administered, the amount of drug which reaches an intended therapeutic site, and the drug's therapeutic effectiveness. This has led to developments in estimating pharmacokinetic parameters using primarily data obtained from measurements of drugs in blood, that can be correlated with a drug's effectiveness. These

Space



Time



FIG 1 Imaging methods Their significance for pharmaceutical development and usage

approaches, because of the nature of such observations, have been of limited success in predicting response to anticancer drugs. Our laboratory has developed noninvasive approaches that aim at measuring drugs at their effector sites (5), as shown in Figure I

Pharmacokinetic Imaging

The ability to predict the drug time course in the solid tumor of an individual living system, and subsequently the exposure to the active moiety in the solid tumor, may prove to be valuable in both animal studies and in treating individual patients. This is because the uptake of substances into solid tumors has a large amount of intersubject and interoccasional variability. These are variances which may be due to perfusion, to the size of the vascular space, to the site of the disease, to the degree of edema, to differences in pH, to the extent of necrosis, or to differences in the content of the extracellular matrix of the solid tumor (6-11).

Nuclear imaging with $^{195\text{m}}\text{Pt}$ labeled cisplatin or carboplatin has been shown in our prior work to be a noninvasive technique which, when combined with pharmacokinetic analysis, can predict the exposure to the active moiety of these drugs in the solid tumor (12). This type of noninvasive method provides a quantitative and kinetic measure of the total amount of platinum within a defined region of interest, such as the solid tumor. Since these measurements consist of the sum of all $^{195\text{m}}\text{Pt}$ -containing compounds (drug and metabolites), compartment modeling must be employed to estimate the amount of the active moiety, and we have shown that in the case of $^{195\text{m}}\text{Pt}$ -cisplatin, such estimates are possible (12,13-15).

This is because the biotransformation of platinated drugs is a chemically, rather than an enzymatically controlled process. Thus, the biotransformation of cisplatin (and carboplatin, and presumably that of most other platinated drugs) is not dependent on the biochemical determinants of the individual living system. Not only does this eliminate the inter-subject variability, but it also eliminates the interspecies variability. It does not, however, eliminate those unique host characteristics, such as perfusion (16), that will be discussed below.

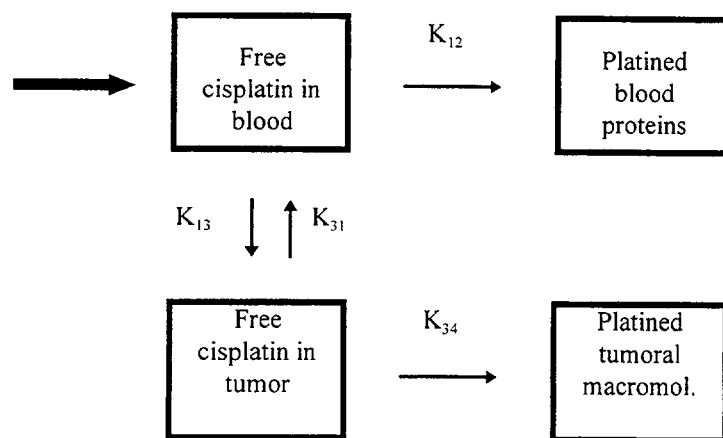


FIG. 2. Compartmental model to describe cisplatin (carboplatin) in animal and human tumors.

Our initial report on the synthesis of $^{195\text{m}}\text{Pt}$ -cisplatin had been presented to the first IAEA meeting on Radiopharmaceuticals in 1973 (17), its usefulness in animal studies reported at the 1985 meeting (1), its clinical usefulness as an agent for pharmacokinetic imaging in 1989 (18) and a method for an automated synthesis in 1992 (19). While these methods of syntheses have now been expanded to other platinated drugs (20), they are still burdened by the limited specific activity of $^{195\text{m}}\text{Pt}$, which is usually between 0.5 and 1 mCi/mg. While this specific activity is fully sufficient for pharmacological studies, it is 3 orders of magnitude lower than what would be required for the use of such drugs at the tracer (radiopharmaceutical) level. There is still a challenge to develop a method for the production of $^{195\text{m}}\text{Pt}$ of much higher specific activity than can be achieved by the neutron activation of highly enriched ^{194}Pt . And while there are other radionuclides of Platinum available, $^{195\text{m}}\text{Pt}$, with its 94% photon yield, its gamma energy very similar to that of ^{201}Tl , its 3-electrons/decomposition, and its $t_{1/2}$ of 4 days, is the ideal radionuclide for studies of the fate of the platinated drugs.

A comparison of the pharmacokinetic imaging profiles between cisplatin and carboplatin (14,15), suggests that both can be readily analyzed by a 4 compartment model, where the tumor space contains both the free platinated drug and platinated macromolecules, and the vascular space similarly the free drug and platinated proteins. While cisplatin will platinate plasma proteins very rapidly, the reaction of carboplatin with such macromolecules is much slower. Reasonably good estimates of the rates constants can be estimated from the noninvasive measurements of the tumor and blood sampling.

The fate of the drug is, however, just one of the key considerations that need to be understood of how drugs are directed to tumors, and how noninvasive methods, especially nuclear, can be used to monitor what happens in a specific individual. The other aspect that needs to be monitored is the functional status of the tumor. And radiopharmaceuticals lend themselves uniquely to perform such measurements.

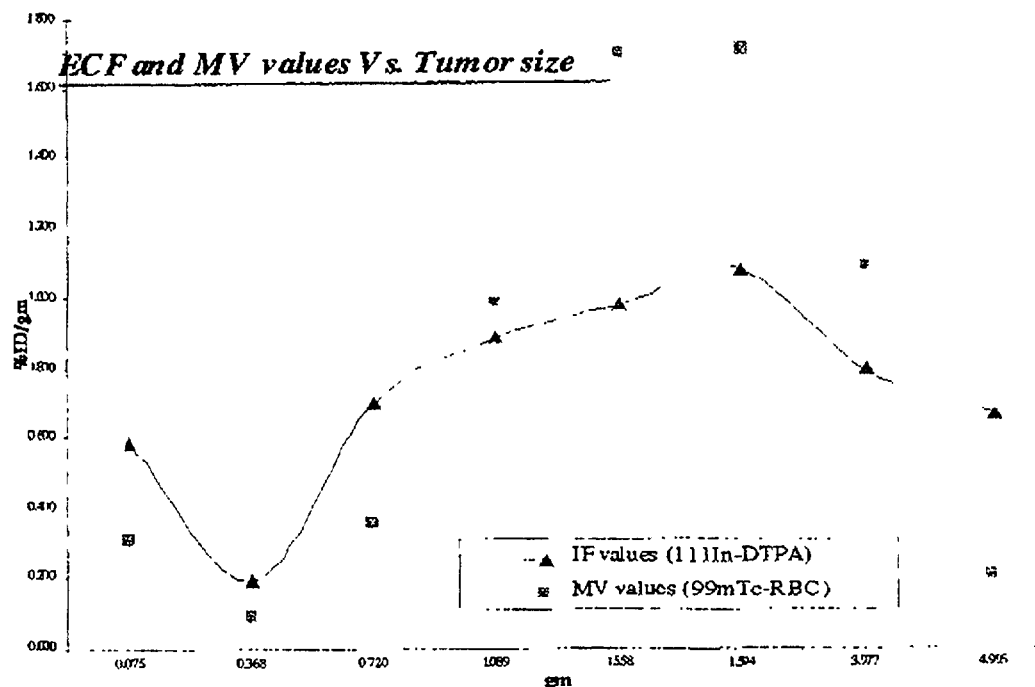


FIG. 3. Measurements of the microvascular and extracellular fluid spaces as a function of tumour size in Walker 256 adenocarcinomas in rats.

Functional Imaging

In studies that are ongoing, the functional status of rat tumors (Walker 256) was studied using ^{99m}Tc -RBC's to measure the vascular space, and ^{111}In -DTPA to measure the extracellular space. Both radionuclides could be readily measured sequentially, revealing that the functional characteristics of this tumor model undergo significant modifications as the tumor grows.

As the tumor starts increasing in size, there is a slow and steady increase in both the microvascular bed as well as the interstitial fluid space. By the time such tumors have reached a size of 1.5-2 grams, such "orderly" growths has been disrupted, and significant spatial heterogeneity occurs. The microvascular bed collapses and becomes extremely leaky, to the point that even blood cells may "ooze" out into a chaotic space that is neither truly vascular nor truly extravascular. Regional heterogeneity results, making it difficult to properly treat such tumors because of the inability of drugs to be delivered to the tumor cells. What is more, manipulations of the living system may affect such tumors further. Anesthetics, in particular, especially those that modify renal function, have a significant effect on the pharmacokinetics properties of the platinated drugs (21).

Conclusion

Based on these observations, we are proposing a new approach to chemotherapeutic planning: using radiolabeled drug, in either pharmacological or trace (radiopharmaceutical) doses, to estimate which fraction of that drug will target the desired effector sites. And using markers for tumoural properties (blood flow, perfusion, etc) that will determine drug transport, to assess the properties of the host.

Such knowledge can then be used to select the methods of drug administration that is likely to be the most effective for optimizing drug delivery, and, thereby, enhancing the therapeutic effectiveness of that drug. This use of radiopharmaceuticals for drug and for functional monitoring (Pharmacokinetic and Functional Imaging) complements the current uses of radiopharmaceuticals for diagnostic studies and for delivery of therapeutic uses.

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**LABELLING AND EVALUATION OF NEW STABILISED
NEUROTENSIN (8-13) ANALOGUES FOR SPET**



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Abstract

Neurotensin(8-13) analogues were biologically stabilised by replacement of the peptide bond between amino acids 8 and 9 by the reduced $\psi(\text{CH}_2\text{-NH})$ isostere. DTPA analogues for In-111 labelling and 2-bromo-phenyl-acetyl analogues for radioiodination, showed receptor affinities in the low nanomolar range in combination with a biological half live in human plasma up to 275 minutes. Biodistribution studies in male Wistar rats of metabolically stabilised and non-stabilised ^{111}In -DTPA-NT(8-13) analogues showed a major clearance from the blood through the kidneys. ^{125}I -labelled Neurotensin (8-13) analogues showed accumulation up to 2.2% of the injected dose per g tissue in the liver which might be an important disadvantage when diagnosis of tumours in the gut is aimed. It is strongly suggested that stabilised Neurotensin(8-13) analogues whether labelled with In-111, I-123 and in the near future with Tc-99m, may act as new potential peptidergic radiopharmaceuticals for SPET diagnosis of different NT-receptor positive tumours like non-endocrine pancreas carcinoma, small cell lung carcinoma or colon adeno carcinoma. It is

enticing to speculate that metabolically stabilised Neurotensin(8-13) analogues labelled with an appropriate isotope might be useful in therapy of different human cancers.

1. INTRODUCTION

Neurotensin (NT) is a linear tridecapeptide (p-Glu-Leu-Tyr-Gln-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu-OH) which has been first isolated from bovine hypothalamus and bovine small intestine [1-2]. Functional characterisation of this neuropeptide revealed its involvement in intracellular communication in the central nervous system and the gut.

Recent studies revealed the involvement of NT in clonal growth of different human cancers and cancer cell lines. NT and NT receptor (NTR) expression is seen in human non-endocrine pancreas carcinomas, colon adeno carcinomas, meningiomas, astrocytic tumor and small cell lung carcinoma cell lines and may play a role in human prostatic cancer development. Consequently, radiolabelled NT may act as an attractive vector for tumour targeting, a strategy already successfully explored for the somatostatin analogue octreotide, which has become a routine radiopharmaceutical in nuclear medicine. An important advantage of developing radiopharmaceuticals from peptides as NT is their size. As peptides are small molecules, they are rather fast and easily to synthesise and purify. Moreover, the small size of peptides is of the utmost importance for a relatively fast blood clearance by the kidneys, thus leading to low background radioactivity. In this way, radiolabelled peptides are powerful alternatives to monoclonal antibodies showing slow blood clearance, which is an important drawback in scintigraphy of target specific tumours [3].

An important restriction in using natural peptide analogues in developing radiopharmaceuticals is there in vivo stability. NT has an in vivo stability in rat and human of only a few minutes [4-5]. In rat, NT is mainly inactivated by a combination of three metallo-endopeptidases which cleave the peptide on three different peptide bonds [6-7]. A very potent enzyme involved in this inactivation is the metallo-endopeptidase (EP) EP24.15 [8] acting on the amide bond between Arg8 and Arg9.

Different approaches have resulted in developing metabolically stabilised peptides. One of the approaches is to replace the metabolically unstable peptide CO-NH function by a pseudo-peptide isosteric bond unable to be hydrolysed [9-10]. When this peptide function is also involved in receptor interaction, it is important to have access to substitutions that maintain either the amine $\psi(\text{CH}_2\text{-NH})$ the carbonyl $\psi(\text{CO-CH}_2)$ or both $\psi(\text{CO-CH}_2\text{-NH})$ functions in order not to lose receptor affinity [11].

Structure-activity studies have shown that the C-terminal hexapeptide NT(8-13) is the minimal fragment required for biological activity [12]. It was shown that $^3\text{H-NT}(8-13)$ binds to human brain homogenates to the same receptor sites as $^3\text{H-NT}$ but with a 4 times higher affinity: K_i values of 2nM for $^3\text{H-NT}$ and 0.5nM for $^3\text{H-NT}(8-13)$ were obtained [13]. These receptor sites have been further defined in a low affinity and a high affinity subtype, the latter being responsible for biological activity [14].

Our group developed different metabolically stabilised NT(8-13) analogues by introducing $\psi(\text{CH}_2\text{NH})$ pseudo-peptide bonds between amino acids 8 and 9. Introducing $\psi(\text{CH}_2\text{NH})$ pseudo-peptide bonds at other places in the peptide gave rise to an important drop in receptor affinity. On the other hand, amino acid

replacement of Arg by Lys can lead in higher receptor affinity [15] but also in higher biological effectiveness.

This study investigates the biological consequences of introducing a pseudo-peptide bond, amino acid replacement and attachment of different chelating groups for radiolabelling to different NT(8-13) analogues concerning binding potential, in vitro and biological stability, biological activity and in vivo kinetics.

It is shown that stabilised NT(8-13) analogues show high potency in developing new radiopharmaceuticals for SPET diagnosis of different NTR positive tumours on the one hand and therapy of the same pathologies on the other hand.

2. EXPERIMENTAL

2.1. Peptide synthesis

Both stabilised and non-stabilised NT(8-13) analogues were prepared by solid phase synthesis on a standard Merrifield resin using Boc-main chain protection. Boc-amino acids were coupled by the DCC/HOBt method.

For Iodine labelling, 2-bromo-phenyl acetic acid was coupled to the peptide as described previously [15]. For ^{111}In labelling, DTPA was coupled to the peptide by coupling DTPA-tetra-T-butylester to the peptide with TBTU/hydroxybenzotriazole.

After cleavage from the resin, both types of peptides were purified by RP-HPLC followed by Mass Spectroscopy and LC-MS analysis.

The synthesis of the metabolically stabilised NT(8-13) analogues was performed as described by Sasaki and Coy [16]. The incorporation of the ψ ($\text{CH}_2\text{-NH}$) pseudopeptide bond was performed by reductive amination of a Boc-amino-aldehyde by the solid-phase resin-bound peptide in the presence of NaBH_3CN as described in detail by Sasaki and Coy [16].

The following NT(8-13) analogues have been synthesised and HPLC purified: DTPA-NT(8-13), DTPA-Lys- ψ (CH_2NH)-Arg-NT(10-13), DTPA-Lys-Arg-NT(10-13), DTPA-Lys- ψ (CH_2NH)-Lys-NT(10-13), DTPA-Lys-Lys-NT(10-13), DTPA-Arg- ψ (CH_2NH)-Lys-NT(10-13), 2-Br-Phe(ac)-NT(8-13), 2-Br-Phe(ac)-Arg- ψ (CH_2NH)-Arg-NT(10-13), 2-Br-Phe(ac)-Lys-Arg-NT(10-13), 2-Br-Phe(ac)-Lys-Lys-NT(10-13) and DOTA-NT(8-13).

2.2. Radiolabeling procedures

2.2.1. DTPA-NT(8-13) analogues: labelling with In-111

The radiosynthesis of non carrier added (n.c.a.) ^{111}In -DTPA-NT(8-13) was performed in a kit-formulation. 1.1 ml 0.02 N HCl and $^{111}\text{InCl}_3$ in 0.02 N HCl (provided by Mallinckrodt, The Netherlands) were added to 10 μg DTPA-NT(8-13), 4.96 mg trisodiumcitrate, 0.37 mg citric acid, 10 mg inositol and 2 mg of 2,5-dihydroxybenzoic acid. After 30 minutes at room temperature, the reaction mixture was diluted with 1 ml of semi-preparative eluent ($\text{H}_2\text{O}/\text{ACN}/\text{TFA}$, 14/86//0.1, pH=1.9) and injected on the semi-preparative HPLC. The radiolabelled peptide was collected at 25.5 min and the collected eluent was diluted with an equal volume of doubly-distilled water. The ^{111}In -DTPA-NT(8-

13) was pre-concentrated on a Baker Bond Octadecyl 100mg mini-column and recovered in 1ml of EtOH/PBS buffer 50/50 pH 7.4 (PBS: 0.14M NaCl, 19mM Na₂HPO₄ and 2.4mM NaH₂PO₄) as described by Terriere et al.[17]. Labelling yields of more than 98% were obtained. The overall yield of the radiosynthesis was about 75% with a radiochemical purity of at least 99.0% and a specific activity of >1700 TBq/mmol.

2.2.2. 2-Bromo-phenylacetic acid NT(8-13) analogues: radioiodination

Radioiodination was performed by using the Cu⁺ assisted non-isotopic nucleophilic exchange reaction [18] on the 2-Bromo-phenylacetic acid (2-Br-Phe(ac)) NT(8-13) analogues. To 1mg of the precursor peptide dissolved in 10µl 100%AcOH were added 500µl of stock solution (1mg SnSO₄, 25mg 2,5-dihydroxybenzoic acid, 35mg citric acid.H₂O and 500µl glacial acetic acid in 4500µl of water) and 60µl of the copper solution (32.5mg CuSO₄.5H₂O dissolved in 10ml water). After N₂ flush during 5-10 minutes, the radioiodine solution was added and the reaction mixture was heated at 140°C for 1 hour. Purification was effected by semi-preparative reversed phase HPLC (H₂O/ACN/TFA: 14/86/0.1 v/v mixture, pH 2 on a Vydac 218 TP54 C18 column). Monitoring UV detection at 254nm was used in combination with NaI(Tl) radiometric detection. An overall labelling yield of 50-72% and a radiochemical purity of at least 99% were obtained.

2.3. In vitro and metabolic stability testing

In vitro biological stability of the ¹¹¹In-DTPA-NT(8-13), ¹¹¹In-DTPA-Lys8- ω (CH₂-NH)-Arg9-NT(10-13) and 2-¹³¹I-phenyl acetic acid NT(8-13)

analogues were compared. In vitro stability at pH 7.4 at room temperature was evaluated by analytical HPLC. Metabolical stability testing was performed by using fresh human plasma. Incubation at 37°C was followed by denaturation using a 5% tri-chloro acetic acid / methanol 50/50 (v/v) solution (1/1 plasma). After centrifugation (2000rpm/2min), small aliquots of the supernatants were HPLC analysed.

2.4. Drug competition studies

For receptor competition assays, guinea pig (Pirbright, 300g) bulbus olfactorius membranes were used. After decapitation, the brains were rapidly removed and the bulbus olfactorii were dissected. Tissue samples were homogenised in 15ml of Tris-HCl buffer (50mM, pH 7.4) using a Ultraturrax homogeniser. The homogenate was centrifuged at 16000RPM for 10minutes in a refrigerated Sorvall centrifuge. The pellet was twice rehomogenised and recentrifuged as described above. The final pellet was suspended in Tris-HCl buffer in a dilution of 10mg of original wet tissue/ml. Competition of the binding of 1nM ^3H -Neurotensin on 4mg of tissue in a final volume of 500 μl was under study.

Analogous binding studies were performed using the human HT29 colon adeno carcinoma cell line. Cells were incubated in a modified Krebs-Ringer Hepes buffer (111mM NaCl, 4mM KCl, 2.5mM CaCl_2 , 1.2mM MgSO_4 , 1.2mM KH_2PO_4 , 20mM Hepes, 0.1% Glucose, 1mM EDTA and 0.1mM Bovine Serum Albumine) at pH 7.4 at a final concentration of 5×10^6 cells per ml. Competition of the binding of 1nM ^3H -Neurotensin was performed on 2×10^6 cells in a final volume of 500 μl .

Incubation at 25°C for 30minutes was followed by rapid filtration under reduced pressure through pre-soaked (2h in pH 7.4 buffer added 1μM of NT) Whatman GF/B glass fiber filters. Filters were rinsed twice with 2ml of Krebs Ringer Hepes buffer with no serum albumin for the HT29 cells and Tris-HCl buffer for the Bulbus Olfactorius homogenates. The filters were placed in plastic scintillation vials containing 2ml of Instagel Gold MV Scintillation fluid. Counting was performed in a Packard Scintillation spectrometer. Inhibition constants (K_i) were calculated by using the following equation (Cheng and Prusoff, 1973):

$$K_i = IC_{50} \times K_d / (K_d + L)$$

where K_d is the dissociation constant obtained from equilibrium binding experiments and L the concentration of 3H -NT.

2.5. Biological activity Studies

Biological activity of the non-radiolabelled NT(8-13) analogues was evaluated. Assays were based on the biological potency obtained from the peptides to relax pre-contracted isolated longitudinal smooth muscle strips of the guinea pig jejunum (isotonic contraction and relaxation). Pirbright Guinea pigs of female sex (250g) were killed by decapitation. The jejunum was removed and rinsed. Segmental strips of 2cm length were dissected. These strips were suspended in an organ bath of 20ml and connected to an isotonic transducer under a preload of 1g (Displacement Transducer Control Unit, Janssen Scientific Instrument Division). The organ bath was filled with De Jalon solution (KCl, 5.6mM; $CaCl_2 \cdot 2H_2O$, 0.54mM, $NaHCO_3$, 6mM; NaCl, 155mM and Glucose, 2.8mM) kept at 37°C and gassed with a 95% O_2 and 5% CO_2 mixture. Total

contraction at the start of the experiment was obtained by using 3×10^{-6} M carbachol. After a contact time of 30s, the organ baths were refreshed and the contraction procedure was repeated at intervals of 15min until reproducible result were obtained. Test compounds were used at a concentration of 3×10^{-8} M. Relaxations of the test compounds were expressed as percentage inhibition of the total contraction induced by 3×10^{-8} M Neurotensin.

2.6. In vivo Biodistribution Studies

Hannover male Wistar rats were injected intravenously in the tail with 20 μ Ci of n.c.a. radiolabelled peptide. Biodistribution and clearance of ^{111}In -DTPA-NT(8-13), ^{111}In -DTPA-Lys ψ (CH₂-NH)ArgNT(10-13) and ^{125}I -Phenyl acetyl NT(8-13) was under study. Rats were sacrificed by decapitation at 15, 30, 45, 60 and 240 minutes after injection respectively. Blood was collected at time of decapitation. Organs including heart, lungs, kidneys, spleen, jejunum, ileum, colon ascendens and liver were quickly removed, washed and dried. Urine was collected by puncture of the bladder. All samples were weighed and counted in a single channel gamma counter. Results were expressed as % of the injected dose per gram tissue.

3. RESULTS

3.1. Radiolabelling procedures

For labelling with In-111, DTPA was substituted as chelating group on the 8 position of NT(8-13). Under the above described conditions, radiolabelling

Table I. Biological Stability in Human Plasma.

Analogue	t ½ (*)
NT	1.5
¹¹¹ In-DTPA-NT(8-13)	10
2- ¹³¹ I-Phenylacetyl-NT(8-13)	12
¹¹¹ In-DTPA-NT-Lys8-(CH ₂ NH)-Arg9-(8-13)	275

*Biological halflife in human plasma in minutes.

yields of 98% is obtained. The overall labelling yield as obtained after semi-prep HPLC purification and recovery is 75% in combination with a radiochemical purity of at least 99% and a specific activity of >1700 TBq/mmol.

Radioiodination using the copper assisted nucleophilic non isotopic displacement reaction on the brominated precursor molecules in reducing and acidic conditions yielded various labelling yields between 50 and 72%. After HPLC purification an overall labelling yield of 30-50% is obtained with a radiochemical purity of >99% and high specific activity.

3.2. In vitro and in vivo evaluation

Biological stability in human plasma of ¹¹¹In-DTPA-NT(8-13), ¹¹¹In-DTPA-Lys8-ψ(CH₂-NH)-Arg9-NT(10-13) and 2-¹³¹I-phenyl acetic acid NT(8-13) is listed in Table I. Introduction of the pseudo-peptide CH₂-NH isostere

between amino acids 8 and 9 gives rise to an important improvement in biological stability from 10 to 275min.

Table II shows the K_i values obtained from drug competition studies using ^3H -NT both for binding on Bulbus Olfactorius (B.O.) and HT29 cells. The dissociation constant K_d for ^3H -NT was calculated from equilibrium saturation experiments; 0.7nM and 3.5nM for the B.O. and HT29 model respectively. As obtained from Scatchard plots, total binding capacity (B_{max}) values are 25 and

Table II. Inhibition of the binding of ^3H -Neurotensin on Bulbus Olfactorius (B.O.) homogenates and HT29 cells.

Analogue	K_i (nM) B O ⁽¹⁾	K_i (nM) HT29 ⁽²⁾
Neurotensin	1.5	1.0
DTPA-NT(8-13)	19.0	62.0
^{115}In -DTPA-NT(8-13)	n.t.	3.9
DTPA-NT-Lys8-(CH ₂ NH)-Arg9-(8-13)	13.0	n.t.
DTPA-NT-Lys8-(CH ₂ NH)-Lys9-(8-13)	7.4	n.t.
DOTA-NT(8-13)	n.t.	17.0
2-Br-Phenylacetyl-NT(8-13)	5.0	4.2
2-Br-Phe(ac)-NT-Arg8-(CH ₂ NH)-Lys9-(8-13)	4.2	n.t.

⁽¹⁾ Ligand : 1 nM ^3H -Neurotensin , S.A. = 92 Ci/mmol 4mg of fresh tissue homogenate was used in a final volume of 500 μl . K_d = 0.7nM.

⁽²⁾ Ligand : 1 nM ^3H -Neurotensin , S.A. = 92 Ci/mmol. 2×10^6 cells were used in a final volume of 500 μl . K_d = 3.5nM.

Results of duplicate experiments. (n.t.) : not tested

Table III. Biodistribution in Wistar rats.

¹¹¹In-DTPA-Lys8ψ(CH₂-NH)Arg-NT(10-13)

Organ	%ID/g			
	15'	30'	60'	240'
Blood	0.4	0.3	0.2	0.0
Kidney	1.8	1.8	1.0	0.3
Liver	0.1	0.1	0.1	0.0
Heart	0.1	0.1	0.1	0.0
Spleen	0.1	0.1	0.1	0.1
Lung	0.4	0.3	0.2	0.1
Duodenum	0.2	0.1	0.1	0.0
Jejunum	0.2	0.2	0.1	0.0
Ileum	0.1	0.2	0.1	0.0
Colon	0.2	0.1	0.1	0.1

2-¹²⁵I-Phenylacetyl-NT(8-13)

Organ	%ID/g			
	15'	30'	60'	240'
Blood	0.3	0.2	0.2	0.0
Kidney	3.5	5.7	2.3	0.3
Liver	0.7	2.2	1.1	0.1
Heart	0.1	0.1	0.1	0.0
Spleen	0.1	0.2	0.1	0.0
Lung	0.3	0.4	0.2	0.0
Duodenum	0.2	0.8	0.8	0.1
Jejunum	0.2	0.7	0.6	0.2
Ileum	0.1	0.8	0.8	0.2
Colon	0.1	0.1	0.1	0.0

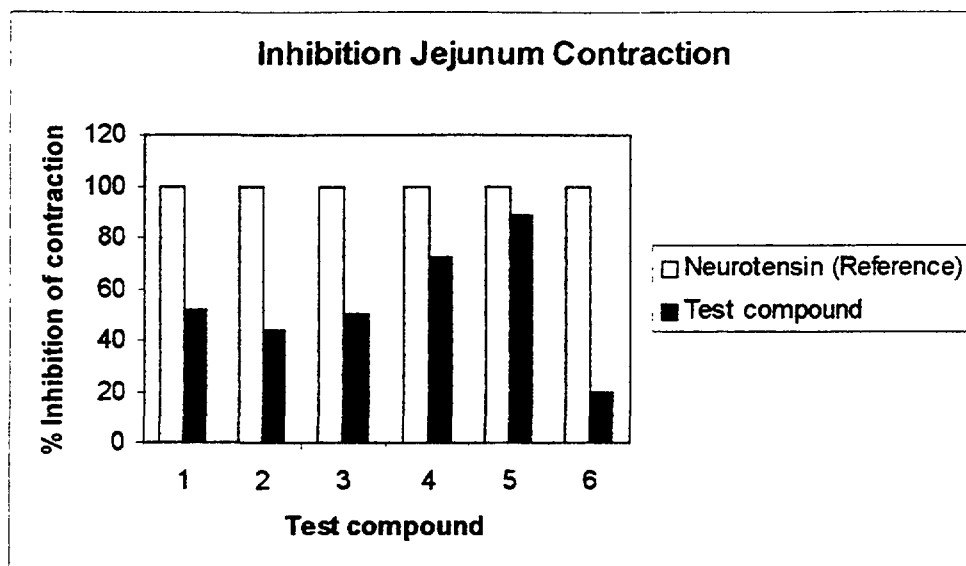


FIG. 1: Inhibition of the contraction of different NT(8-13) analogues as percentage of the contraction induced by parent NT.

All test compounds were used at 3×10^{-8} M. Mean values of duplicate experiments are shown. The following test numbers were screened (values \pm SD): 1. DTPA-NT(8-13) ($55 \pm 14\%$), 2. 2-Br-Phe(ac)-Lys ψ (CH₂-NH)Arg-NT(8-13) ($44 \pm 19\%$), 3. DTPA- Lys ψ (CH₂-NH)Arg-NT(8-13) ($50 \pm 14\%$), 4. DTPA- Lys ψ (CH₂-NH)Lys-NT(8-13) ($72 \pm 11\%$), 5. 2-Br-Phe(ac)-Arg ψ (CH₂-NH)Lys-NT(8-13) ($89 \pm 6\%$), 6. 2-Br-Phe(ac)-NT(8-13) ($20 \pm 1\%$).

37 fmoles/mg tissue binding low affinity and high affinity sites for the B.O. model and 42 fmol/ 10^6 cells binding only high affinity sites for the HT29 model. All analogues show K_i values in the low nanomolar range. DTPA-NT(8-13) analogues show 5 to 10 fold lower affinities in comparison to the 2-bromo-phenyl acetic acid-NT (8-13) analogues.

Relaxation of the guinea pig jejunum induced by different stabilised and non-stabilised NT(8-13) analogues are expressed as percentage of the inhibition induced by native NT. Results are reproduced in Figure 1. No analogue showed total antagonistic effects. Biological effects were between 20 and 89% of the

effect induced by parent NT for the 2-Br-Phe(ac)-NT(8-13) and the 2-Br-Phe(ac)-Arg- ψ (CH₂NH)-Lys respectively. The use of partial agonists in developing peptidergic radiopharmaceuticals should be preferred above the use of full agonists in order to minimize biological effects. On the other hand, a minimal agonistic effect should be required in order to provoke internalisation which might enhance diagnostical and/or therapeutical qualities of the tracer.

Biodistribution in Wistar rats with 20 μ Ci of ¹¹¹In-DTPA-NT-Lys8 ψ (CH₂NH)Arg-NT(10-13) and 2-¹²⁵I-Phenylacetyl-NT(8-13) are reproduced in Table III. Both types of radiolabelled NT(8-13) analogues show fast blood clearance by the kidneys up to 90% of the injected dose within 4h. The radioiodinated analogue shows an important clearance by the liver up to 2.2 % of the injected dose per gram tissue which might be an important disadvantage when visualisation of tumours in the gut is aimed.

4.0. CONCLUSION

The results of our studies show that both DTPA and 2-bromo-phenyl-acetic acid NT(8-13) analogues show very high and receptor specific binding properties which makes these peptides very interesting in developing new radiolabelled vectors for SPET diagnosis or therapy. On the other hand, the finite biological stability of these peptides is an important disadvantage limiting all properties. The use of DTPA-NT(8-13) analogues with a reduced peptide bond of the ψ (CH₂-NH) type between amino acids 8 and 9 being Arg or Lys, combines high biological stability with binding properties similar to those of their non-stabilised analogues. Using neutral charged radioiodinated phenyl acetic acid NT(8-13)

analogues as radiopharmaceuticals might be limited when visualisation of the gut is aimed as these analogous show an important clearance by the liver as seen in rats. Analogous results were seen in biodistribution studies using neutral charged radiolabelled Somatostatin analogues [19], suggesting the need of a charged peptide in order to avoid clearance by the liver.

NT(8-13) analogues with other chelating groups like DOTA for Y-90 labelling and 2-Methyl-Gly-L-Ser-L-Cys-Gly (Resolution Pharmaceuticals, ON) for labelling with Tc-99m are currently under development.

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RADIOMETRIC ASSAY

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CURRENT STATUS AND FUTURE DEVELOPMENTS IN RADIOLABELLED IMMUNOASSAYS

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Abstract

Radioisotopes are used extensively in medical practise and their use in RIA or IRMA usually represent a small proportion of the total. Radiolabelled immunoassays based on ^{125}I constitute a simple didactic, cost effective and robust technology which is still regarded as the reference method in many clinical applications. The IAEA has implemented many successful programmes using the "bulk reagent" approach, involving 68 countries in all the different regions. The main achievements have been in technology transfer with self sufficiency in production for some countries; training of large numbers of staff; quality control and quality assurance schemes; devolution of screening programmes for neonatal congenital hypothyroidism.

Alternatives to the use of radioisotopic tracers are constrained by many factors and are often only available in restricted commercial packages. They are often not suitable for technology transfer programmes and often lack any didactic component in addition to a relative high cost.

The production of radiolabels using ^{125}I is both simple and adaptable. In addition expertise in their preparation and purification is widespread even in developing countries. Together with the ease of producing antibodies, the facts have made ^{125}I -radiolabelled immunoassays ideal for investigative procedures for many research activities (30,31) particularly in the medical context where radioisotopes are commonly used.

In conclusion, even a superficial examination of public health statistics for various countries throughout the continents indicates a need for a simple, inexpensive and robust analytical tool. In this light, there is a predicted continuing role for radiolabelled immunoassays.

CURRENT STATUS AND FUTURE DEVELOPMENTS IN RADIOLABELLED IMMUNOASSAYS

1. Introduction

Radiolabelled-immunoassays use reagents incorporating radioisotopes as tracers to monitor the distribution of free and bound antigen in radioimmunoassays (RIA) or free and bound antibody in immunoradiometric assays (IRMA). Radioisotopes have been used as tracers to monitor both chemical and biological systems since 1913, when George Charles de Hevery used a natural radioisotope of lead to investigate the solubility of lead salts in water and later the uptake of lead into growing plants.

The available natural radioisotopes were somewhat limiting in terms of experimental use. The invention and application of the cyclotron, i.e. charged particle accelerator, in the early 1930's, did much to enhance the availability and range of others. However, manufactured radioisotopes became plentiful only with the development of nuclear reactors in the 1950's, which produced fission products using neutron bombardment.

Radioisotopes have been widely used in medicine. The list is extensive, with more than 100 different ones having been used since 1945. The most significant medical radioisotopes are ^{51}Cr , ^{131}I , ^{32}P , ^{59}Fe and ^{99}Tc . Radioisotopes are used in therapy, diagnosis 'in vivo' and 'in vitro', and sterilisation of medical supplies. The latter accounts for the most extensive use of gamma radiation.

Radioisotopes were introduced into immunoassays in 1960 [1]. Although earlier immunoassays, e.g. using red blood cells and subsequent haemagglutination [2] were similar in principle to the radiolabelled assays, the use of a radioisotope proved a major advance and improved detection immeasurably. Development of radioimmunoassays can be traced back to the mid-fifties. At this time, work was proceeding in two centres, one in London and the other in New York.

Solomon Berson and Rosalyn Yalow were investigating the metabolic fate of intravenously administered ^{131}I -labelled insulin at the Veterans Administration Hospital in New York. Their studies showed that, although the insulin disappeared rapidly from the blood of normal subjects and diabetic patients not treated with insulin, it persisted for a longer period in the blood stream of patients who had received insulin therapy for more than a few weeks. This proved to be due to the presence of antibodies to insulin in those patients receiving therapy. Their initial studies demonstrated that the binding of the ^{131}I -insulin to antibody was inhibited in a quantitative manner by the presence of unlabelled insulin. These simple findings were to be the basis of their method now familiar as an RIA.

In the other centre, the radioisotope unit of the Middlesex Hospital Medical School, Roger Ekins was working on a new theory for the measurement of endogenous hormones at levels consistent with those found in blood. Using radiolabelled

thyroxine, produced "in vivo" by a patient with thyroid carcinoma metastases, and thyroxine binding globulin as a high affinity binding reagent, Ekins was able to demonstrate the measurement of thyroxine in serum. This technique, called saturation analysis [3], was a general procedure utilising a specific binding protein and thus included all immunoassays.

Attempting to optimise sensitivity and precision in radiolabelled-immunoassays lead Miles and Hales in 1968 [4] to formulate the principles of assays using labelled antibodies, referred to as 'immunoradiometric' assays (IRMA) where the label was a radioisotope. The use of highly purified antibodies coupled to a radioactive tracer increased sensitivity and improved precision. Subsequent developments, in 1970, incorporating an additional antibody coupled to a particle or other solid-phase, and referred to as the 'two-site' IRMA or 'sandwich' assay [5,6,7] further improved precision and hence sensitivity. The exponential growth in application of radiolabelled-immunoassays was accompanied by numerous symposia, workshops and colloquia seeking to clarify and consolidate both theoretical and practical aspects. The proceedings of many of these meetings have been published [e.g. 8-16] and are a useful source of information. In addition, there have been a number of reviews e.g. [17-20].

1.1. Measurement of radioactivity in radiolabelled-immunoassays

Measurement of radioactivity in radiolabelled immunoassays

depends on the conversion of energy from the primary ionising particle into light, which is then used to generate photoelectrons from the cathode of a photomultiplier tube. Such Detectors are commonly referred to as scintillation counters.

There are two types of scintillation counter used for radioimmunoassays, as follows:

- the scintillation crystal or 'gamma' counter;
- the liquid scintillation or 'beta' counter, the common name clearly indicates the type of radiation each is appropriate for.

2. Application Of Radioisotopes To Immunoassay

A number of different radioisotopes have been used as tracers in radioimmunoassays including ^{57}Co , ^{75}Se , although the current practice is to use either ^{125}I or ^3H , with the occasional use of ^{57}Co as a dual label in conjunction with ^{125}I where two different substances are measured in a single tube. The first published radioimmunoassay [1] used ^{131}I which has a relatively short half life and consequently a higher activity, i.e. higher number of disintegrations per unit time. This was useful as it enabled the measurement of small aliquots of reaction medium, dictated by the use of paper electrophoresis strips for separating free and antibody bound analyte; the paper strips having a limited loading capacity. ^{131}I was also readily available at a relatively high level of radioisotope purity.

Because antibody binding sites are extremely specific, it would seem logical that the best choice of radioisotope would be one that replaces its non-radioactive isotope in the tracer molecule; for example, replacing ^3H for hydrogen or using ^{125}I for molecules containing iodine as in thyroxine or triiodothyronine. In practice, if the substitution is made in a part of the molecule away from the antibody binding site, the choice of radioisotope can be governed by other considerations, such as half life, availability, high activity and radiochemical purity. The larger the tracer molecule, the easier this is achieved.

When ^{125}I with good radiochemical purity became commercially available, it rapidly became the radioisotope of choice representing a practical combination of high activity and reasonable reagent shelf life. ^{125}I , with its half life of 60 days, has higher activity than those radioisotopes with a longer half life, and a longer shelf life than those radioisotopes with a shorter half life.

Because the iodine atom is relatively large, about the size of a benzene ring, it proved unsuitable for many small molecules, such as steroids, as it easily interfered with antibody binding. The exceptions were those few molecules like thyroxine with endogenous iodine. ^3H , another readily available radioisotope, became widely used for small molecules.

The use of ^3H introduces certain constraints, particularly the need for relatively expensive liquid scintillation detection. In 1968, an eloquent technique was published [21], which permitted the use of ^{125}I to label small molecules without compromising immunoreactivity. It involved the synthesis of a suitably modified analogue, incorporating the radioactive iodine into an appropriate group like a histidyl or a tyrosyl group, attached to the molecule through a side chain at a point not directly bound by the antibody. In practice, this technique proved successful for virtually all small molecules requiring only the judicious identification of appropriate positions to introduce side chains for the analogues. This approach has even allowed the use of extremely large molecular tracers, such as enzymes; with relative molecular weights greater than 50,000 dalton, attached to analytes with molecular sizes only a few hundred dalton.

In recent years, ^{125}I has become the most commonly used radioisotope in RIAs and IRMAs. Tritium (^3H) is now used for relatively few assays, but is particularly useful where the incorporation of iodine is not practical because of a lack of suitable analogues or because it leads to an unstable tracer.

The widespread use of ^{125}I was a potent factor in the introduction and success of the multi-head detector. Because of the relatively low energy of ^{125}I , much smaller crystals could be used, in turn requiring less lead shielding to reduce the background, allowing for several heads to be assembled in a

single instrument. Up to this time, gamma counters were traditionally expensive instruments with large crystals to measure a variety of radioisotopes, a lot of lead shielding, the ability to detect energy wavelengths over a continuously variable range, and complex automated sample handling facilities. The use of a manually operated, simple instrument dedicated to the measurement of low energy radioisotopes like ^{125}I was extremely economic in terms of cost and time and proved to be very popular with many laboratory users. In 1977, at St Bartholomew's Hospital, a single 16-headed gamma counter, with subsequent high throughput, replaced eight automated gamma spectrophotometers, with a capital cost saving of approximately 20 fold. Being a manual instrument, it was also not subject to the frequent breakdown problems associated with the complex automated sample handling facilities. In essence, gamma counting is extremely efficient and economic when compared with other forms of detection.

2.1. Dual Radioisotopic Assays

Gamma radiations from different radioisotopes display different energy spectra. It is possible to take advantage of this difference and discriminate the radioactivity associated with specific radioisotopes. The concept of assaying two components in a biological medium simultaneously using tracers labelled with two different radioisotopes was introduced in 1966 [22]. This 'dual' radioimmunoassay measured both growth hormone and insulin using ^{125}I and ^{131}I . Dual assays are perhaps best exemplified by the ICN SimulTRAC^R range using ^{125}I and ^{57}Co . ^{57}Co

was introduced originally as an appropriate tracer for vitamin B₁₂ (cobalamin) which has an endogenous cobalt atom. These types of assay are most appropriate for the measurement of those analytes that are measured in the same sample; such as thyroid stimulating hormone TSH/free thyroxine (free T₄), luteinising hormone (LH)/follicle stimulating hormone (FSH) and vitamin B₁₂/folate.

2.2. Homogeneous Assays

Because the radioactive process is unaffected by external conditions, radiolabelled assays have often been considered as unsuitable for non-separation protocols, i.e. homogeneous assays. However, the detection of low-energy radioisotopes such as tritium [23] can be interfered with or altered.

Amersham's scintillation proximity assay (SPA) is an example of homogeneous immunoassays, involving the use of beads impregnated with scintillant which are coupled to binding proteins, such as antibodies. In principle, the bound radioactive tracer allows emitted radiation energy to interact with the scintillant and thereby produce light which is detected in conventional liquid scintillation counters. The energy from unbound or free radioactive tracers, being at a distance from the bead, does not react with the fluorimicrospheres.

Homogeneous radiolabelled immunoassays are also possible if scintillant is localised in or at the surface of a microtitre plate e.g. Wallac ScintiStrip or DuPont NEM

Flashplate. In the ScintiStrip a scintillant is an integral part of the polystyrene used to manufacture the microtitre plate, whereas for the Flashplate it is precoated onto the surface of the 96-well plate. These products are available coated with second antibody or other specific binding protein for use with a primary antibody of choice.

Using these principles, homogeneous radiolabelled immunoassays have been developed for a variety of uses including steroid hormones [24] and prostaglandins [25]. These assays have demonstrated sensitivities from 10^{-9}M to 10^{-10}M .

3. National and Regional Reagent Schemes

Initially, the use of radioimmunoassays was limited to a few research centres and analyses of patients' samples was uncommon. In 1972, the Lancet published a series of letters from clinical endocrinologists expressing concern about the difficulties in obtaining access to these specialised radioimmunoassays. In response, the health department of the U.K. government agreed to fund a national immunoassay service.

The funds were used to organise and expand facilities in existing research centres who were then able to undertake the analyses of patients samples sent in by general hospitals. Initially, the scheme covered many radioimmunoassays including TSH, T4, Prolactin, LH, FSH and growth hormone (GH) which at the time were considered to be specialised services. In subsequent years, all of these were found to have general clinical use with a need to provide them on a local rather than

a central basis. The need for devolution of immunoassay services had been envisaged as an integral part of the scheme as and when the use of specialised assays extended into general application. By 1975, the reported growth in immunoassay analyses in clinical practice encouraged chemical pathologists from about twenty district (i.e. local) hospitals to request that a unit be established at St Bartholomew's Hospital. This unit (Netria) was to be responsible for the devolution of expertise and reagents from the research centre to them.

The strategy adopted by Netria included a variety of activities as follows:

- Provision of Training Courses and Workshops
- Distribution of Bulk Matched Reagents
- Equipment Evaluation
- Co-ordinated Approach to Equipment and Reagent Purchase
- Co-ordinated Representation to Funding Authorities
- Quality Control Scheme (now superseded by NEQAS)
- Research and Development

The rationale for the Netria scheme was influenced by the need to maintain considerations of the high quality of results. Therefore the factors on which such devolution must depend include the training of graduates and technicians from participating laboratories, pressure on appropriate authorities to make available funds for isotope counters and other equipment, the provision of necessary reagents and the introduction of quality control.

Funding for the scheme was on a pro-rata basis, with each "customer" laboratory contributing in proportion to their use of services. The budget covered all items of expenditure, including overheads, i.e. salaries, rent, rates, services and running costs.

The charge of each laboratory was calculated on the units of primary antibody used. All other reagents and services such as training were included in this one charge. The price per unit was based on the simple calculation of dividing the total expenditure incurred in running the unit by the total amount of reagents distributed in terms of number of assay tubes.

Netria was administered by a Steering Committee comprising:

- Regional Scientific Officer
- Director of NETRIA
- Professor of Chemical Endocrinology (Medical College)
- Representative from Finance Department
- Representative from Regional Treasurer's Department
- Chairman of Regional Committee for Biochemistry

All management decisions were referred to this Committee. The success of the Netria scheme was undoubtedly related to the presence of this steering committee and its role in acting as an interface to resolve difficulties, provide a co-ordinated

strategy and direct expert advice to the appropriate authorities.

The scheme rapidly expanded to cover the whole of the country and in 1994 reached a peak in distribution of 2.5 million assay tubes.

In 1982, an initial contact was made by a representative of the IAEA to discuss the general availability of Netria's bulk matched reagent programme. The Agency's regional activities towards the promotion of radiolabelled-immunoassays (RIA and IRMA) in human health commenced in 1986 using the bulk matched reagent concept together with extensive training programmes, transfer of all aspects of the technology, and funding support for equipment purchase. During 1986 to 1997, through the Agency's programmes, Netria has supplied bulk reagents to 68 countries throughout Africa, East Asia and the Pacific, Latin America and West Asia. The focus of these programmes was to increase the analytical reliability of RIA procedures used for clinical diagnosis and research investigations whilst reducing the unit cost of assays. The clinical scope concentrated initially on thyroid-related diseases and later included tumour marker assays. Major projects to establish national screening programmes for congenital neonatal hypothyroidism using Netria's reagent have been successful in some countries in all regions. Bulk reagents from the CIAE in China have also been distributed for hepatitis B screening.

These activities in particular have been responsible for the successful establishment of a number of national reagent programmes [26, 27, & 28]. Although the bulk reagent schemes have benefited the large central laboratories, they have had a limited impact on devolution within a country. The difficulty has often been perceived to be one of methodological choice. Many local laboratories prefer the antibody coated tube technology found in imported commercial kits. Until now, this technology has not been compatible with local bulk reagent production.

The main achievements of these IAEA activities are the following:

- Technical expertise in reagent preparation.
- Training in IRMA and RIA methodology and quality control.
- Capital investment to upgrade laboratories with items of equipment.
- EQAS established in some regions.
- Distribution of locally produced reagents to other countries within same region.
- Schemes to screen for neonatal hypothyroidism.

4. Alternative Techniques Or Non-Isotopic Immunoassays

In recent years so called alternative or non-isotopic (i.e., non-radioisotopic) immunoassays have become available. In fact, the use of non-radioisotopic labels predated the introduction of radioimmunoassay by at least a decade. The recent renewed interest in them and the growing tendency

towards their use that has been observed, even in some developing countries particularly, is primarily related to the following factors:

- The perceived harmful effects of using radioisotopes.
- The increasing need for automation in some countries, particularly in the industrialised world, and the belief that this would be simpler to achieve when non-isotopic labels are used.
- The requirement in some instances to increase assay sensitivity and the theoretical possibility of achieving this by the use of some alternative labels.
- Constraints on the use of radiolabelled reagents under national or local regulations involving some form of licensing and supervision.

The alternative labels in current use may be summarised as follows:

- Enzyme substrates with calorimetric measurement
Fluorimetry, Luminescence or Electrochemical
- Fluorophores using simple fluorimetry, polarisation fluorimetry, or time resolved fluorimetry
- Luminescent compounds
- Agglutination measured by turbidimetry, nephelometry or by eye
- Electrochemical by potentiometric modulation or electroreactive labels
- Evanescent waves detected by surface plasmon resonance or total internal reflection fluorescence

Many of these alternatives are only suitable for specific situations. Some techniques have been adopted for determination of relatively high concentrations, e.g., agglutination, and others are particularly suited to

immunosensors and real time dynamic measurements, e.g., evanescent waves.

Immunoassay, regardless of the choice of labels, use a variety of systems including liquid phase reagents or solid phases such as coated beads or tubes. The appropriate use of different systems can enhance or restrict the performance of assays. In some cases a perceived advantage of alternative labels may really be attributable to a change in the system itself.

With respect to the specific application of radioimmunoassay, viable alternatives are limited to relatively few choices which, from a practical point of view, are as follows:

- Time resolved fluorescence (e.g., Delfia)
- Enzyme-enhanced luminescence (e.g., Amerlite)
- Chemiluminescence (e.g., Immulite)
- Amplified enzyme cycling (e.g., Dako or Murex)
- Enzyme using simple colorimetry

With the exception of enzyme calorimetric techniques all of the above have theoretical sensitivities exceeding those of radiolabelled methods. They all have been adopted by various commercial companies and there are commercially developed automated systems using one or other detection system. Most of these techniques are subject to patent legislation and the technology associated with reagent preparation and application is therefore, restricted.

Reviewing automation and its impact on the practice of immunoassay, several factors emerge. Automation ranges from application to single stages such as pipetting, modular automation applicable to several stages or complete systems. The complete systems are invariably supplied with integral reagents only available from the same commercial source.

4.1. Comparisons

The perceived health hazards of radioimmunoassays have been the subject of singular criticism for many years. Given the nature of ^{125}I this is surprising and obviously misinformed.

It is possible to derive a figure for the average radiation dose received by a laboratory worker performing radioimmunoassays using ^{125}I labelled reagents, assuming a reasonable throughput of up to 200 assays (approximately) 40 samples per assay each in one year. The predicted annual dose of approximately 0.03 mSv compares with the dose of 0.4 mSv that would be received from a single transatlantic flight (see Table I).

Moreover, the general public is subjected to a background radiation dose many times higher than either of the above. For example, it is known that the annual background radiation in the UK is 2.5 mSv and the dose from an x-ray can be up to 1 mSv. By comparison, reagents with alternative labels are not without toxicity particularly some enzyme substrates which are carcinogens or mutagens. It may also be pointed out that whereas radiation is easily detected, toxic substances are not, (until it is too late!).

Table I.

A comparison of doses

Source		Dose
Annual background dose in:		
Kerala, India	(Monazite)	3.7-28 mSv
Brazil	(Monazite)	5 mSv
France	(Granite)	1.6-2.2 mSv
Cornwall, U.K.	(Granite)	7.8 mSv
Average annual dose in U.K.		2.5 mSv
Dose from single transatlantic flight		0.04 mSv
Dose from an x-ray examination		up to 10 mSv
Average annual dose for radioimmunoassayist		approx. 0.03 mSv

On the question of improved sensitivity, it is interesting to note that the sensitivities of non-isotopic techniques are usually limited by factors such as background signal detection, a requirement for chemical purity of reagents which is technically too demanding, and difficulties with labelling procedures. In general the potential improvement in sensitivity of non-isotopic methods has not been achieved in practice.

The main use of alternative techniques has been seen in the introduction of automated systems. Commercial suppliers of diagnostic immunoassay have developed such systems. Most of these are examples of complete automation with an integrated reagent supply, in the form of packs or cartridges, compatible only with the equipment supplied. Although these may be ideal for laboratories with limited technical expertise or staffing constraints, they are much more expensive than current radiolabelled options and have lost inherent flexibility with respect to the choice of reagents or suppliers.

The equipment costs for a typical example using one of the most popular automated systems would be at least U.S. \$100,000 including maintenance contract.

This contrasts significantly with the estimated costs for equipping a complete radioimmunoassay laboratory at U.S. \$50,000. Reagents for RIA/IRMA if obtained in bulk form, would cost approximately US \$ 0.2 per tube, compared with approximately US \$ 0.9 (thyroid function); US \$ 2.0 (fertility); US \$1.9 (Hepatitis B); using the above automated system.

It was noted that suitable equipment for a RIA laboratory is now available (at a cheaper price) in countries such as China and this would further reduce the cost.

A further disadvantage of automated systems is that they are essentially "black box" technology which is neither

didactic nor in principle transferable. Radioimmunoassay technology on the other hand has been eminently suited for training as was evident from the success of the IAEA training programmes.

A non-isotopic alternative, which is an exception to the black box technology mentioned above, is considered to be enzyme-labelled colorimetric methods, particularly when coupled with the use of the micro-titre plate format. This general technique has proven performance with characteristics at least equivalent to all others in practice. Supplies of suitable equipment are available from many sources. It is also possible to achieve a high degree of automation using a modular flexible approach incurring only a small increase in cost. It was noted that all the technology required is generally available and it would thus be appropriate for technology transfer and training programmes. However, the reagent cost would be higher than that for radio labelled immunoassays and the technique is less amenable to bulk reagent based concepts.

The main advantage of non-isotopic methods is that their practice avoids the requirements for licensing of laboratories or supervision under national regulations or legislation. Many user laboratories find this constraint an obvious disadvantage of radiolabelled techniques.

Another advantage of non-isotopic tracers is their enhanced stability. In many examples the shelf life is on

average at least six months, whereas ^{125}I labelled tracers last on average two months. However, It is not unusual for some ^{125}I -tracers to be stable for 6 months or more.

A particular advantage of radioisotopes like ^{125}I is that detection is not influenced or affected by either sample or reagent quality. Thus, sample degradation such as hemolysis or conditions like lipaemia, while representing a potential problem in any optical based detection system, do not interfere with ^{125}I Gamma Counting. The same can be noted for other environmental factors like dust, water quality, temperature or interfering substances including drugs. In fact highly purified water, such as is used in HPLC, may be required as standard in most of the optical systems especially those using enzymes or fluorescence.

The comparative use of radioisotopic and non-isotopic products in the UK is not consistent with either other industrialised countries or other regions. Fig. 1 shows the increase in the number of U.K. laboratories using alternative systems for a typical analyte (T4) over eleven years. The figure shows a fall in those using ^{125}I to 17% of the total. Nonetheless, it is significant there are signs that this trend is levelling out. Other data clearly indicate that the change was entirely related to the introduction of automation and not any other aspects related to non-isotopic technology. Other figures show that the pattern of change varies around the world.

In disciplines like serology, immunology or virology there is a preference for simple enzyme labels using the micro-titre plate format.

5. Concluding Perspectives

In 1995 the department of technical co-operation of IAEA published an independent review of its activities to promote RIA in human health during the period 1986-1995 [29]. This report confirmed the achievement of the objective to increase the reliability and quality of tests at the same time as reducing the cost of assays. Although figures for the cost reduction vary greatly from country to country, the cost of an assay using imported kits was, on average, in the order of \$2-3 per sample. Using bulk reagents, this was reduced to \$0.5. A further reduction in cost could well accompany the final establishment of the local production of reagents.

In addition to the reduced cost, the report identifies the high analytical reliability and robustness of RIA/IRMA bulk reagents compared to other technologies, and therefore expects their use to continue in developing Member States for the immediate future.

5.1. Economic and social factors

Although there are considerable variations world-wide in the provision of health services, it is difficult to make accurate comparisons. However, in relation to the use of

diagnostic tests, the two sets of statistics shown in Tables II & III are illustrative of the vastly differing needs.

Table II shows the relative number of doctors for selected countries in terms of population per physician. "Physician" here refers to medical staff qualified according to WHO standards and excludes "traditional health" practitioners.

TABLE II

Comparative Statistics: Population Density of Physicians

COUNTRY	POPULATION PER PHYSICIAN
Argentina	376
Bangladesh	5,196
Brazil	681
Chile	875
China	630
Colombia	914
Egypt	552
Ethiopia	30,195
France	362
Germany	305
India	2,173
Indonesia	7,102
Iran	1,600
Jordan	651
Malaysia	2,301
Morocco	2,945
Pakistan	2,064
Peru	944
Portugal	403
South Korea	817
Sri Lanka	4,745
Sudan	10,000
Thailand	4,259
Tunisia	1,640
Uganda	20,720
UK	667
Uruguay	286
USA	385
Vietnam	2,490
Zaire	15,584
Zambia	11,414
Zimbabwe	6,909

Nonetheless, these statistics can be used to illustrate the comparative importance of a diagnostic test in different countries. In many developed countries there are sufficient physicians to carry out the necessary extensive clinical examinations. In these circumstances, the analytical test provides results which are largely confirmatory to the diagnosis already made. However, as the proportion of physicians decreases, the extent of clinical attention and thus confidence in the diagnosis must also fall. In these countries a simple, reliable laboratory test performed by non-medically qualified staff may play a more primary role in the diagnostic process.

The data in Table III shows comparative statistics of total expenditure on health in U.S \$ per capita for the same countries. This figure includes both public (e.g. governmental) and private monies. It is difficult to directly compare these data because of variations in local cost of living rates and, of course, the very high cost of private health care in some countries which is not necessarily translated into quality of care. Nonetheless, the range of figures is considerable and undoubtedly reflects the need for cost reduction in many countries. Also, it should be remembered that for many countries equipment and reagents are usually only available in the international market where prices reflect the full monetary exchange rate and not the relative local cost.

TABLE III**Comparative Statistics: Health Expenditure**

COUNTRY	TOTAL HEALTH EXPENDITURE PER CAPITA U.S. \$
Argentina	137
Bangladesh	6
Brazil	146
Chile	100
China	11
Colombia	51
Egypt	28
Ethiopia	4
France	1,869
Germany	1,511
India	21
Indonesia	12
Iran	244
Jordan	55
Malaysia	71
Morocco	26
Pakistan	12
Peru	61
Portugal	383
South Korea	365
Sri Lanka	18
Sudan	34
Syria	41
Thailand	72
Tunisia	76
Uganda	8
Uruguay	123
UK	1,039
USA	2,765
Vietnam	3
Zaire	5
Zambia	17
Zimbabwe	39

An evolution of the world-wide business for radiolabelled-immunoassays reveals a very heterogeneous market with no consistent trend. There is an obvious drop in use in a few countries, however in some, for example, China, there is a dramatically expanding market in kits incorporating radiolabels, both in terms of total numbers and range of

analytes. The majority of countries at the lower end of health expenditure (Table III) have a real need to keep costs of reagents and equipment to a minimum. For many there may well be no economic alternative to a bulk reagent programme and, in particular, the use of radioisotopic tracers.

5.2. The role of research

It would be quite wrong to assume that all analytical procedures undertaken in health laboratories relate to diagnostic tests with proven clinical validation. Of course, such tests start out as research methods and gain clinical import over a period of years. Thus at any given time, there exists a spectrum of assays with a range of clinical effectiveness. Indeed, the clinician-laboratory interface could be seen as an important entity in the evolutionary didactic improvement in medicine. In this respect it is interesting to note that radiolabelled immunoassays remain a method of choice for the medical research worker even in developed countries. The fact that in the U.K. over 50% of clinical laboratories measuring GH use immunoassays with a radiolabelled tracer is obviously out of step with the trend for other routine services. It is a matter of observation that this difference is a reflection that most of this work (ie GH analyse) is carried out by active research groups.

The production of radiolabels using ^{125}I is both simple and adaptable. In addition expertise in their preparation and purification is widespread even in developing countries.

Together with the ease of producing antibodies, the facts have made ^{125}I -radiolabelled immunoassays ideal for investigative procedures for many research activities [30,31] particularly in the medical context where radioisotopes are commonly used.

Work done in recent months in our department has greatly simplified the technology required to produce antibody coated tubes. This means that provided production and distribution can be undertaken on a sufficiently large scale, coated tube technology can now be integrated into bulk reagent schemes. This would resolve one of the major difficulties experienced in the IAEA programmes [29].

It is also possible to source coated tubes from a commercial source. Again, provided that these can be purchased on an international or regional scale, a commercial supply of coated tubes would prove cost effective. Notwithstanding, it is important to establish that any given coated tube is compatible with other bulk reagents already being produced.

In conclusion, even a superficial examination of public health statistics for various countries throughout the continents indicates a need for a simple, inexpensive and robust analytical tool. In this light, there is a predicted continuing role for radiolabelled immunoassays.

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DEVELOPMENT OF SIMPLE IMMUNORADIOMETRIC ASSAYS USING AVIDIN COUPLED TO POLYSTYRENE BEADS AS A COMMON SOLID PHASE



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Abstract

In this paper, we describe the preparation and application of avidin coupled polystyrene beads as a common solid phase for use in immunoradiometric assays (IRMAs). The assay system is based on two matched commercial monoclonal antibodies, of which, the capture antibody is biotinylated using biotinamidocaproate N-hydroxysuccinimide ester and the detection antibody is radiolabeled with ^{125}I by conventional Chloramine-T method. Avidin was immobilized on the polystyrene beads through a primary coat of bovine serum albumin using glutaraldehyde activation method. Various factors, such as, concentration of reagents, incubation time, etc. were optimised to obtain a simple assay protocol consisting of only two pipetting steps, namely, that of a mixture of the two labelled antibodies (radiolabelled and biotinylated) and of the standard or sample. The advantage of the Avidin-Biotin system is the improved sensitivity, economy of antibody and the possibility to use a common solid phase in assays for different analytes. Using the polystyrene beads along with the novel decanting device, it has been possible to achieve the convenience of the 'coated-tube' technology without the expensive automation necessary for large scale preparation of antibody coated tubes. This protocol has been successfully applied to Prolactin, LH and FSH assays. The sensitivity of the Prolactin assay is $8\mu\text{IU/mL}$ (0.3 ng/mL), that of the FSH assay is 1mIU/mL and that of the LH assay is 0.9 mIU/mL . The intra-assay and inter-assay variations were $<10\%$. Shelf life of the avidin coupled beads was found to be about 8 months and that of the biotin labelled antibodies upto 18 months.

1. INTRODUCTION

Immunoassays like radioimmunoassays (RIA) and immunoradiometric assays (IRMA) have provided an accurate, elegant but simple tool for the measurement of a variety of biologically important substances in body fluids. Of the two assay variations mentioned above, the IRMA has been found to be superior in terms of sensitivity, specificity and ruggedness. An important requirement in sandwich IRMA is the solid phase for immobilising the capture antibody. The quality of the solid phase has a great influence on the sensitivity, range and cost of the assay. The antibody coated tube is the most preferred solid phase on account of its performance and convenience. However, the large scale manufacture of these tubes is expensive and requires great skill and control at all stages of antibody coating. An alternative to the 'coated tubes' is to use magnetisable particles. Though not as 'user-friendly', these solid supports are less expensive and can be easily prepared on a large scale even with modest facilities [1,2,3]. The avidin-biotin interaction has been widely applied to a variety of immunological techniques [4,5,6]. For use in IRMAs, avidin is coupled to the solid phase and the capture antibody is biotinylated. The use of the avidin-biotin system offers improved sensitivity and the possibility of having a common solid phase in IRMAs for different analytes. The use of finely divided solid phases, such as magnetisable particles involves pipetting the antibody suspension, which could be a source of imprecision,

hence, 'Single piece' solid phases like plastic beads would be a better alternative. This paper deals with the preparation and application of avidin coated polystyrene beads. The beads are relatively inexpensive and several thousands of them can be handled without the need for expensive automation or instrumentation. Moreover, with the help of a very simple and inexpensive device, we can achieve the convenience of the coated tubes, namely, that of decanting the contents from the tubes at the end of the assay. This technique has been successfully applied to immunoradiometric assays for Prolactin (PrL), LH and FSH. The respective capture antibodies have been labelled with biotin and detection antibodies with ^{125}I . After a careful study of various parameters such as incubation conditions, concentration of reagents, stability of the reagents and assay protocols, a simple assay format consisting of only two pipetting steps was established. The assays so developed have been evaluated by conventional procedures.

2. MATERIALS AND METHODS

2.1 Materials

Purified, matched pairs of monoclonal antibodies for each hormone were purchased from Boehringer Mannheim, Germany, ^{125}I was purchased from Dupont, USA, purified avidin from egg white, biotinamidocaproate N-hydroxy succinimide ester, glutaraldehyde, BSA, glycine were purchased from Sigma Chemical Co., 8 mm diameter, 'spec finish' polystyrene beads were from Precision Plastic Ball Co., USA, RIASTAR Packard automatic gamma counter from USA was used for assaying the radioactivity.

2.2 Methods

2.2.1. Immobilisation of avidin on polystyrene beads

Preparation of avidin coated polystyrene beads was carried out as reported earlier [7] with some minor modifications. The beads were coated with bovine serum albumin (BSA) by incubating them in 0.05 M phosphate buffer, pH 7.5, containing 0.2% BSA for 2h with gentle stirring. They were then washed thoroughly and placed in a 2% solution of glutaraldehyde for 6 h. The activated beads were washed free of the aldehyde and immersed in a solution containing avidin (50 μg /mL), not optimised, however, far in excess and incubated overnight at room temperature. The beads were then washed thoroughly and remaining active sites blocked using 0.05 M phosphate buffer containing 0.2% glycine and 2% skimmed milk powder [8,9]. Finally, the avidin coupled beads were washed several times and stored at 4°C, immersed in 0.05 M phosphate buffer containing 0.2% BSA, 0.1% Tween-20, 0.9% NaCl and preservatives.

2.2.1. Biotinylation of capture antibody

The capture antibodies for each hormone, identified earlier by "cross-matching" and tested for cross-reactivity, were labelled with biotin (10). Biotinamidocaproate N-hydroxy succinimide ester was dissolved in dimethylformamide (1mg / mL). 10 μL of this solution was added, with constant stirring to 1 mg of the monoclonal antibody dissolved in 0.1M bicarbonate buffer, pH 8.6. After incubating for 2 h, another 10 μL of the biotin solution was added and the reaction was carried out for another 2 h. The biotin labelled antibody was separated from the unreacted biotin by dialysis and diluted with 0.05 M phosphate buffer containing 0.2% BSA, 0.1% Tween-20, normal mouse serum and preservatives and stored at 4°C. The biotinylated antibody was tested periodically by setting up assays with the avidin coated beads.

2.2.2. Radiolabelling of antibody

Purified monoclonal antibody was radiolabelled with ^{125}I by the conventional procedure [11] to obtain a specific activity of about $12 \mu\text{Ci} / \mu\text{g}$ and purified by gel filtration over Sephadex G-100 (35cm x 1cm) column. Purified fractions were pooled, aliquoted and stored at -20°C . At the time of assay, each vial is reconstituted to get approximately 1.5×10^6 counts per minute / mL.

2.2.3. Hormone standard preparation

The standard hormone solutions were prepared in horse serum, calibrated against the respective WHO reference preparation, aliquoted and stored frozen.

2.2.4. Assay procedures

The effect of various assay parameters such as concentrations of biotinylated and radiolabelled antibodies, length of incubation, etc have been studied to arrive at a simple and reproducible assay format. With a view to improving the precision and simplifying the assay procedure, the biotin labelled and radiolabelled antibodies were mixed together and added as a single reagent in one pipetting step. Thus, the developed assay consists of adding an avidin coated bead, standard (or sample) and the mixture of the two labelled antibodies to the assay tube. At the end of the assay, 1mL of wash buffer is added to each tube (except in total tube), stirred, the contents decanted and the radioactivity associated with the bead measured.

3. RESULTS AND DISCUSSION

In the work reported here, we have used the avidin-biotin system for developing IRMAs for Human Prolactin, LH and FSH. The method of coupling avidin to polystyrene beads was found to be simple and reproducible. These beads are relatively inexpensive and several thousands of them can be handled at a time without the need for automation or expensive instrumentation at any stage of coating. Stability of these beads was studied over a period of 8 months. Fig.1. shows the results of an assay carried out immediately after coating the bead (curve A) and that of an assay done eight months later (curve B). This study showed the stability of the beads over the period of storage, without any significant change in the assay performance. The main advantage of using avidin coupled beads was the feasibility of a common solid phase for all assays.

The biotinamidocaproate N-hydroxy succinimide ester has been used in order to take advantage of the spacer arm, which reduces the steric hindrances generally associated with the binding of four biotinylated protein molecules to one avidin molecule [12,13]. Biotinylation of the antibody is technically simple. Labelling of the protein with biotin depends upon the reaction stoichiometry as well as on the overall concentrations of the protein and the biotin ester, that is, a higher labelling yield is observed with smaller reaction volumes. The solution of the biotin ester should be freshly prepared just before labelling. For the labelling reaction, the volume of the biotin ester solution not exceed 5% to 10% of the total reaction volume. The reaction mixture should be kept stirring during the addition of the ester as well as during the course of the reaction. The shelf life of the biotinylated antibody was about 18 months at 4°C .

TABLE I. TYPICAL STANDARD CURVE DATA**Prolactin assay**

Prolactin (ng/mL)	0	2.5	5	10	25	50	100
%B/T	0.06	2	3.7	5.4	10.6	18	25

FSH assay

FSH (mIU/mL)	0	2	4	9	18	36	72	144
%B/T	0.05	0.34	0.51	2.3	4.1	10.8	17.3	37.4

LH assay

LH (mIU/mL)	0	2.5	5	10	25	50	100	200
%B/T	0.08	0.21	0.39	0.94	2.2	4.4	6.9	14.9

The Chloramine-T method of iodination followed by purification by gel filtration resulted in labelled antibody of high specific activity, purity and stability. The tracer could be stored for atleast 60 days at 4°C at assay dilution. Specific activity of 10 μ ci / μ g has been found to be quite adequate for the assay.

Typical standard curves obtained for each assay are tabulated in Table -I. Various assay parameters were optimised for each assay by conventional assay procedures as shown in Table-II after different assay protocols were studied with a view to simplifying the assay and improving the precision. A simple assay format consisting of only two pipetting steps was established by adding the two labelled antibodies as a single reagent, the other pipetting being that of the standard (or sample). The stability of the mixture was studied and found to be satisfactory over the complete shelf life of the tracer antibody (60 days) as shown in Fig.2. Curve A shows the standard curve with the freshly prepared antibody mixture and Curve B shows that obtained towards the end of

TABLE II. CHARACTERISTICS OF PROLACTIN AND LH ASSAYS

Parameter	Prolactin assay	LH assay
Incubation period	3h, room temperature	3h, room temperature
Sensitivity	0.3ng/mL	0.9mIU/mL
Recovery test	86-110%	89-104%
Dilution test	90-107%	90-110%
Intra-assay variation	<5%	<10%
Inter-assay variation	<10%	<10%
Hook effect	No hook effect upto 200 ng/mL	No hook effect upto 300 mIU/mL

TABLE III. COMPARISON OF AMOUNT OF CAPTURE ANTIBODY REQUIRED IN CONVENTIONAL IRMAs (WITH ANTIBODY COUPLED DIRECTLY TO THE MAGNETISABLE PARTICLES) AND THE AVIDIN-BIOTIN BASED IRMAs

IRMA System	Capture antibody required µg/tube
Conventional IRMAs [1,3]	
Prolactin	1.2
FSH	1.5
LH	0.8
Avidin-Biotin Based IRMAs	
Prolactin	0.25
FSH	0.60
LH	0.66

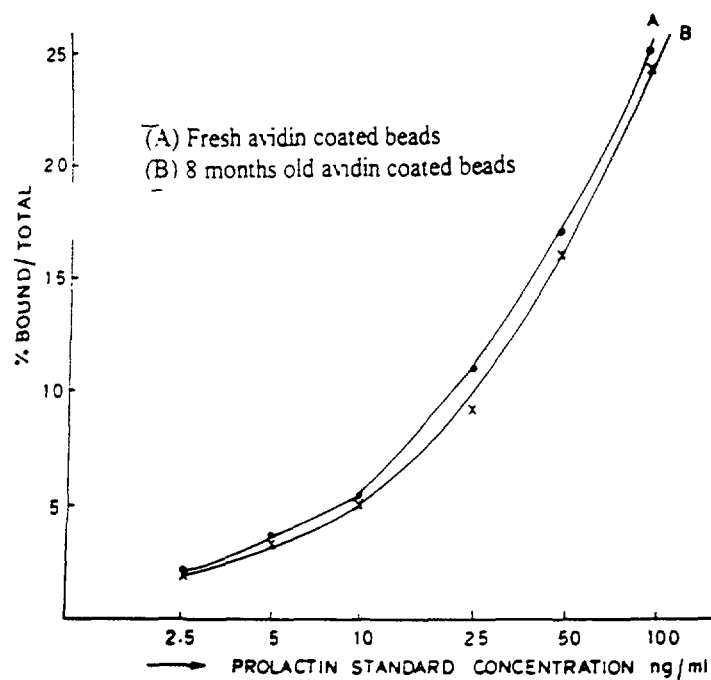


Fig. 1. Stability of avidin coated beads elicited by representative standard curves of Prolactin IRMA.

(A) Fresh avidin coated beads.

(B) 8 months old avidin coated beads.

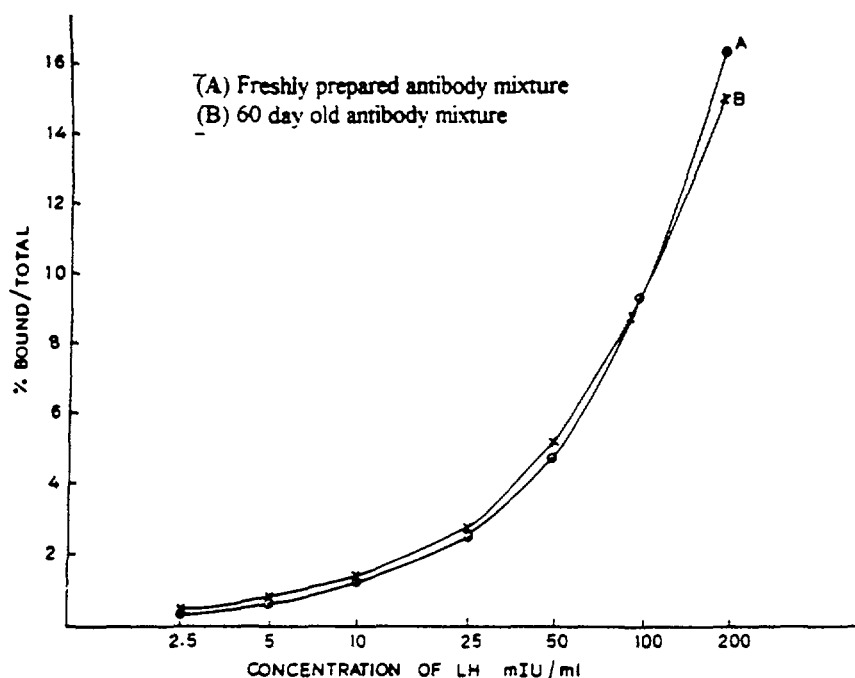


Fig. 2 . Stability of mixed labelled antibody reagent elicited by representative curves of LH IRMA.

(A) Freshly prepared antibody mixture.

(B) 60 day old antibody mixture.

the shelf life of the antibody mixture. The length of incubation period was also studied at 3, 5 and 24 hours with stirring and overnight without stirring. The assays were found to be similar for incubation at 3 h with stirring and overnight without stirring, with respect to sensitivity and other standard curve parameters.

In the conventional "bead assays" the free fraction is aspirated out. In the assays reported here, the convenience of the "coated tube" has been achieved by making use of a very simple, inexpensive and universally available device, namely, a paper clip. The assay tubes, placed in a decanting rack, are fitted with clips that have been partially pried apart. To carry out the separation, wash buffer is added to the tubes, the contents stirred and the rack inverted to discard the solution, as is done with the coated tubes.

The Avidin-Biotin system is generally associated with a high non-specific binding (NSB) [8]. However, we have found that the NSB largely depended upon the quality of the tracer, the NSB in all our assays has been $< 0.1\%$. The use of Avidin-Biotin system has also reduced the amount of capture antibody required as shown in Table-III. This assay format has been successfully applied to Prolactin, LH and FSH.

4. CONCLUSION

The avidin-biotin based IRMAs, described in this paper, are simple, accurate and reproducible. The advantages of using the avidin-biotin system include, improved sensitivity, economy of the monoclonal antibodies and the possibility of having a common solid phase for different assays.

Using the polystyrene beads as the solid phase, along with the simple decanting device, one can achieve the convenience of the "coated-tube" assays, without the need for expensive instruments or automation at any stage of the antibody coating procedure.

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**MAGNETIC PARTICLE SEPARATION TECHNIQUE:
A RELIABLE AND SIMPLE TOOL FOR RIA/IRMA
AND QUANTITATIVE PCR ASSAY**



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Abstract

Five types of magnetic particles without or with aldehyde, amino and carboxyl functional groups, respectively were used to immobilize first or second antibody by three models, i. e. physical adsorption, chemical coupling and immuno-affinity, forming four types of magnetic particle antibodies. The second antibody immobilized on polyacrolein magnetic particles through aldehyde functional groups and the first antibodies immobilized on carboxylic polystyrene magnetic particles through carboxyl functional groups were recommended to apply to RIAs and/or IRMAs. Streptavidin immobilized on commercial magnetic particles through amino functional groups was successfully applied to separating specific PCR product for quantification of human cytomegalovirus.

In the paper typical data on reliability of these magnetic particle ligands were reported and simplicity of the magnetic particle separation technique was discussed. The results showed that the technique was a reliable and simple tool for RIA/IRMA and quantitative PCR assay.

1. INTRODUCTION

In procedures of radioimmunoassay (RIA), immunoradiometricassay (IRMA) and quantitative polymerase chain reaction (PCR), separation of determined target molecules is an essential step. In addition to being technically demanding and time-consuming, the step is the major source of imprecision in the assays. Application of solid phase separation techniques is an important improvement in analytical methodology. Introduction of magnetic particle separation technique is more interesting because the magnetic particles bound to the target molecules can be quickly and clean separated from mixture solution, thus providing a solid phase methodology which avoids many disadvantages of other solid phase techniques while retaining their advantages.

In the paper six types of magnetic particles with or without functional groups were used to immobilize antibodies or streptavidin by three types of model, forming five types of magnetic particle ligands. The solid phase ligands were applied to the procedures of RIA/IRMA or quantitative PCR assay. Reliabilities of the separation techniques were briefly reported.

TABLE I . MAGNETIC PARTICLE AND MAGNETIC PARTICLE LIGAND

Magnetic particle and magnetic particle ligand	Description
Magnetic Particle	
1. Fe ₃ O ₄ particle (Magnetic nucleus, MN)	Prepared by a chemical precipitation method, 10nm ± 34 % in diameter
2. Polyacrolein magnetic particle (AMP)	Aldehyde functional groups were coated on MN by ⁶⁰ Co-irradiation polymerization of acrolein
3. Silanized magnetic particle (SMP)	Amino functional groups were coated on MN by acidic aqueous silanization using 3-amino-propyltrimethoxysilane
4. Carboxylic polyacrylamide magnetic particle (CAMP)	Carboxyl functional groups were coated on MN by emulsion polymerization of methacrylic acid and acrylamide (CAMP) or of methacrylic acid and styrene (CSMP)
5. Carboxylic polystyrene magnetic particle (CSMP)	
6. Magnetic Affinity Particle (MAP)	Purchased from Paesel/Lorei Co., Germany, 0.5~1.5μm in diameter, amine-terminated
Magnetic Particle Ligand	
1. Magnetic second antibody-P (MSA-P)	Donkey anti-rabbit (D×R) serum was immobilized on MN by physical adsorption (P)
2. Magnetic second antibody-C (MSA-C)	D×R serum was immobilized on AMP by chemical coupling (C)
3. Magnetic first antibodies-C (MFA-C)	Anti-T ₃ , T ₄ or TSH serum was immobilized on SMP, CAMP or CSMP by chemical coupling (C)
4. Magnetic first antibody-I (MFA-I)	Anti-T ₃ rabbit serum molecules were immobilized on the D×R serum molecules of MSA-C by immuno-affinity (I)
5. Magnetic particle streptavidin (MP•SA)	Streptavidin (SA) was immobilized on MAP by chemical coupling

2. MATERIALS AND METHODS

2.1. Magnetic particles and magnetic particle ligands

In Table I were listed six types of magnetic particles and five types of magnetic particle ligands, which were developed in the former researches^[1-3] except that the Magnetic Affinity Particle (MAP) was from a commercial source and the magnetic particle streptavidin (MP•SA) was prepared in our laboratory according to the procedure presented by the manufacturer of MAP.^[4]

2.2. Use of magnetic particle antibodies in RIAs and IRMAs

Magnetic second antibody of MSA-P or MSA-C was used as an immuno-separation reagent in RIAs. After the reaction between an antigen and its first antibody was completed, add the magnetic second antibody into the reaction solution and incubate for 10 min. The magnetic solid phase was separated by a magnet, and then washed and counted. The procedures of incubation, separation and washing may be finished within 20 min.^[1]

Magnetic first antibody of MFA-C or MFA-I were used as an immuno-separation reagent while as an immuno-reaction reagent in RIAs and/or IRMAs. Add the magnetic first antibody to a sample serum and incubate until the reaction was finished. Separate and wash as above, and then continue following assay procedures.^[1,2]

2.3. Use of MP·SA in quantitative PCR assay

MP·SA was used to separate specific PCR products. The separation was based on the extremely high affinity of streptavidin (SA) to biotin (B). A nested PCR (nPCR) procedure was performed.^[5] In the second PCR run one of B-labelled primer and [α -³²P] dATP were used. The amplified specific products which were labelled by B at an end of one strand of the double strands while by [α -³²P] dATP into the DNA sequences were separated by adding MP·SA to the amplified mixture and incubating for 15 min. Magnetically separate and wash the magnetic solid phase. The no B-labelled strand of the specific products was eluted by using NaOH solution from the magnetic solid phase for 5 min and then counted. The procedures of separation may be finished within 1 h.

3. RESULTS

3.1. Application of magnetic particle antibodies to RIAs/IRMAs

Magnetic second antibodies of MSA-P and MSA-C have been successfully applied to RIAs for tri-iodothyronine (T₃), reverse T₃(rT₃), free T₃(fT₃), thyroxine (T₄), free T₄(fT₄), thyroid-stimulating hormone (TSH), thyroglobulin (TG) and TG-antibody (TG-Ab).^[1] In Table II was listed the correlation of levels of quality control (QC) serum obtained with MSA-C assay and liquid phase double antibody (LDA) assay (As a control). The results showed a better correlation of the results between both of assays thus the reliability of MSA-C. The MSA-P presented the similar performance to MSA-C but unsatisfactory stability of storage at surrounding temperature.^[1]

TABLE II . CORRELATION OF LEVELS OF QC SERUM OBTAINED WITH LDA(X) AND MSA-C (Y) ASSAYS*

RIA item	Y = a + bX			
	N	a	b	r
T ₃	156	0.043	0.977	0.988
T ₄	159	2.266	0.973	0.993
TSH	159	0.371	0.897	0.982

* Tests of the correlation were performed on three levels of QC serum monthly from 1992 to 1996. N: number of determination, r: correlation coefficient

TABLE III . PARAMETERS OF STANDARD CURVE OF IRMA FOR BLOOD SPOT TSH USING MAGNETIC ANTI-TSH ANTIBODIES

Magnetic particle immobilized by anti-TSH antibody	Binding, %		LogY = a + bLogX			QC serum, μ IU/ml		
	B ₀	B ₁₆₀	a	b	r	L	M	H
Latex-M (Control)	0.29	8.94	1.82	0.941	0.9975	6.3	26.3	52.5
CSMP	0.29	8.25	1.79	0.940	0.9982	8.7	27.8	53.6
CAMP	0.29	7.14	1.73	0.943	0.9996	9.8	29.1	58.3
SMP	0.29	7.56	1.81	0.891	0.9955	10.1	31.8	52.9

B₀ and B₁₆₀: ¹²⁵I-binding at TSH of 0 μ IU/ml and 160 μ IU/ml, respectively; L, M and H: Low, middle and high values

Magnetic first antibodies of MFA-C and MFA-I could be applied to RIAs of T_3 , fT_3 , T_4 , fT_4 or blood spot T_4 .^[1,2] A standard curve of T_3 RIA obtained by using MFA-I was shown in Fig. 1 and compared to those by using MSA-C and LDA under the same conditions. The results showed that the standard curve of MSA-C was comparable with that of LDA, but the standard curve of MFA-I showed a lower slope than that of LDA. The difference may result from unsatisfactory suspension ability of the magnetic first antibody since it needed to react for a longer time (1 h) than the magnetic second antibody (10 min).

The anti-TSH antibodies immobilized on SMP, CAMP and CSMP could be applied to IRMAs of TSH and blood spot TSH.^[2] In Table III were listed parameters of standard curves of the blood spot TSH obtained by using these magnetic anti-TSH antibodies and were compared to that obtained by using Latex-M (A commercial magnetic anti-TSH antibody purchased from Rhone-Doulenc Co., France). The results showed that the anti-TSH antibody based on CSMP presented better performances similar to Latex-M and should be recommended.

3.2. Application of MP·SA to quantitative PCR assay

Biotinylated PCR products were able to be separated quantitatively by MP·SA as shown in Table IV. The results showed that ^{32}P -binding of MP·SA decreased to the level of its non-specific binding when secondary separation was done.

In a quantitative PCR assay initial amount of determined templet is determined by detection of accumulated amount of ^{32}P -labelled PCR products from a standard curve which may be prepared by using a standard templet. The standard curve was established by an external standard method for quantification of human cytomegalovirus (HCMV) using MP·SA separation technique^[5], as shown in Fig. 2. The standard curve was a straight line on Log to Log scales between initial templet amounts from 1.3×10^{-4} amol to 1.3×10^{-1} amol and its intercept, slope and correlation coefficient (r) were 1.071, 0.517 and 0.986, respectively.

Three levels (low, middle and high) of QC templet were determined by the method and the results were listed in Table V. The results showed that coefficients of variation (CVs) were less than 39% for intra-assay and less than 73% for inter-assay, and were decreasing with increasing of levels of QC templet. Compared the detected average values (\bar{X}) with the reference values (X_0), there were differences up to three times between both of relevant values. Above results were comparable to that reported in some literatures^[5] and the method could be applied to relative quantification of HCMV.

4. DISCUSSION

An ideal magnetic solid phase should fulfill the following requirements: 1) Its preparation should be simple, reproducible and inexpensive and should use readily available reagents and equipments. Its ligands should be immobilized by covalent linkage, preferably without prior active step, 2) It should have high lig-

TABLE IV. SEPARATION OF ^{32}P -BOUND PCR PRODUCT

Item	^{32}P -binding, % *
Non-specific binding	0.23 ± 0.04
Specific binding, First	$3.92 \sim 8.38$
Second	0.15 ± 0.06

* Ratio of ^{32}P -counts separated to added

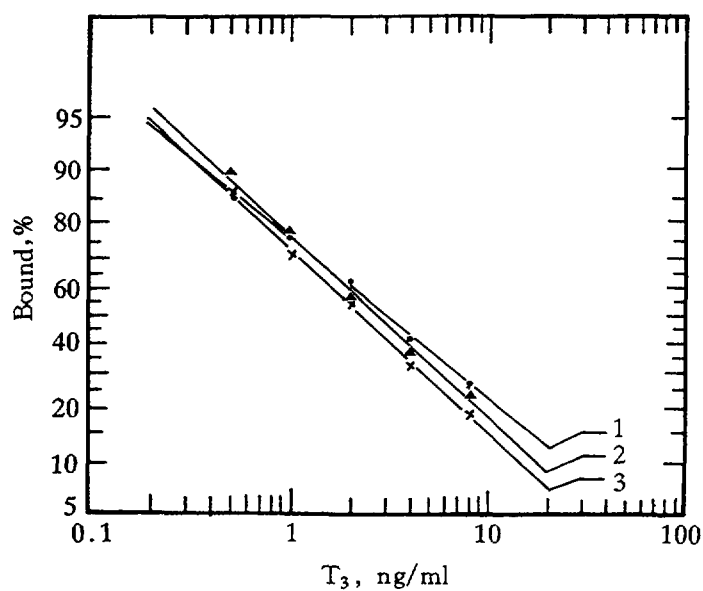


Fig.1. Standard curves of RIA for T_3 using MFA-I (“·”, curve 1), MSA-C (“▲”, curve 2) and LDA (“×”, curve 3) * assays. 1 ng/ml is equal to 1.54 nmol/L for T_3

* As control

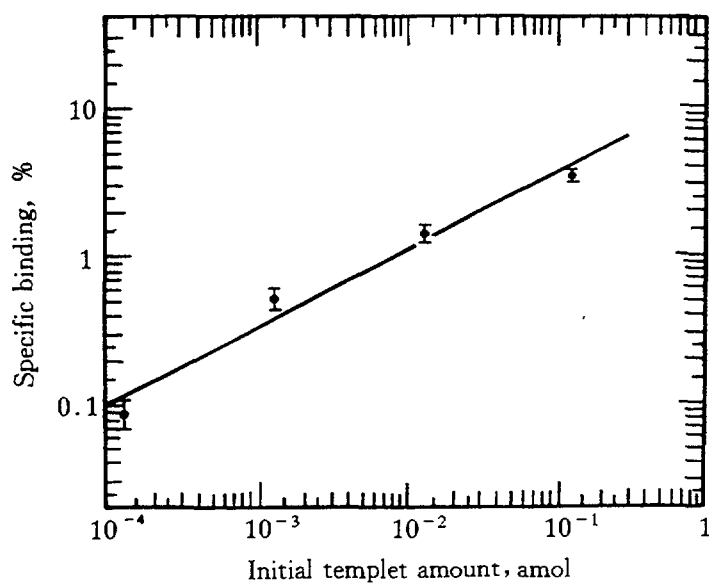


Fig.2. Standard curve for quantification of HCMV by nPCR using MP-SA separation technique

TABLE V. QUANTITATIVE RESULT OF QC TEMPLET BY NPCR

QC templet		Intra-assay			Inter-assay		
No	X_0 , amol	N	\bar{X} , amol	CV, %	N	\bar{X} , amol	CV, %
L	2.6×10^{-4}	4	1.3×10^{-4}	38.9	5	1.6×10^{-4}	72.5
M	2.6×10^{-3}	4	7.8×10^{-3}	17.8	5	4.4×10^{-3}	54.9
H	6.4×10^{-2}	4	6.6×10^{-2}	14.5	5	6.6×10^{-2}	39.0

X_0 and \bar{X} : Reference level and detected average value; CV: Coefficient of variation

and-bound capacity but low non-specific binding, thus providing a complete and clean separation of target molecules from impurities. It can remain stable suspension for a longer time during incubation and keep a high magnetic response to ensure rapid separation, 3) It should be stable in aqueous solution at 4°C and/or surrounding temperature without change of its physical, chemical and affinity characteristics, 4) It should be applicable to the majority of assay items and suitable for automation of the assay procedures.

It is difficult to meet all these requirements, where unsatisfactory suspension ability is a main obstacle to application of the magnetic solid phase. Magnetic materials, which have to be used, have a high density hence the size of magnetic particle should be controlled. But too small particle used will require a longer time of separation or a stronger magnetic field. The density of magnetic particle can be decreased by means of coating a polymer with low density on surface of magnetic nucleus while amount of the magnetic material in the particle should be enough to ensure its high magnetic response. It is possible to resolve these problems technically.

To date, the magnetic solid phase technique is being increasingly applied to many scientific aspects. In China the technique is being applied to invitro assays clinically instead of the traditional methods. Introduction of the technique has made our work get twice the result with half the effort and seemed to make radionuclide-based operation easy be accepted since its simplicity, time-saving and suitability to automation. With improving of the performance of the magnetic solid phase, the unique technique will undoubtedly have a substantial future impact on the practice of radionuclide-based laboratory diagnoses.

5. CONCLUSION

A second antibody immobilized on polyacrolein magnetic particles through aldehyde functional groups and first antibodies immobilized on carboxylic polystyrene magnetic particles through carboxyl functional groups were recommended to apply to RIAs and/or IRMAs. Streptavidin immobilized on commercial magnetic particles through amino functional groups was successfully applied to separating specific PCR products for quantification of HCMV. The results showed that the magnetic particle separation technique was a reliable and simple tool for RIA/IRMA and quantitative PCR assay.

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MEDICAL ISOTOPE PRODUCTION

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Abstract

Recent developments in both reactor and accelerator production of radioisotopes finding applications in nuclear medicine and in biomedical research are summarised. The priorities for the production of 48 different cyclotron radioisotopes; and for 42 reactor produced radioisotopes finding biomedical applications are identified. Each includes 5 generator systems. The rapid expansion of cyclotron based radioisotope production and automated synthesis of short-lived radiopharmaceuticals with the positron-emitting radionuclides continues to gain momentum. Recent feasibility studies of the cyclotron production of ^{186}Re , $^{99\text{m}}\text{Tc}$ and of ^{99}Mo are cited as examples of motivation to develop accelerator alternatives to use of nuclear reactors for medical radioisotope production. Examples of SPET and PET radiopharmaceuticals labelled with ^{131}I , ^{123}I , ^{124}I , ^{18}F , and with therapeutic radionuclides are highlighted.

INTRODUCTION

Nuclear reactors have played a key role in the production of radioisotopes required for medical, industrial, agricultural applications, education in the nuclear sciences and research. Millions of people worldwide have benefited from the $^{99}\text{Mo} \rightarrow ^{99\text{m}}\text{Tc}$ generator for diagnostic imaging, and ^{131}I for the treatment of cancer. Table I lists the important reactor-produced biomedical radioisotopes. Advances in accelerator and medical imaging technology are driving the demand for radioisotopes and radiopharmaceuticals required by nuclear medicine.

Circumstances such as public perception arising from concern for the environment either from radiation accidents or long term storage of nuclear waste, as well as the operating and replacement costs for aging reactors are factors influencing the prospects of future availability of radioisotopes. This is reflected in recent decisions taken to initiate the de-commissioning of a few research TRIGA reactor(s) that were installed in hospitals during the 1960's.

TRENDS

The number of cyclotron installations at national laboratories, universities and teaching hospitals has expanded [1], since 1970 due to commercial availability of user-friendly cyclotrons. (Fig. 1). There are >200 cyclotrons operating in 1998, with the highest concentration in the United States, European Union and Japan. National accelerator programmes often include use of parasitic beam for LINAC production of selected radioisotopes (e.g., ^{26}Al , ^{67}Cu , ^{68}Ge , ^{72}Se , ^{82}Sr , ^{109}Cd , ^7Be).

The reactor produced radioisotopes in highest demand for endotherapeutic radiopharmaceuticals are: ^{32}P , ^{67}Cu , ^{89}Sr , ^{90}Y , ^{103}Pd , $^{117\text{m}}\text{Sn}$, ^{153}Sm , ^{165}Dy , ^{166}Ho , ^{186}Re , ^{188}Re and ^{198}Au . The $^{188}\text{W} \rightarrow ^{188}\text{Re}$ generator and ^{186}Re have great potential for cancer therapy, particularly in the form of organo-rhenium radiopharmaceuticals. The $^{166}\text{Dy} \rightarrow ^{166}\text{Ho}$ *in vivo*

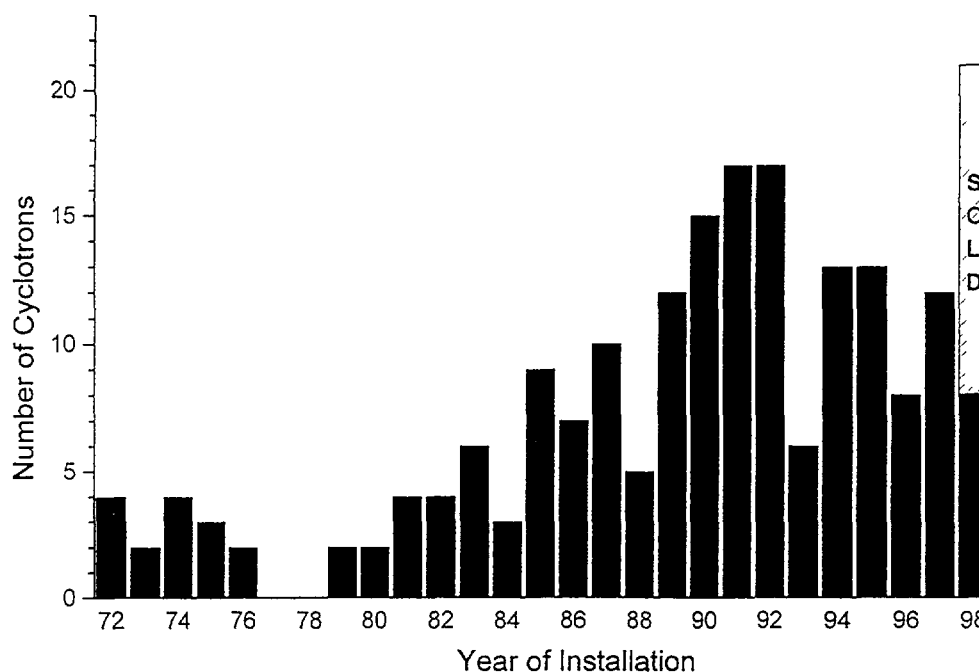


Fig. 1. Number of cyclotron installations commissioned between 1972 and 1997, and a projection for 1998 based upon the number of identified cyclotron orders.

biomedical generator has been suggested as offering advantages for bioconjugated monoclonal antibodies. Both ^{188}W and ^{166}Dy are produced by double neutron capture reaction, and therefore production is limited to a few high power nuclear reactors. The applications of neutron-rich radioisotopes include: treatments of cancer with radioimmunospecific radiopharmaceuticals, bone pain palliation, radiation synovectomy for treatment of rheumatoid arthritis, bone marrow ablation for treatment of myeloma.

It is essential to develop accelerator technology for the production of neutron rich radioisotopes that are needed for therapeutic and industrial purposes. Since 1993 there has been considerable progress at a few laboratories to meet this objective, and to reduce reliance on research nuclear reactors for medical radioisotopes. For example, there are 16 cyclotrons that will be dedicated to operating 24 h at 7 d per week year round only for the production of ^{103}Pd . It is used as a brachytherapy source for treatment of prostate cancer. Interestingly, the worldwide shortage of highly enriched ^{102}Pd was the driving force to abandon (n,γ) production of ^{103}Pd . The cyclotron production of ^{186}Re , ^{64}Cu and ^{183}Ta has been demonstrated. There is a technological challenge to develop cyclotron targets that can withstand 1 to 5 mA beam currents, as will be required for certain of the nuclear reactions that have a small cross section. Current targetry technology limits beam currents to ~ 1.2 mA.

Cyclotron radioisotope production uses nuclear data such as decay schemes, excitation functions and thick target yields. Recently a multi-institutional study [2,3] was completed to evaluate the feasibility of the cyclotron production of $^{99\text{m}}\text{Tc}$, and of the ^{99}Mo - $^{99\text{m}}\text{Tc}$ generator. There are various conflicting reports in the scientific literature. The study was motivated because of the continuing concern about the future supply of ^{99}Mo which is produced with nuclear reactors. Both theoretical calculations using the Hybrid-ALICE code, and experimental measurements of the $^{100}\text{Mo}(p,pn)^{99}\text{Mo}$ nuclear reaction with 30-50 MeV protons indicated that the approach was not a viable alternative. However, detailed

TABLE I. IMPORTANT REACTOR - PRODUCED BIOMEDICAL RADIOISOTOPES

^{99}Mo - $^{99\text{m}}\text{Tc}$	^{131}I	^{32}P	^{133}Xe	^{60}Co	^{153}Sm
^{188}W - ^{188}Re	^{169}Y	^{153}Gd	$^{117\text{m}}\text{Sn}$	^{186}Re	^{165}Dy
^{166}Dy - ^{166}Ho	^{166}Ho	^{90}Sr	^{89}Sr	^{47}Sc	^{59}Fe
^{199}Hg - ^{199}Au	^{198}Au	^{192}Ir	^{82}Br	^{51}Cr	^{55}Fe
^{125}Xe - ^{125}I	^{64}Cu	^{177}Lu	^{42}K	^{109}Cd	^{105}Rh
^{212}Bi	^{213}Bi	^{33}P	^{24}Na	^{137}Cs	^{75}Se
^{14}C	^3H	^{35}S	^{47}Ca	^{152}Eu	^{170}Tm

TABLE II. PRIORITIES FOR PRODUCTION OF ACCELERATOR RADIONUCLIDES

Application	Radionuclide
PET and 511 KeV SPET	
Emphasis on:	^{11}C , ^{13}N , ^{15}O , ^{18}F
Emerging	^{64}Cu , ^{124}I , $^{82}\text{Sr} \rightarrow ^{82}\text{Rb}$
Research Interest	^{38}K , ^{45}Ti , $^{62}\text{Zn} \rightarrow ^{62}\text{Cu}$, ^{73}Se , ^{75}Br , ^{76}Br , $^{82\text{m}}\text{Rb}$, $^{94\text{m}}\text{Tc}$
SPET	
Clinical	^{67}Ga , ^{111}In , ^{123}I , ^{201}Tl
Therapeutic	^{64}Cu , ^{67}Cu , ^{103}Pd , ^{186}Re , ^{211}At
Standards and Sources	^{22}Na , ^{57}Co , ^{139}Ce
Commercial -Medical	^{18}F , ^{13}N , ^{67}Ga , $^{81}\text{Rb} \rightarrow ^{81\text{m}}\text{Kr}$, ^{103}Pd , $^{123}\text{Xe} \rightarrow ^{123}\text{I}$, $^{201}\text{Pb} \rightarrow ^{201}\text{Tl}$
Emerging	^{124}I
Various	^{22}Na , ^{57}Co , ^{88}Y
Others	^7Be , ^{10}C , ^{26}Al , ^{28}Mg , ^{48}V , ^{75}Se , $^{87}\text{Y} \rightarrow ^{87\text{m}}\text{Y}$, ^{93}Mo , ^{99}Mo , ^{109}Cd , $^{99\text{m}}\text{Tc}$, ^{147}Gd , ^{195}Au , ^{206}Bi

TABLE III. OUTLINE OF ^{123}I -RADIOPHARMACEUTICALS

Tumours	Peptides and proteins Receptor-specific ligands Meta-iodobenzylguanidine (m-IBG) Hypoxia agents α -methyl-iodotyrosine 5-Iodo-2'-deoxyuridine
Heart	m-IBG Receptor-specific ligands, Fatty acids, e.g., BMIPP
Brain	Neuroreceptor specific ligands, e.g. IDEX, β -CIT, FP-CIT, Blood flow tracers
Infection and Inflammation	Monoclonal antibodies Cytokines
Other	Atrial natriuretic peptide Serum amyloid P component (SAP) Growth factors

measurements of the excitation function for the $^{100}\text{Mo}(p,2n)^{99\text{m}}\text{Tc}$ nuclear reaction determined that the 22-12 MeV proton energy range could be considered to produce a few Curies of instant $^{99\text{m}}\text{Tc}$ for local use provided that a ^{100}Mo target of high isotopic enrichment is used. The peak of the excitation function is between 16-18 MeV. The method may be considered by Member States that do not presently have a nuclear reactor, and have to rely upon imported ^{99}Mo in order to have $^{99\text{m}}\text{Tc}$ for nuclear medicine.

The priorities for the production of 48 different cyclotron radioisotopes including 5 generator systems are summarised in Table II. A classification as to applications include medical uses (diagnostic and therapeutic radiopharmaceuticals, stents for treatment of coronary restenosis); radioactive standards and calibration sources; industrial purposes, and environmental tracers, and research.

DESIGN OF RADIOPHARMACEUTICALS

The cyclotron produced radioisotopes used in nuclear medicine have a short half life and decay with a high abundance of photons (100 to 200 KeV) that are efficiently detected by medical imaging instruments such as SPET (Single Photon Emission Tomography) and gamma camera; or that decay with positron emission that permit quantitative imaging with PET (Positron Emission Tomography).

Iodine-123 is gradually displacing the use of ^{131}I for diagnostic applications. The major drawback to wider use of ^{123}I is the expensive targetry system involving the use of isotopically enriched ^{124}Xe as the target system. Table III outlines the range of ^{123}I -radiopharmaceuticals used for various applications [4].

Fluorine-18 ($T_{1/2} = 110 \text{ m}$), ^{11}C ($T_{1/2} = 20 \text{ m}$), ^{15}O ($T_{1/2} = 2 \text{ m}$), and ^{13}N ($T_{1/2} = 10 \text{ m}$), are the most popular PET radioisotopes. Over 1000 compounds have been labelled to study specific biochemical processes and physiologic function. Numerous of receptor-specific ligands, small molecules, growth factors are being evaluated [5]. The European concerted action [6] on new radiotracers is an excellent example of coordinated research efforts for quality assurance and technology transfer. The new frontier for radiopharmaceutical development is based upon the collaboration of radiopharmaceutical scientists and molecular biologists.

Radioisotope production has been optimised, but there is a continuing need for automation in radionuclide processing and radiopharmaceutical synthesis. Considerable synthesis automation is commercially available for the preparation of synthetic precursors and PET radiopharmaceuticals. The clinical applications in oncology, cardiology and neurology presently rely upon 25 different PET radiopharmaceuticals. However, most clinical PET centres routinely use only 2 to 5 PET radiopharmaceuticals. ^{18}F FDG is the most popular PET radiopharmaceutical. There is an opinion the nuclear oncology with ^{18}F FDG accounts for >80% of clinical PET applications.

An additional advantage of emission tomography is that low dose of short lived cyclotron radioisotopes is used to do individual patient radiation treatment planning prior to administration of a high dosage of reactor produced radioisotopes for endotherapeutic treatments. For example, the use of the positron emitter ^{124}I for PET studies as the prelude to high dose administration of ^{131}I ; ^{64}Cu and ^{67}Cu ; or ^{86}Y and ^{90}Y .

PROGRAMME DEVELOPMENT CONSIDERATIONS

The first question arising when an institution is considering to purchase a cyclotron relates to the decision of the scope of the program envisioned for radioisotope production. The next questions focus on all aspects of the radiochemistry, hot cell processing, automation for provision of radioisotopes, labelled synthetic precursors and preparation in the required radionuclidic purity and radiochemical form for use. Considerable information is available in topical books and review articles published during the past 5 years [7,8,9,10]. The Cyclotron Directory to be published in 1998 contains very useful information concerning cyclotron programs worldwide[1].

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DEVELOPMENT AND JUSTIFICATION OF A SCHEME FOR COMMERCIAL SUPPLY OF RADIOPHARMPREPARATION ^{99m}Tc USING CENTRALIZED Zr-Mo GEL-GENERATOR

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Abstract

Zr-Mo gel-generator ^{99m}Tc on the basis of activated ^{99}Mo is used in modern medical diagnostics as a convenient, safe and reliable local source of ^{99m}Tc production in the form of sodium pertechnetate. The methodology and technological process of the wasteless reactor gel-technology of radiopharmpreparation (RPhP) ^{99m}Tc production with the use of centralized Zr-Mo gel-generator has been developed in the Institute of Power Engineering Problems / National Academy of Sciences of Belarus in co-operation with Scientific Research Institute of Oncology and Medical Radiology of Ministry of Health of the Republic of Belarus. As a result of performed clinical tests the Ministry of Health had approved the clinical application of RPhP ^{99m}Tc produced according to this technology. Zr-Mo gel-generator at full load of 150-370 GBq of ^{99}Mo can provide with ^{99m}Tc solution such a region as the Republic of Belarus and export the preparation.

An optimization of initial irradiation of MoO_3 target in a nuclear reactor as well as conditions of the gel-generator operation is important for definition of the price of produced ^{99m}Tc .

The technological process of delivery of sodium pertechnetate (^{99m}Tc) produced according to Zr-Mo gel-technology at centralized generator has been modeled where the main technological steps were considered, such as irradiation of target in reactor, loading into container, transport to the centralized generator, charging of the generator, gel preparation, operation of the centralized generator, and delivery of final product to the hospitals.

In developed mathematical model with two-component presentation of ^{98}Mo activation reaction velocity on neutrons the activity value is presented in the form of two multipliers: the first multiplier describes the dependence on neutron thermal flux density, spectrum hardness, enrichment of ^{98}Mo target, target geometry; the second multiplier sets time dependence. On the basis of this model the different irradiation conditions and target parameters have been analyzed.

The modeling of the technological process of sodium pertechnetate (^{99m}Tc) delivery allows to perform an optimization of the generator operation conditions when ^{99m}Tc producing and delivering it to the hospitals with the least loss of ^{99m}Tc activity depending on eluent volume and elution profile.

The significantly increased needs of the Republic of Belarus in radiopharmpreparation (RPhP) ^{99m}Tc and high costs of supplied commercial generators have led to the necessity of development of the own generator $^{99}\text{Mo} \rightarrow ^{99m}\text{Tc}$ which would retain the ease of operation of extraction generators and at the same time utilize ^{99}Mo of low and medium specific activity. Such approach allows to use cheap initial raw material in the form of non-enriched MoO_3 and medium neutron flux reactors, $(3-5) \cdot 10^{13}$ neutron $\text{cm}^{-2} \cdot \text{s}^{-1}$ that are available in the Republics of the former USSR. The concept of centralized Zr-Mo gel-generator with activity of (200-370) GBq as to ^{99}Mo is assumed as a basis.

The methodology and technological process of the wasteless reactor gel-technology of RPhP ^{99m}Tc production on the basis of centralized Zr-Mo gel-generator has been developed in the Institute of Power Engineering Problems/ National Academy of Sciences of Belarus in co-operation with Scientific Research Institute of Oncology and Medical Radiology of Ministry of Health of the Republic of Belarus. As a result of performed clinical tests the Ministry of Health had approved the clinical application of RPhP ^{99m}Tc produced according to this technology.

The distinctive advantages of the technology developed are:

- using of native molybdenum as a start target (MoO_3);
- absence of deleterious and toxic impurities in the final product (nitrates, organics, etc.);
- the application of modified method of ^{99m}Tc elution from ^{99}Mo with the help of Zr-Mo-gel that allows to shorten the number of process stages and simplify the technology. It is very important at its remote realization;
- the unit is readily automated and remote-controlled;
- simplicity of design and compactness offer a wide application of the unit.

The originality of gel-technology of ^{99m}Tc production and centralized generator design is protected by patents of the Republic of Belarus and of the Russian Federation [1. 2]. The centralized generator design is presented in Fig. 1.

The generator initial activity depends on the target activity. The justification of optimal conditions of initial MoO_3 target irradiation in nuclear reactor core is very important for determination of produced ^{99m}Tc price. It can be stated that the flux of more than 10^{14} neutron $\text{cm}^{-2} \cdot \text{s}^{-1}$ solves the problem of obtaining of required activity as to ^{99}Mo . But not all the reactors have the experimental channels with fluxes more than 10^{14} neutron

$\text{cm}^{-2} \text{s}^{-1}$ and the cost of irradiation in them is several times higher than the irradiation in the experimental channels with fluxes $8 \cdot 10^{12} - 5 \cdot 10^{13} \text{ neutron}/(\text{cm}^2 \cdot \text{s})$. The proportionally more irradiation time is required for obtaining of higher activity at neutron fluxes $5 \cdot 10^{12} - 5 \cdot 10^{13} \text{ neutron cm}^{-2} \text{s}^{-1}$ that increases the irradiation cost. The mathematical model with two-component presentation of activation reaction velocity on neutrons for the MoO_3 target has been developed that allows to analyze the influence of different irradiation conditions and target properties on specific activity value as to ^{99}Mo [3].

In two-component presentation the activation reaction velocity according to [4] looks as follows:

$$\hat{\sigma} \Phi = (\zeta \sigma + q \gamma I) \Phi, \quad (1)$$

where σ is thermal section and I is a resonance integral of ^{98}Mo ; $\hat{\sigma}$ is the cross-section of reaction $^{98}\text{Mo} (n, \gamma) ^{99}\text{Mo}$; $\gamma = \omega / \Phi$ is spectrum hardness; Φ is thermal neutron flux density; ω is density of resonance neutron flux; ζ, q - are the coefficients of thermal and resonance blocking.

The dependence of ^{99}Mo specific activity on neutron flux density Φ , and irradiation time in the reactor is shown in Table I.

The activity of ^{99}Mo considerably depends on neutron spectrum hardness in the reactor - γ . In thermal reactors spectrum hardness varies within $0,1 \leq \gamma \leq 0,4$. As higher the value of spectrum hardness γ as higher the resulting activity value. This dependence is presented in Table II.

The time of irradiation is defined by neutron flux and required specific activity, but the irradiation cost should be taken into account, also. The activity value can be presented in the form of two multipliers: the first multiplier describes the proportional dependence of physical characteristics of the irradiation process (neutron flux density, spectrum hardness, enrichment, etc.) but does not depend on time; the second multiplier sets only time dependence and is the exponential approximation to asymptotic (maximum) value. This dependence is presented in Table III. It is evident that 96% of ^{99}Mo maximum activity is attained at irradiation for 300 hours.

The irradiation cost is in direct proportion to irradiation time. Let the irradiation time of 70 hr which results in 52% of ^{99}Mo maximum activity to be a unity value then

Table I. ^{99}Mo specific activity (GBq/g) depending on neutron flux density and irradiation time in reactor

Time, hr	Φ , neutron/(cm ² ·s)			
	$5 \cdot 10^{12}$	10^{13}	$5 \cdot 10^{13}$	10^{14}
70	3.3	6.7	32.9	65.9
100	4.1	8.1	41.1	81.8
200	5.6	11.1	55.1	110.6
300	5.9	12.2	60.7	121.0
400	6.3	12.6	62.2	124.3

Table II. Dependence of ^{99}Mo specific activity (GBq/g) on spectrum hardness and irradiation time for unblocking target

Irradiation time, hr	Spectrum hardness			
	0,1	0,2	0,3	0,4
50	14.4	25.9	37.0	48.5
70	18.1	32.9	47.4	62.2
100	22.6	41.1	37.0	77.3
200	30.7	55.1	79.9	104.3

Table III. Ratio of ^{99}Mo activity to ^{99}Mo maximum activity depending on irradiation time

Irradiation time, hr	$A_{^{99}\text{Mo}}(t) / (A_{^{99}\text{Mo}})_{\max}$
50	0,4088
70	0,5209
100	0,6505
200	0,8778
300	0,9573
400	0,9851

irradiation during 300 hours will result in 96% of ^{99}Mo maximum activity and will cost 4,3 times more. Therefore, in the first case 52% of ^{99}Mo maximum activity is obtained whereas in the second case only 22% of target maximum activity is obtained for the same cost.

It is evident, that the final selection of irradiation time is determined by the number of final product ($^{99\text{m}}\text{Tc}$) users and the conditions of centralized gel-generator operation that determine the RPhP radiation characteristics.

When eluting from generator specific activity of $^{99\text{m}}\text{Tc}$ depends on elution time and elution effectiveness which is defined as ratio between eluted activity of $^{99\text{m}}\text{Tc}$ and activity of ^{98}Mo .

The elution effectiveness depends on many parameters of gel preparation, such as acidity, process temperature, time of gel maturation, etc. It depends on thorough carrying out of different technological stages of the process of Zr-Mo gel production as well. Moreover it depends on eluent volume. With increasing of the eluent volume the elution effectiveness increases, but the specific activity of eluate decreases.

It was experimentally determined that the pH range 6,3÷6,8 is the optimum range for pH of gel [1, 2]. The experience of the generator operation have shown that while long operation the washing of H^+ ions from gel took place which leads to the increase of pH and, as a result, to the gel instability, to the increase of $^{99\text{m}}\text{Tc}$ yield, and to Mo coming to the preparation simultaneously. Therefore, to keep an optimum pH value is one of the technological problems. This was achieved by the conditioning of gel with acidic solution, water and saline solution in every 3-4 days of generator operation.

Elution profile is the other parameter effecting the $^{99\text{m}}\text{Tc}$ yield value. The dependence $^{99\text{m}}\text{Tc}$ activity on eluate volume is called the elution profile (or elution curve) of the generator. The elution profile is one of the parameters affecting the $^{99\text{m}}\text{Tc}$ yield and it is defined experimentally. The elution profile depending on the elution solution volume for Zr-Mo gel-generator taken from [5] is presented in Table IV.

It was experimentally shown that the total generator effectiveness as a function of eluent volume increases with the increase of eluent volume. But the eluate specific volumetric activity decreases with the increase of eluent volume. The using of experimental elution curve allowed to model the generator operation depending on eluent volume.

The $^{99\text{m}}\text{Tc}$ specific volumetric activities for two eluent volumes - 50 ml (average yield is equal to 0,73) and 100 ml (average yield is equal to 0,96) are presented in Table V.

Table IV. Elution profile for Zr-Mo gel-generator

Eluate volume, ml	20	40	60	80	100	120
^{99m}Tc yield, %	30	51	73	84	91	96

Table V. Dependence of ^{99m}Tc specific volumetric activity (GBq/ml) on elution solution volume (target initial activity 110 GBq)

Number of elution	Eluate volume, ml	
	50	100
1	1.03	0.68
2	0.84	0.54
3	0.67	0.43
4	0.53	0.34
5	0.42	0.26
6	0.33	0.21
7	0.26	0.17
8	0.21	0.13
9	0.17	0.11
10	0.13	0.08
11	0.10	-
12	0.08	-

In both cases the target activity as to ^{99}Mo is 220 GBq and the generator initial activity after its loading and gel preparation is 110 GBq.

The number of elutions is determined by minimum value of ^{99m}Tc specific activity that is equal to 74 MBq/ml according to [5]. It follows from above that gel elution with 50 ml of eluate is considered to be the optimum condition in spite of the fact that 100 ml of eluate elute the generator practically completely and give the higher activity, but sodium pertechnetate specific activity is lower by a factor of 1.5. Different elution conditions with different elution volumes allow to obtain different ^{99m}Tc activity at the same activity of ^{99}Mo . Therefore, the generator operation conditions will be determined by requirements of the hospitals to which pertechnetate will be delivered.

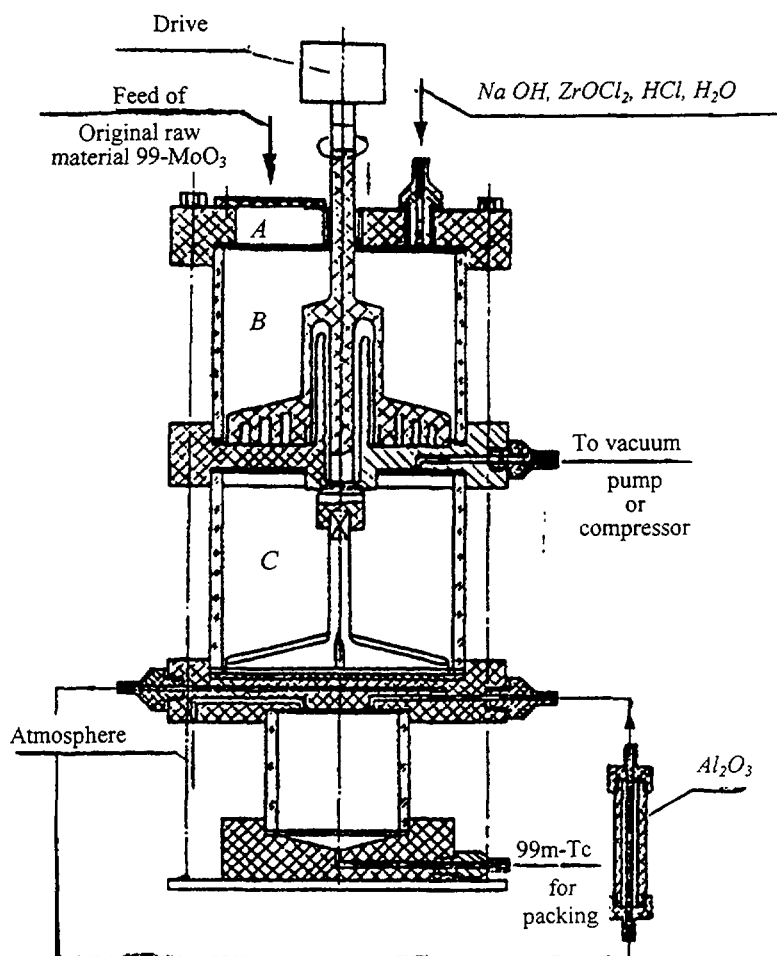


Fig. 1. Centralized generator of 99mTc .

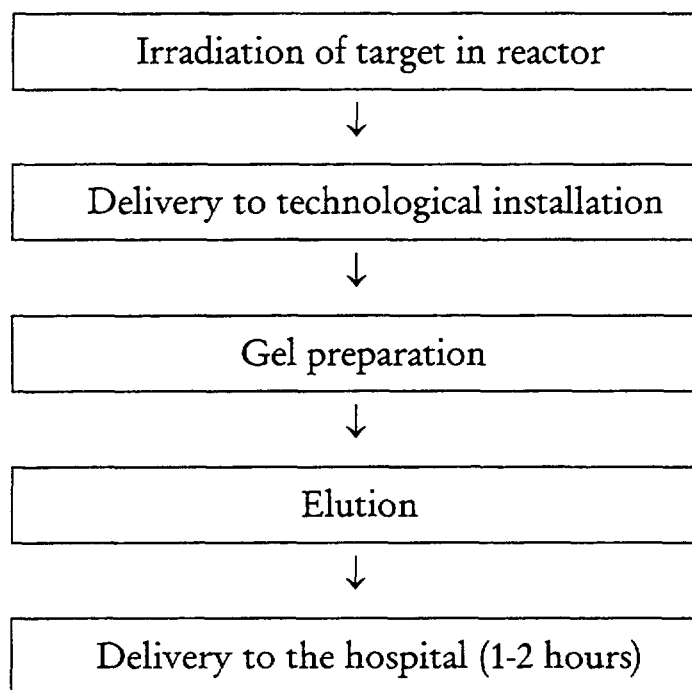


Fig. 2. Scheme of technological steps sequence for 99mTc production.

^{99m}Tc solutions always contain nuclide ^{99}Tc . It's quantity changes significantly depending on generator radiation characteristics. The experimental investigations showed that after preparation of gel the ratio $^{99}\text{Tc}/^{99m}\text{Tc}$ in generator system was high enough (180). After the first elution this ratio reduced till 50. That is why during the development of routine technique the decision was made that the first elution to be considered as waste and it should not be given to hospitals. For further elutions this ratio drops to 10.

The investigations performed have allowed to justify the technological regime of sodium pertechnetate ^{99m}Tc production on the basis of Zr-Mo gel with MoO_3 start target. An operation according to this regime leads to the characteristics of radiopharmpreparation of sodium pertechnetate solution ^{99m}Tc , produced on the basis of Zr-Mo gel, to be in agreement with the requirements of Pharmacopoeia Standard.

For the defining of the most favorable conditions for ^{99m}Tc production and its delivery to the hospitals with the least loss of ^{99m}Tc activity it was necessary to model the technological process of sodium pertechnetate (^{99m}Tc) delivery where the main technological process stages were included, such as irradiation of target in reactor, reloading into container, transportation to the centralized generator, charging of generator, gel preparation, centralized generator operation (obtaining of final product, sodium pertechnetate, by elution with saline solution) and delivery of final product to the hospitals.

The scheme of technological works sequence is presented in Fig. 2.

Table VI. Ratio of ^{99m}Tc activity to maximum activity depending on time between elutions

Time, h	$^{99m}\text{Tc} / ^{99m}\text{Tc}_{\text{max}}$
5	0,500
10	0,817
15	0,947
20	0,994
23	1,0

Table VII. ^{99}Mo activity and $^{99\text{m}}\text{Tc}$ specific volumetric activity depending on effectiveness and elution time during the operation of one generator for eluent volume of 50 ml

Number of elution of $^{99\text{m}}\text{Tc}$	Yield of $A_{^{99}\text{Mo}}$ of $A_{^{99}\text{Mo}}$, %	$A_{^{99}\text{Mo}}$ at the moment of $^{99\text{m}}\text{Tc}$ elution, GBq	$A_{^{99}\text{Mo}}$ in final preparation, MBq/ml time interval between elutions	
			t = 6 h	t = 23 h
0		102.7		
1	74	81.5	653	856.1
2	73	64.7	580	664.0
3	63	51.3	378.2	462.3
4	75	40.7	291.6	427.5
5	78	32.3	239.8	352.1
6	79	25.7	191.9	283.4
7	80	20.4	152.6	228.0
8	86	16.2	120.9	192.2
9	88	12.8	96.7	156.3
10	88	10.2	76.9	124.1

The increase of $^{99\text{m}}\text{Tc}$ activity in generator system $^{99}\text{Mo} - ^{99\text{m}}\text{Tc}$ with time, t , may be expressed through the ^{99}Mo initial activity (^{99}Mo activity after unloading from the reactor):

$$A_{^{99\text{m}}\text{Tc}}(t) = A_{^{99}\text{Mo}}(T_1) \gamma_1 \frac{\lambda_{^{99\text{m}}\text{Tc}}}{\lambda_{^{99\text{m}}\text{Tc}} - \lambda_{^{99}\text{Mo}}} (e^{-\lambda_{^{99}\text{Mo}} t} - e^{-\lambda_{^{99\text{m}}\text{Tc}} t}), \quad (2)$$

where $A(T_1)$ is the ^{99}Mo activity after unloading from the reactor, γ_1 is the $^{99\text{m}}\text{Tc}$ yield at ^{99}Mo decay.

If the $^{99\text{m}}\text{Tc}$ quantity produced at the first elution is taken as a unit, then at every next elution the following $^{99\text{m}}\text{Tc}$ activity may be obtained:

$$A_{^{99\text{m}}\text{Tc}}(n) = \left(\frac{\lambda_{^{99}\text{Mo}}}{\lambda_{^{99\text{m}}\text{Tc}}} \right)^{\frac{\lambda_{^{99}\text{Mo}}(n-1)}{\lambda_{^{99\text{m}}\text{Tc}} - \lambda_{^{99}\text{Mo}}}}, \quad (3)$$

where n is a number of elution.

The limited activity of ^{99m}Tc which can be obtained for the infinite quantity of elutions is equal to:

$$\Sigma_{\infty} = \frac{1}{1 - \left(\frac{\lambda_{^{99}\text{Mo}}}{\lambda_{^{99m}\text{Tc}}} \right)^{\lambda_{^{99m}\text{Tc}} - \lambda_{^{99}\text{Mo}}}} \approx 4.69, \quad (4)$$

In Table VI the ratio of ^{99m}Tc activity to maximum activity depending on time between elutions is presented. These data show that elution may be carried out for some times per day not waiting for maximum accumulation of ^{99m}Tc (in ≈ 23 hours) because 50% of maximum activity is attained in 6 hours.

In Table VII the calculation results of ^{99}Mo activity and eluate specific activity with using of experimentally determined ^{99m}Tc technological yield during generator operation for two accumulation times, $t = 6$ hours and $t = 23$ hours, are presented.

Total activity of ^{99m}Tc is 2,78 GBq for time interval between elutions 6 hours. and 3,745 GBq - for time interval 23 hours.

Produced sodium pertechnetate solution after filtration was packed and sent for sterilization. The preparation was sterilized for 20 min. After sterilization the preparation was ready for application. The activity change of ^{99m}Tc while transport of the preparation to hospitals is determined by radioactive decay. For example, if the transport takes 2 hours then about 20% of sodium pertechnetate initial activity is lost.

Thus this mathematical model allows to determine concentration and activity of ^{99}Mo and ^{99m}Tc at any time moment of technological process of RPhP ^{99m}Tc production according to available physical parameters of irradiation. With the help of this model it is possible to perform the optimization of conditions of ^{99m}Tc production when irradiating the target in the reactor.

Moreover, modeling of the technological process of delivery of sodium pertechnetate (^{99m}Tc) produced by Zr-Mo gel-technology at centralized generator where all the main process stages are included (irradiation of target in reactor, reloading into container, transportation to the centralized generator, charging of generator, gel preparation, centralized generator operation and delivery of final product to the hospitals) allows to determine the most favourable conditions of ^{99m}Tc production and delivery to the hospitals with the minimum activity loss.

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**EVALUATION OF PREPARATION AND PERFORMANCE OF
GEL COLUMN $^{99}\text{Tc}^{\text{m}}$ GENERATORS BASED ON
ZIRCONIUM MOLYBDATE — ^{99}Mo**



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Abstract

In view of the importance and relevance of zirconium molybdate (ZrMo) based gel generators for $^{99}\text{Tc}^{\text{m}}$, developed first by Australian scientists, particularly for developing nations having production capability for neutron activated ^{99}Mo , work has been carried out in our centre towards optimisation of the preparatory conditions. Appropriate facilities have been set up for safe, reliable and regular manufacture of such $^{99}\text{Tc}^{\text{m}}$ gel generators on a small scale based on our earlier successful attempts on the approach for process standardisation. The results of our extensive evaluation, including for clinical use, are reported here. Following the regular procedure standardised by us earlier, 9 lots of ^{99}Mo were converted into ZrMo gel adopting aseptic practices and two different techniques for drying the ZrMo cake - heated air at $60\text{--}80^\circ\text{C}$ and microwave drying at 385 watts. The ZrMo granules obtained after further processing were loaded onto generator assemblies. 10 g molybdenum was handled in each batch of gel conversion, while typically 2 g Mo was used per generator column, except in the case of demonstration of higher capacity generators. The generator performance was evaluated systematically over nearly 15 days, in terms of rapidity and smoothness of elution, $^{99}\text{Tc}^{\text{m}}$ elution yield and quality of eluted pertechnetate. The consistent results of over 60% (Max. 90%) yield of $^{99}\text{Tc}^{\text{m}}$, $<10^{-3}\%$ ^{99}Mo breakthrough, $>98\%$ radiochemical purity of pertechnetate, <10 ppm each of Mo , Zr and Al in the eluates, compatibility for formulating even sensitive $^{99}\text{Tc}^{\text{m}}$ compounds etc. indicated satisfactory quality of pertechnetate, comparable to that obtained by conventional generator systems. 12 such generators, containing upto 18.5 GBq ^{99}Mo , supplied for clinical use and evaluated at two hospital radiopharmacies showed satisfactory generator performance. The applicability for preparing larger capacity generators for centralised radiopharmacies and feasibility to achieve reduced process time and ease of control by using microwave oven drying have been established.

1.0 Introduction

A novel concept $^{99}\text{Tc}^{\text{m}}$ generator based on gel column matrix of zirconium molybdate - ^{99}Mo (ZrMo , Zr^{99}Mo) was reported by Australian scientists for retaining the advantages of ease and reliability of column chromatography generators, and at the same time utilising neutron activated ^{99}Mo of medium specific activity [1-3]. While the process in principle might appear easy to carry out, it has been found to be rather difficult to ensure reproducible results, apart from a need for special facilities for carrying out complex cumbersome radiochemical process steps [4,5]. The actual optimisation of the preparatory conditions and facilities, for safe, reliable and regular manufacture of such $^{99}\text{Tc}^{\text{m}}$ gel generators poses several challenges. However, as $^{99}\text{Tc}^{\text{m}}$ continues to reign supreme as the most preferred diagnostic tracer, simple, inexpensive, user-friendly delivery systems for $^{99}\text{Tc}^{\text{m}}$ are essential [6]. In view of the importance and relevance of the Zr^{99}Mo gel concept approach in this regard, particularly for developing nations having production capability for neutron activated ^{99}Mo , considerable volume of work has been

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carried out in our Centre, both independently and as a part of an IAEA's Coordinated Research Project (CRP) [7-12]. Our investigations led to standardisation of basically two methods of drying the $Zr^{99}Mo$ cake, which is the crucial step in the gel generator preparation; one involving the use of heated air at 60-80°C [7-9] and the other based on microwave heating at 385 watts [11,12]. The successful production of $Zr^{99}Mo$ suitable for column operation and elution of $^{99}Tc^m$ from these attempts enabled preparation and testing of $^{99}Tc^m$ gel generators. This paper describes the preparation of a series of such generators and the evaluation of their performance, including in hospital radiopharmacies.

2.0 Materials and Methods

All the chemicals used were from commercial sources and mostly of GR / AR grade. The type of glass columns used has been previously described [8,9]. Acidic alumina used in purification cartridge was the same as that used in BRIT's column chromatography generators for $^{99}Tc^m$. Kits for $^{99}Tc^m$ compounds were available ex-stock in-house. ^{99}Mo was obtained as sodium molybdate in NaOH solution, 150mg Mo / ml and radioactive concentration 30-60 mCi ^{99}Mo / ml from the Radiochemicals Programme Unit, BRIT. In order to maintain the ^{99}Mo activity to the desired level, spiking with fission molybdenum was resorted to when the neutron activated ^{99}Mo was of inadequate activity. Fission product ^{99}Mo (fission moly) suitable for preparing $^{99}Tc^m$ for medical use was obtained from National Technology Products, A.E.C., South Africa, 2-3 Ci / ml in 0.2N NaOH. In a few instances, carrier molybdenum solution in NaOH, 150mg Mo / ml was prepared and directly mixed with the required quantity of fission molybdenum.

An ionisation chamber (NPL, UK) pre-calibrated with ^{137}Cs was used for the assay of radioactivity. The activity of ^{99}Mo in the eluates was assayed in a NaI(Tl) counter, set to count under the 740 keV photopeak of ^{99}Mo and in comparison to a carefully prepared and standardised reference source of ^{99}Mo [6]. Colorimetric tests for Zr, Al, Mo were carried out as limit tests [13,14].

2.1 Zirconium molybdate - ^{99}Mo ($ZrMo$, $Zr^{99}Mo$)

The general procedure for preparation of $Zr^{99}Mo$ involved careful mixing of equimole quantities of sodium molybdate - ^{99}Mo solution (10g Mo) and zirconyl chloride solution at 60°C, digestion of the precipitate, rapid filtration, drying under controlled conditions by two different methods, fragmentation of dried cake, re-drying and dispensing the free flowing of $Zr^{99}Mo$ granules into glass columns and has been described in detail in another paper [9].

The first method of drying involved passing heated compressed air (60-80°C) over the precipitate of $Zr^{99}Mo$ for 16-18 hours. In the second method of drying, $Zr^{99}Mo$ precipitate was transferred to a petri dish, placed in a microwave oven (Kitchen Model BMC - 900T of BPL, India) and dried at 385 watts (2.45GHz) for 35 minutes [11,12]. The promising results reported by us earlier [11] using the micro wave drying technique to reduce the processing time provided the basis for this evaluation study. 10 g Mo on conversion to $Zr^{99}Mo$ resulted in 30 g of granules; 2g Mo (i.e. 6g gel granules) was filled per gel column. Till this stage, all the processing was carried out in 50mm thick lead shielded plants provided with appropriate gadgets like remote handling tongs, glove ports, trolley etc. (Fig.1).

2.2 $^{99}Tc^m$ gel generator

The gel column containing granular $ZrMo$ powder was crimped with aluminium cap and assembled in proto type generator assemblies designed and fabricated in-house (Fig.2). All generators were subjected to washings with 50ml normal saline using a vacuum line. The washing lines were then disconnected and a 6mm lead shield (with a stainless steel (SS) sheath) holding a 2g acidic alumina purification cartridge (replaceable, if required) was connected and placed in position (Fig.3). In-process QC checks were carried out on each

generator after allowing 2 hours in-growth of $^{99}\text{Tc}^m$. Reasonable estimates of the generator activity were available from this elution data on the basis of which the generators were released to users or retained for extensive evaluation as described below.

In order to assess the suitability of this approach for centralised radiopharmacy requirements, a “jumbo” gel generator (containing about 24g of gel and 615mCi of Zr^{99}Mo gel) was prepared and evaluated for comparison with a “standard” gel generator (6g Zr^{99}Mo gel) prepared from the same batch of Zr^{99}Mo gel. For the “jumbo” generator, a larger glass column designed and rendered compatible for use with the lead shielding assembly was employed.

2.3 Evaluation of performance of $^{99}\text{Tc}^m$ gel generator

The generator performance in terms of rapidity and smoothness of elution, pH and clarity of eluate, $^{99}\text{Tc}^m$ elution yield and ^{99}Mo breakthrough (by 6mm thick lead screen test [6]) was ascertained. The quality of pertechnetate eluate was evaluated as per pharmacopoeial specifications. The pharmaceutical purity aspects of sterility and apyrogenicity were checked by carrying out conventional sterility and apyrogenicity tests on decayed eluates. Technetium compounds were formulated using pre-tested ready to use standard kits and evaluated. From the ten lots prepared, one generator per batch was retained for detailed evaluation. This generator was eluted daily and eluates checked for the above parameters systematically over nearly 15 days and a few times thereafter. The generator was carefully dismantled (usually one week after preparation) to assay the radioactivity on the gel column, referred to as representative column and used for back calculations for determining the percentage of $^{99}\text{Tc}^m$ yield and of gel process recovery.

With the concurrence of national authority, 12 generators prepared under optimised conditions using hot air drying method and 2 generators using microwave dried Zr^{99}Mo were supplied for clinical use and evaluated at two hospital radiopharmacies (HRPh), Nair Hospital, Mumbai and Radiation Medicine Centre (RMC), BARC, Mumbai. The generators contained upto 18.5 GBq (500 mCi) ^{99}Mo , either (n, γ) ^{99}Mo alone or after spiking with fission moly.

3.0 Results and Discussion

3.1 $^{99}\text{Tc}^m$ gel generator processing

Table-I presents the relevant data of batch production of the generators. All operations were executed smoothly in the facilities set up. The pharmaceutical purity aspects of sterility and apyrogenicity were ensured by adapting aseptic practices throughout the processing stages according to a detailed protocol. The gel yield, both in terms of gel mass and radioactivity of ^{99}Mo , showed reasonable consistency from batch to batch. Typically, 80% utilisation of ^{99}Mo activity processed was easily feasible. In all cases, the first few millilitres of

normal saline washings showed slight opalescence probably due to fines, but the remaining washings and all eluates were clear. The elutions performed after 2 hours growth, as the in-process QC tests for smooth operation of the generator, elution yield, breakthrough of ^{99}Mo and pH of eluates, provided satisfactory index for product release for further use. In all, about 24 hours were required for the completion of entire batch operation while using hot air drying method. In other words, the calibration time for these gel generators could be set earliest at 48 hours from the commencement of the process.

Table-I also indicates the results of processing of generators using microwave drying technique for Zr^{99}Mo cake. It is evident that the total duration of processing has been significantly reduced in this case to about 7 hours, in turn, enabling the calibration to be a day earlier than when using hot air drying method.

Table - I : PROCESS DATA FOR $^{99}\text{Tc}^m$ GEL GENERATORS

Lot No.	^{99}Mo activity at the beginning of the process (mCi) & its source	Number of generators made \$	Activity of the representative generator column (mCi)	$^{99}\text{Tc}^m$ elution after 2 h growth Average $^{99}\text{Tc}^m$ eluted (mCi)	Average ^{99}Mo breakthrough (%)
A	Hot air dried gel (16-18 hours drying time for 30 g gel mass)				
1	^a 1710	4	196	24	< 0.004
2	^c 1793	4	232*	28	0.0014
3	^b 2619	4	468*	63	0.00021
4	^b 2555	4	219	36	0.00003
5	^b 3769	5	484	74	0.00003
6	^b 2286	4	385*	52	< 0.0001
B	Microwave dried gel (12-17 minutes drying time for 6-12 g gel mass)				
7	^b 2598	3	249	48 @	0.0015
8	^c 1059	3	164	58 #	0.00004

a. (n, γ) ^{99}Mo + fission product ^{99}Mo b. Fission product ^{99}Mo + carrier Moc. (n, γ) ^{99}Mo alone

* About 30% additional filling of the column

@ 4 hours growth

18 hours growth

\$ Total gel mass was not used in most instances

Table - II : PERFORMANCE EVALUATION OF ⁹⁹Tc^m GEL GENERATORS

Lot No.	⁹⁹ Mo activity at ref. time	No. of elutions	⁹⁹ Tc ^m eluted (mCi)		⁹⁹ Tc ^m yield (%)		⁹⁹ Mo breakthrough (%)		Radiochemical Purity (%)
			Max.	Min	Min.	Max.	Min.	Max.	
1	165	9	80	13	55	67	0.0001	0.0008	99
2	162	8	92	14	56	66	0.0001	0.0007	99
3	372	8	200	28	58	73	<0.0001	0.0004	99.3
4	197	9	104	13	59	69	0.0001	0.0008	99.1
5	467	10	260	42	66	86	<0.0001	0.0002	98.9
6	322	9	188	36	63	73	<0.0001	0.0003	99.5
7	194	6	72	25	54	79	0.0001	0.003	99.7
8	120	4	58	23	64	85	<0.0001	0.0009	99.9

Note:

All elutions were smooth and rapid.

pH of all eluates was 5-6

Radiochemical purity by PC with 85% methanol.

Mo, Zr and Al content : < 10 ppm each (by spot test)

Table - III : EVALUATION OF $^{99}\text{Tc}^m$ GEL GENERATOR IN TWO HOSPITAL RADIOPHARMACIES

Generator lot No./ ^{99}Mo act. at ref. time (mCi)	No. of elutions		Duration of use (days)		$^{99}\text{Tc}^m$ eluted (mCi)		No. of $^{99}\text{Tc}^m$ formulations @		No. of patients	
	A	B	A	B	A Max-Min	B Max-Min	A	B	A	B
1 / 196	4	6	14	8	97 - 3.6	66 - 10	1	—	3	—
2 / 232	3	8	5	9	125 - 52	100 - 14	5	1	18	2
3 / 468	5	7	10	9	201 - 30	181-20	10	3	46	17
4 / 219	3	2	12	2	160 - 10	121- 72	6	2	17	8
5 / 484	6	9	15	10	269 - 11	280 - 12	6	4	35	30
6 / 385	4	7	11	9	197 - 9	217- 10	8	4	35	20

A: Generator evaluated in Radiation Medicine Centre (RMC), BARC, Mumbai

B: Generator evaluated in Nair Hospital, Mumbai

@ Formulations used : Sodium pertechnetate, Tc- Mebrofenin, Tc- Tetrafosmin, Tc- HSA microspheres, Tc- Sulfur colloid, Tc -DTPA Aerosol, Tc- Phytate, Tc-MDP, Tc-DTPA, Tc-GHA, Tc-RBC, Tc-DMSA.

Table - IV : COMPARATIVE EVALUATION OF PERFORMANCE OF 'JUMBO' GEL GENERATOR AND 'STANDARD' GEL GENERATOR

'JUMBO' GEL (24g) GENERATOR (615 mCi)						'STANDARD' GEL (6g) GENERATOR (158 mCi)				
Day of Elution	Time between elutions (hour)	^{99m} Tc Eluted ^a (mCi)	^{99m} Tc Yield ^a (%)	⁹⁹ Mo Breakthrough (%)	Tc compound, Activity (mCi) and RC purity (%)	Day of Elution	Time between elutions (hour)	^{99m} Tc eluted ^b (mCi)	^{99m} Tc yield ^b (%)	⁹⁹ Mo Breakthrough (%)
1	2	108	96	0.001		1	2	26	90	0.0014
2	20	329	80	0.0003		2	20	88	81	< 0.0002
4 #	48	245	88	0.0002	Tc-MDP; 60 ; 98.7	4	48	60	82	< 0.0003
5	24	179	88	0.0006	Tc-MDP; 97 ; 98.5	5	24	45	85	< 0.0003
6	24	137	86	0.0001	Tc-MDP; 45; 98.5 Tc-Mebrofenin; 6 ; 99	6	24	33	80	< 0.0005
7	24	106	85	0.0003		7	24	28	87	< 0.0005
8	24	86	89	0.0002	Tc- GHA ; 7.5 ; 99 Tc-Mebrofenin; 7.5; 99					
11	72	44	92	0.001		11	96	11	88	< 0.0014
12	24	27	77	0.01		12	24	8	89	0.002
13 #	20	22	87	< 0.0002		13	24	6	86	0.002
14	18	18	94	0.004		14	24	5	87	0.006
15	22	14	95	0.002		15	26	4	88	0.015

alumina bed was replaced.

a : 'JUMBO' gel generator was eluted with 15 ml normal saline.

b : 'STANDARD' gel generator was eluted with 9 ml normal saline.

Table - V : COMPARATIVE EVALUATION OF $^{99}\text{Tc}^m$ GEL GENERATORS PREPARED BY HOT AIR DRYING AND MICROWAVE DRYING TECHNIQUE

Hot air dried Zr^{99}Mo cake; 377mCi generator at ref. time; Process time : 24 hours					Microwave oven dried Zr^{99}Mo cake; 194mCi generator at ref. time; Process time : 7 hours				
Day of elution	Time between elutions (hours)	$^{99}\text{Tc}^m$ eluted (mCi)	$^{99}\text{Tc}^m$ Yield (%)	^{99}Mo breakthrough (%)	Day of elution	Time between elutions (hours)	$^{99}\text{Tc}^m$ eluted (mCi)	$^{99}\text{Tc}^m$ Yield (%)	^{99}Mo breakthrough (%)
1	2	52	76	0.0004	1	4	38	59	0.0002
2	18	152	61	<0.0001	2	20	72	54	0.003
2	4	57	60	0.0001					
5	68	76	73	<0.0001	#5	75	44	62	0.0002
5	4	29	85	0.0003	6	24	36	71	0.0004
8	66	31	74	0.0002	7	24	25	63	0.0006
9	20	26	76	0.0004	8	23	25	79	0.0002

The alumina purification column was replaced.

Note

Eluent volume : 9 ml normal saline (additive free)

Radiochemical purity by PC with 85% methanol : > 98% in all cases.

Mo, Zr and Al content : < 10 ppm each (by spot test)

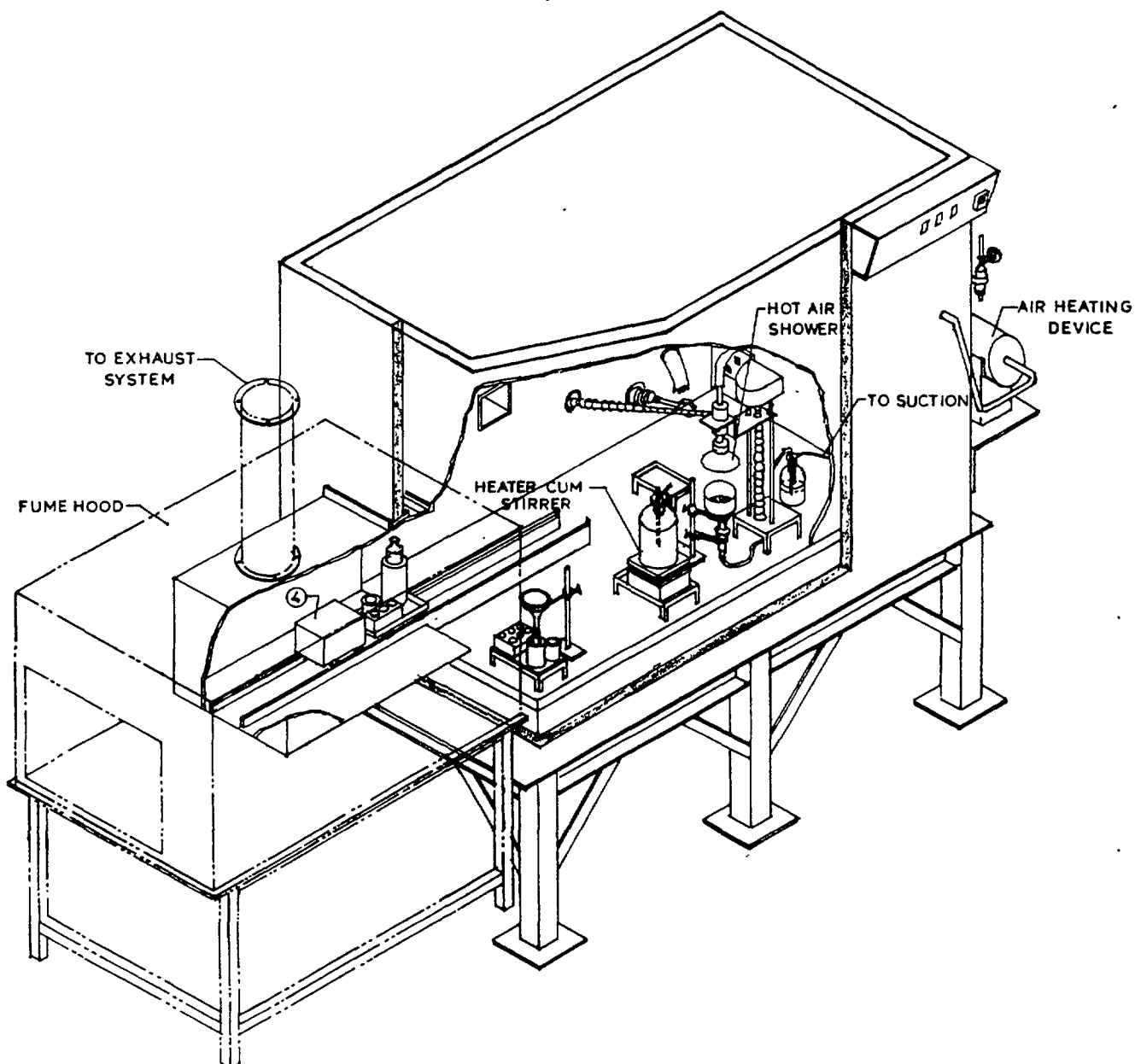


Fig.1 : Isometric view of production plant for $^{99}\text{Tc}^{\text{m}}$ gel generators

3.2 Performance characteristics of $^{99}\text{Tc}^{\text{m}}$ gel generator

Elutions were smooth, rapid and completed in about 2 min. Clear eluates of pH 5-6 were always observed. The consistent results (Table-II) of over 60% (max. 90%) yield of $^{99}\text{Tc}^{\text{m}}$, $<10^{-3}\%$ ^{99}Mo breakthrough, $>98\%$ RC purity of pertechnetate, less than 10 ppm of Mo, Zr and Al in eluates, compatibility for formulating even sensitive $^{99}\text{Tc}^{\text{m}}$ compounds etc. indicated satisfactory quality of pertechnetate in compliance with pharmacopoeial specifications and comparable to that obtained by conventional generator systems. The elution profile studies (Fig.4) showed that $>80\%$ of the available $^{99}\text{Tc}^{\text{m}}$ was eluted in a volume of about 4 ml. The eluates were found to comply with the tests for sterility and apyrogenicity performed post facto.

The findings of generator performance at the hospital radiopharmacy and in clinical studies reported by the two users were similarly satisfactory (Table-III).

Table-IV compares the performance characteristics of the "jumbo" and "standard" gel generators over a period of 2 weeks. It can be seen that the $^{99}\text{Tc}^m$ yields and ^{99}Mo breakthrough are comparable. The elution profile (Fig.4) revealed that the "jumbo" generator could also be eluted in 9ml thereby achieving adequately high radioactive concentration.

Typical life time performance characteristics of two gel generators, one each prepared by hot air and micro wave oven drying technique for ZrMo cake, are shown in Table-V. No significant differences in the $^{99}\text{Tc}^m$ elution yields or in ^{99}Mo breakthrough were observed. Further experiments with the microwave oven drying technique have indicated that a batch size of 20g molybdenum could be easily processed in about 8 hours, thereby enabling production of 8 - 10 generators per batch in the facility set up.

3.3 Prospects of $^{99}\text{Tc}^m$ gel generator system

Assuming an initial specific activity of 500 mCi / g Mo, a gel column of 2g Mo and a calibration time of 2d from start of operations, generators of 500 mCi are feasible. Even on a conservative level, i.e. availability of ^{99}Mo with a specific activity of only 200 mCi / g Mo, clinically useful generator capacities could be achieved. A recent article has also argued the merits of the gel generator viability [15]. Our work also shows that in the case of availability of ^{99}Mo of high specific activity or fission moly (the latter for spiking), one could achieve higher

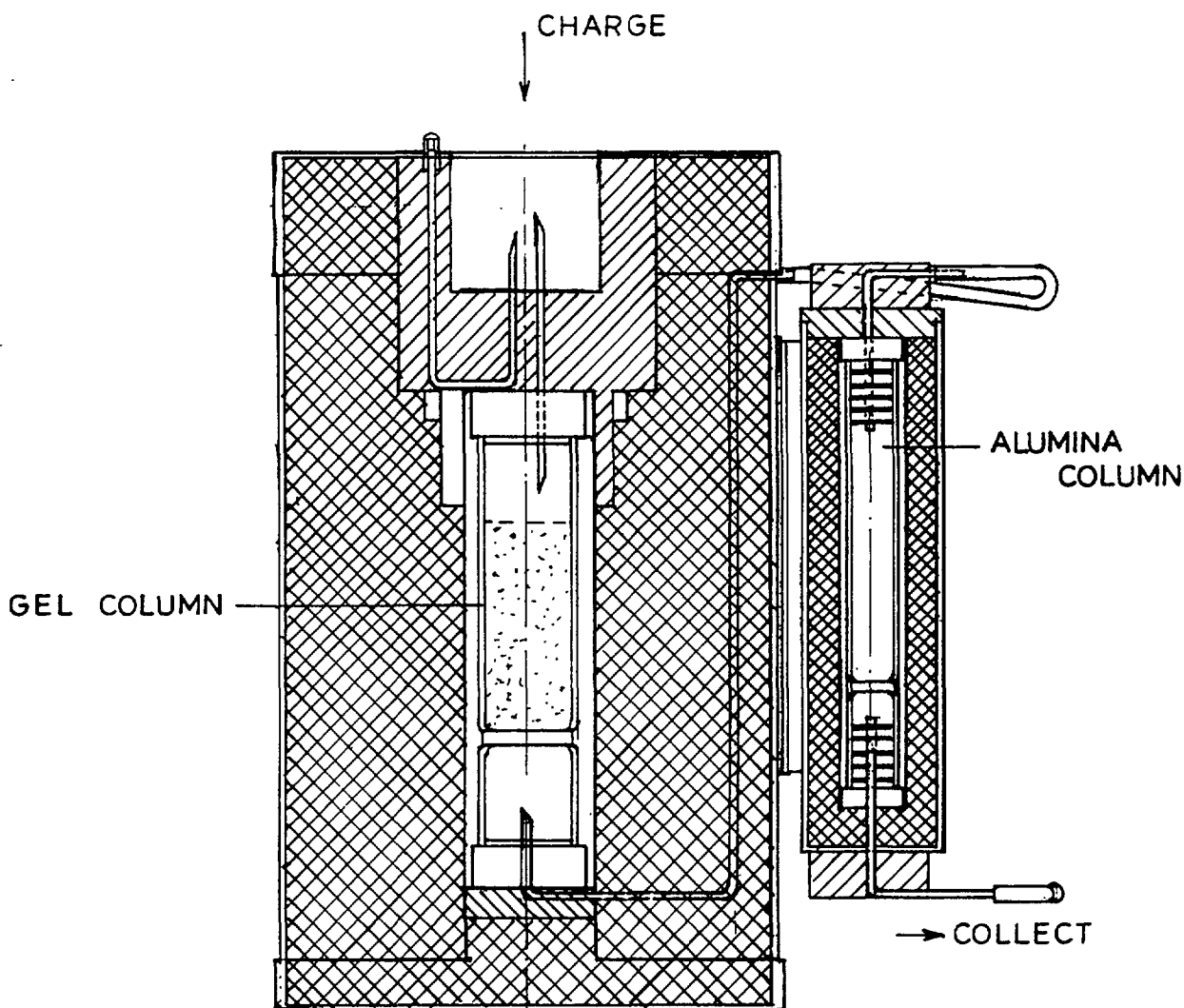


Fig.2 : Cross-section view of $^{99}\text{Tc}^m$ gel generator assembly

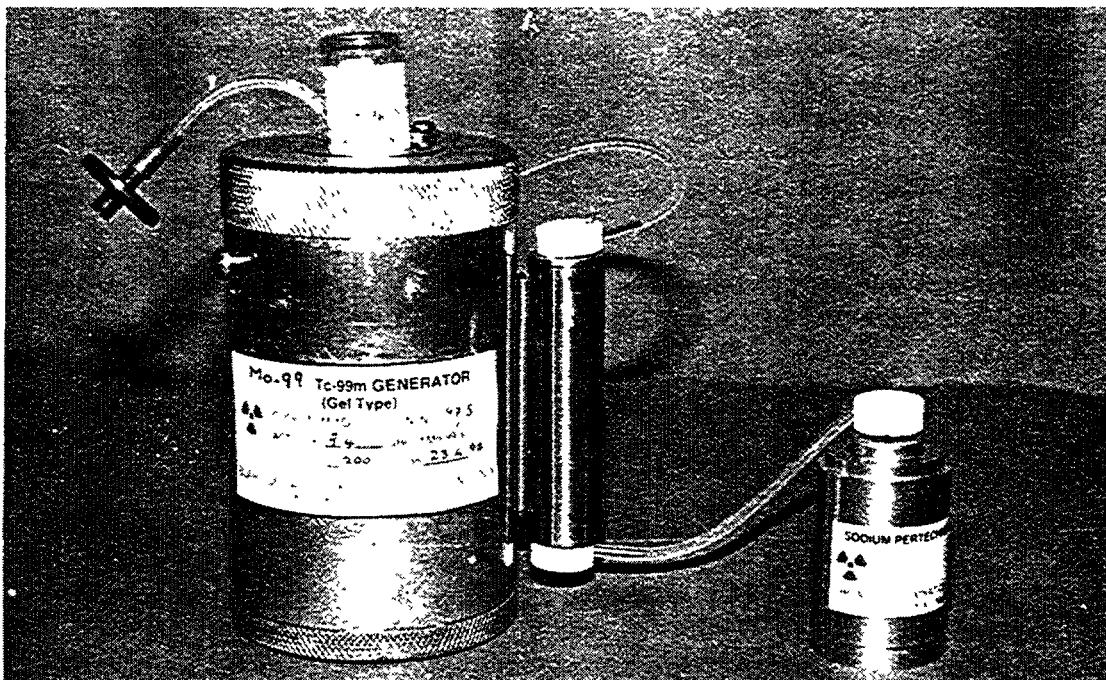


Fig.3 : BRIT's $^{99}\text{Tc}^{\text{m}}$ Gel Generator

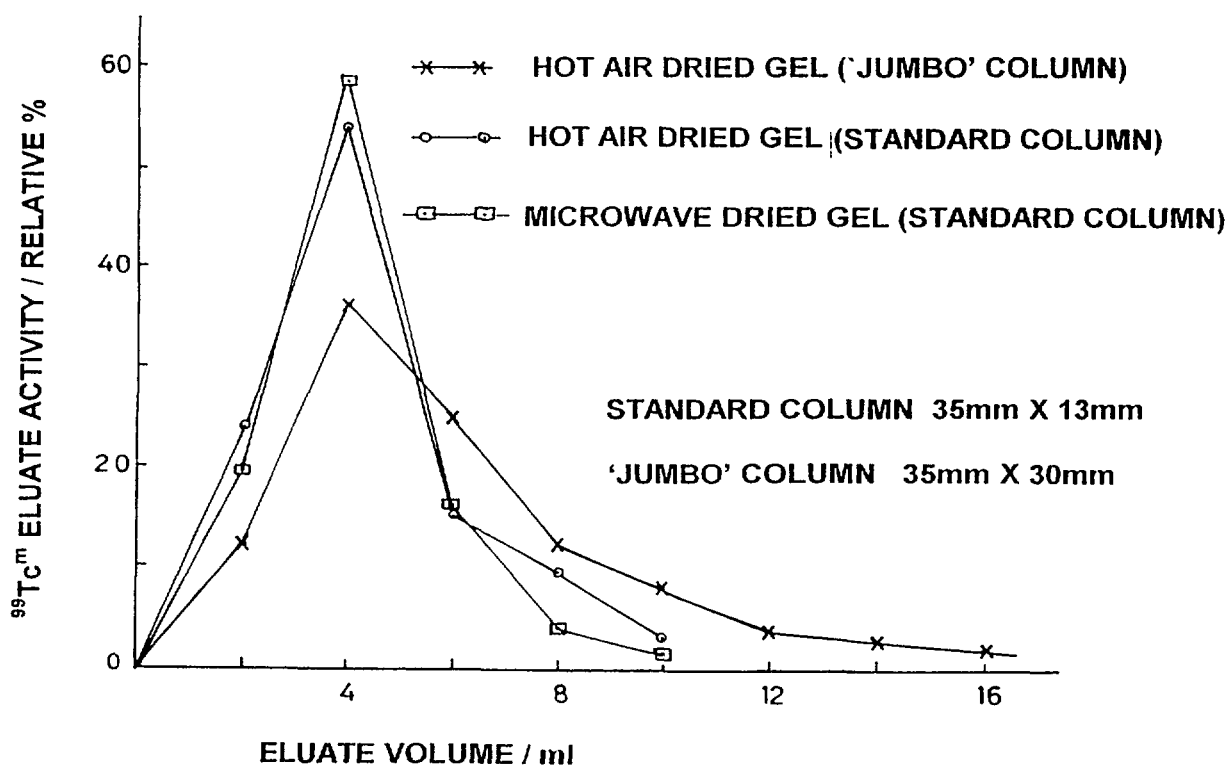


Fig.4 : Elution profile of $^{99}\text{Tc}^{\text{m}}$ from gel generators

activity generators as well as larger scale of production without any increase in the quantities of chemicals to be handled, by filling lesser quantity of ZrMo granules (1-2g) per column. Applicability for preparing larger capacity generators of the "jumbo" type (~25g ZrMo) suitable for centralised radiopharmacy has also been established (Table-IV).

Promising attractive additional features of ease and convenience of manufacture of gel generators, when using microwave drying method, have also been demonstrated; ~7 hours total process time as against ~24 hours for heated air method. We have thus established reliable methods for manufacture on small scale of user-friendly gel column $^{99}\text{Tc}^{\text{m}}$ generators suitable for clinical use and adaptable even by countries with only modest facilities.

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I-124 AND ITS APPLICATIONS IN NUCLEAR MEDICINE AND BIOLOGY

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Abstract

^{124}I decays simultaneously by positron emission (25.6 %) and by electron capture (74.4 %). This dualistic decay allows in principle to use ^{124}I in both diagnostic and therapeutic approaches. In some high-current measurements, ^{124}I was produced via the nuclear reaction $^{124}\text{Te}(p,n)^{124}\text{I}$ using 12.6 MeV protons in yields 25 % below those of the mainly used reaction $^{124}\text{Te}(d,2n)^{124}\text{I}$, but with a very much lower contamination by long-lived ^{125}I and ^{126}I . The minimum obtained value for the sum of all impurities was 0.14 % of the ^{124}I activity, at 6 days after end of bombardment, using 99.8 % enriched $^{124}\text{TeO}_2$ as target material. This yield/purity ratio also permits the production of ^{124}I by low-energy "baby" cyclotrons which could considerably increase the general availability of this nuclide.

$[^{124}\text{I}]\text{IUdR}$ was synthesized by direct electrophilic labelling in good yield (45-65 %), high radiochemical purity (> 95 %) and high specific activity for functional PET imaging of brain tumours. One day after administration to patients and after completion of the "washout", the only remaining activity was that in tumour structures. The comparison with the tumour labelling index showed that PET with $[^{124}\text{I}]\text{IUdR}$ introduces a novel imaging approach: tumour diagnostics by the measurement of cell proliferation.

[¹²⁴I]IodoHoechst 33258 was synthesized by direct electrophilic labelling in yields of 70 % and in a radiochemical purity of 99 %. In cell culture experiments using HTB-40 (human adenocarcinoma line), it was shown to be taken up by the DNA as well as the unlabelled fluorescence dye H 33258. Furthermore, its radiobiological activity was equal to that of the ¹²⁵I-labelled H 33258, but markedly stronger than that of the ¹³¹I-labelled derivative. This suggests a mechanism for Auger-electron induced radiobiological activity as a novel therapeutical approach. *p*-[¹²⁴I]Iodophenylalanine and [¹²⁴I]iodo- α -methyltyrosine are two other compounds labelled with ¹²⁴I that show potential for functional imaging in boron neutron capture therapy.

1. INTRODUCTION

4.15-d ¹²⁴I decays simultaneously by positron emission and by electron capture. Detailed decay data are shown in Table I. Therefore, it should be a prospective labelling radionuclide for both approaches: diagnostic PET investigations, e.g. of tumours, and experimental radiotherapy by means of short-range high-LET Auger electrons.

To date, no clinical studies confirming this dualistic behaviour are available. Despite some single case reports, there are only few PET studies using ¹²⁴I as tracer and also with more patients [1-4]. The number of groups dealing with ¹²⁴I on tumour patients is even smaller [2, 4, 6, 7].

The short range of the emitted Auger electrons in tissue requires investigations with biochemical systems containing the DNA as the actual radiation-sensitive component and consequently a search for DNA ligands as labelling molecules. In order to achieve this, two approaches are promising: (i) application of labelled pyrimidine derivatives, which are incorporated into the DNA *in vivo*, and (ii)

labelling of DNA dyes which bind to the DNA via hydrogen bridges. For diagnostics, from analogies with thymidine [8] it may be concluded that cell proliferation could play a role in the visualization of tumour structures. This would constitute a new approach in PET diagnostics.

The availability of ^{124}I poses a certain problem. It is recommended to produce this nuclide in high yield and purity via the nuclear reaction $^{124}\text{Te}(\text{d},2\text{n})^{124}\text{I}$, by irradiation of isotopically enriched $^{124}\text{TeO}_2$ with 15-MeV deuterons [9], which was recently confirmed [10]. The cyclotrons useful for high-current acceleration of deuterons are mainly old and personnel-intense machines. Since a relatively high amount of the undesired long-lived 60-d ^{125}I is simultaneously formed via this nuclear reaction [10], ^{124}I was tried to be produced by a proton-induced nuclear reaction, the excitation functions of which were measured [11,12] but not verified under realistic high-current production conditions. Once these requirements met, ^{124}I should become available by low-energy "baby" cyclotrons.

2. PRODUCTION OF ^{124}I : UTILIZATION OF "BABY" CYCLOTRONS

For the production of ^{124}I , yields and impurity rates of both nuclear reactions, $^{124}\text{Te}(\text{d},2\text{n})^{124}\text{I}$ ($E_{\text{d}} = 14 \text{ MeV}$), using 89.6 % enriched $^{124}\text{TeO}_2$ and $^{124}\text{Te}(\text{p},\text{n})^{124}\text{I}$ ($E_{\text{p}} = 12.6 \text{ MeV}$), using 89.6 and 99.8 % enriched $^{124}\text{TeO}_2$ as target materials were measured under realistic high-current production conditions. The irradiations were performed at the compact cyclotron CV 28 in Essen, Germany, typically for 10 min at a beam current of 25 μA . The target material was molten onto a 90 % Pt/10 % Ir plate which was cooled from the backside by a separate water circuit. To prevent exfoliation of the brittle glassy target material, parallel furrows of 0.2 mm width and 0.2 mm depth with a distance of 0.1 mm each along the beam direction were cut into the surface of the platinum backing. This target arrangement withstands beam currents of up to 35 μA without measurable evaporation of iodine during irradiation. The target is placed at a 15° angle from the beam direction.

Table I. Diagnostic and radiotherapeutic properties of 4.15-d ^{124}I

	Diagnostic approach	Therapeutic approach
Decay mode	Positron decay (25.6 %)	Electron capture (74.4 %)
Particles emitted per decay	0.256 positrons	9.2 Auger electrons ^a
Particle ranges in tissue	approx. 3.5 mm	approx. 30 nm
Chemical strategy	Labelling of any cell-seeking ligands	Labelling of DNA ligands

^a Ref. [5]

Table II: Yields of ^{124}I produced by different nuclear processes

Nuclear Reaction	Energy Range (MeV)	Enrichment of ^{124}Te (%)	Yield ^a in MBq/ μAh (mCi/ μAh)
$^{124}\text{Te}(\text{d},2\text{n})^{124}\text{I}$	14.0—>0	89.6	15.0 ^b (0.41)
$^{124}\text{Te}(\text{p},\text{n})^{124}\text{I}$	12.6—>0	89.6	11.3 (0.31)
$^{124}\text{Te}(\text{p},\text{n})^{124}\text{I}$	12.6—>0	99.8	13.0 (0.35)

^a corrected for distillation yield

^b averaged on more than 30 production runs

Table III: Characteristic data of the decay and formation curves for contaminants in ^{124}I produced by different nuclear processes

Nuclear Reaction	Incident Energy of Particles (MeV)	Isotopic Enrichment of ^{124}Te (%)	Time Window after EOB for Observing 5 % Impurities	Minimum of Impurities (%) at Time after EOB
$^{124}\text{Te}(\text{d},2\text{n})$	14.0	89.6	1.6 \rightarrow 4.1 d	4.5 % at 2.6 d
$^{124}\text{Te}(\text{p},\text{n})$	12.6	89.6	1.1 \rightarrow 14.0 d	1.6 % at 4.0 d
$^{124}\text{Te}(\text{p},\text{n})$	12.6	99.8	0.7 \rightarrow 33.3 d	0.14 % at 6.0 d

Some minutes after the end of bombardment (EOB), the iodine radioisotopes were distilled from the automatically dismantled target plate in a quartz tube for 6 min at 740°C , transported into a small stainless steel trap by a stream of air (20 ml/min) and eluted in about 100 μL of 0.02 M NaOH solution. Under these conditions, 80 - 95 % of the total activity of iodine radioisotopes was trapped in solution and more than 90 % of the activity was concentrated in the first drop of 20 μL . Details are described elsewhere [10].

The distillation yields and the amounts of the long-lived iodine radioisotopes were determined by measurement of ^{124}I in the target before and after irradiation and before and after distillation. ^{124}I and the iodine radionuclides with high-energy γ -lines were measured in a calibrated Ge(Li) spectrometer, ^{125}I in a planar ultra-pure Ge detector (0.3 mm Be cap).

The yields of the three production reactions are presented in Table II. Generally, the yields of the (p,n) reactions are about 25 % less than those of the (d,2n) reaction. Although the data were corrected for the distillation yield,

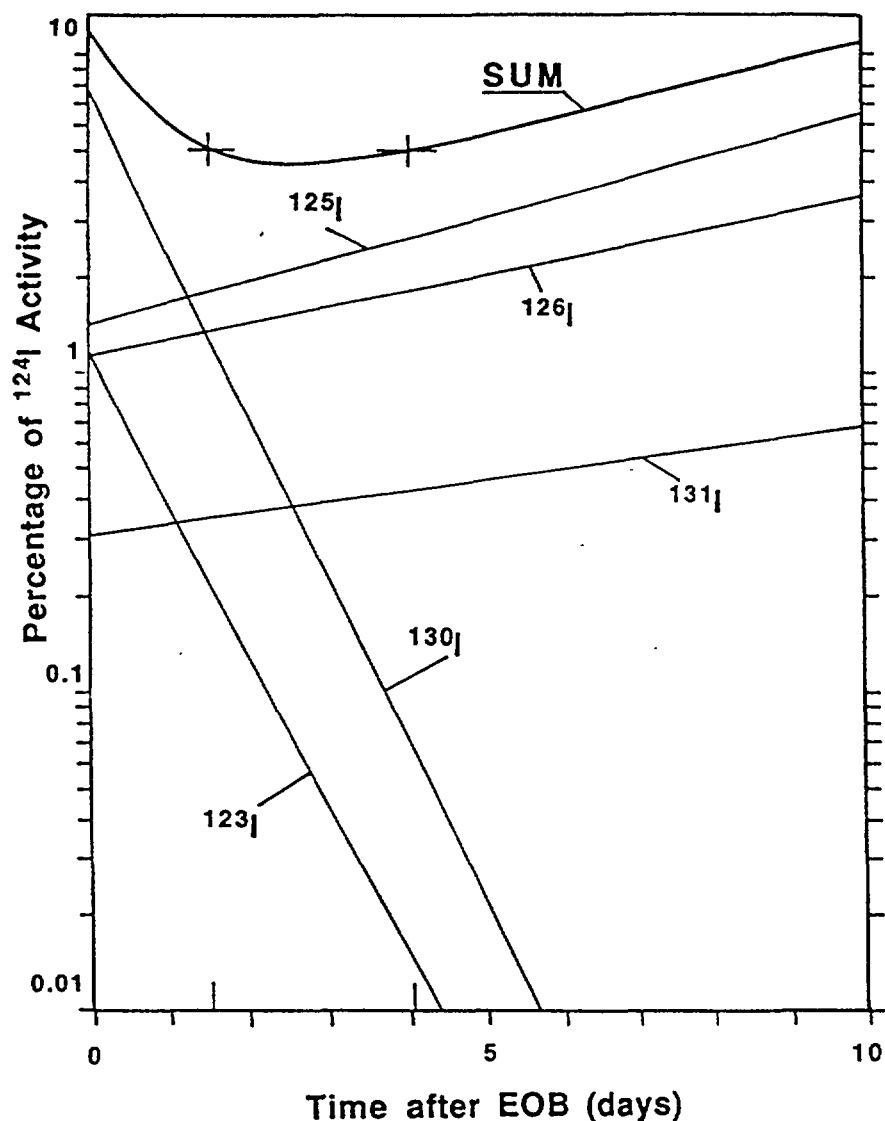


Fig. 1: Impurities in ^{124}I produced via the nuclear reaction $^{124}\text{Te}(d,2n)^{124}\text{I}$ using 89.6 % enriched $^{124}\text{TeO}_2$ and 14.0 MeV deuterons.

comparison between the three reactions is difficult: the yield of the (d,2n) process is a more than two-year average of routine production runs for a clinical trial [13], the yields of the (p,n) reactions were obtained by specially designed experiments using freshly prepared targets, in three independent production runs. The reasonably high yield of the (p,n) reaction may introduce the use of low-energy "baby" cyclotrons for the production of 4.15-d ^{124}I . To date, these cyclotrons are used almost exclusively for the production of short-lived positron emitters such as 20-m ^{11}C , 10-m ^{13}N , etc. Thus, even better use could be made of such cyclotrons by producing ^{124}I overnight when no patients are being treated.

The purity data of ^{124}I are presented schematically in Figures 1 - 3. As can be seen, in all three production reactions a time window can be defined within which the sum of the contaminants does not exceed 5 % of the ^{124}I activity. These windows are presented in Table III. The 5 % limit for the sum of contaminating activities was required by the Swiss Regulatory Authorities granting permission for the clinical trial using [^{124}I]IUdR [4,13]. Since the amount of ^{124}I also decreases by decay, it is advisable to administer ^{124}I immediately after the contaminants reach the limit of 5 % of ^{124}I activity. It is noteworthy, however, that also for the production

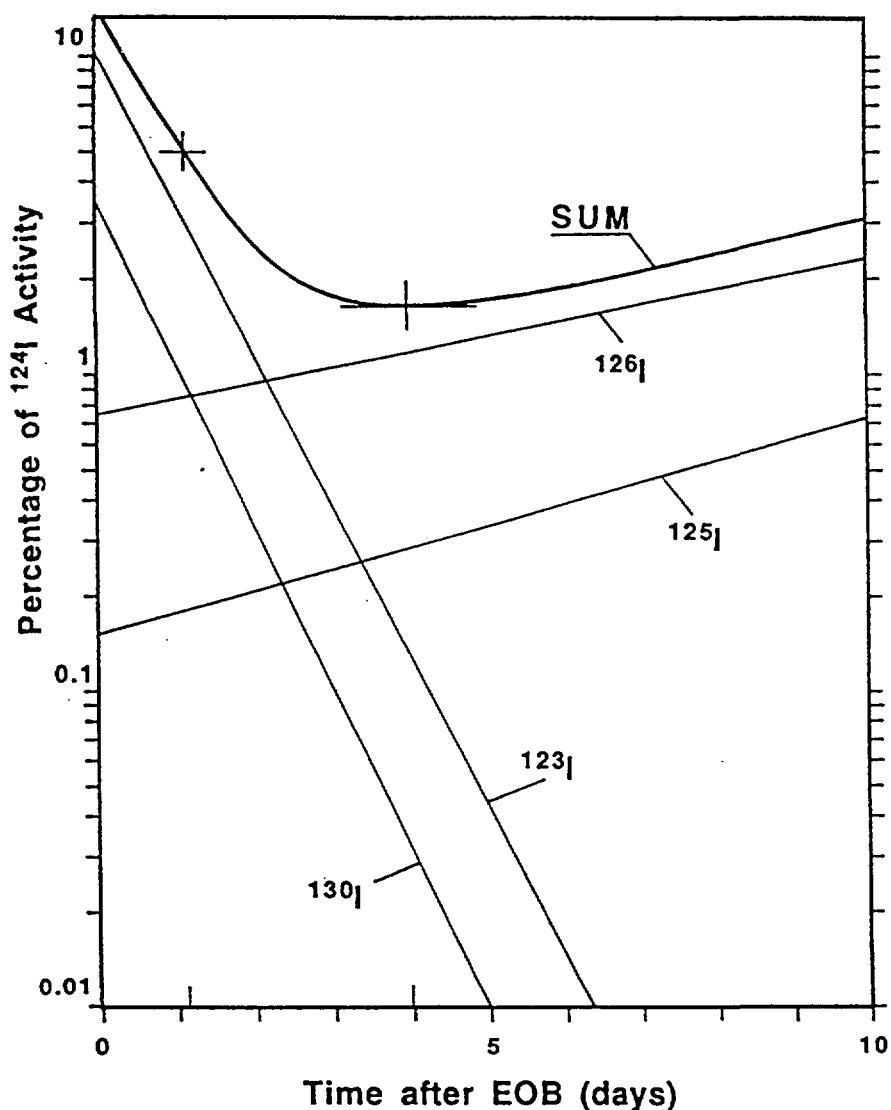


Fig. 2: Impurities in ^{124}I produced via the nuclear reaction $^{124}\text{Te}(p,n)^{124}\text{I}$ using 89.6 % enriched $^{124}\text{TeO}_2$ and 12.6 MeV protons.

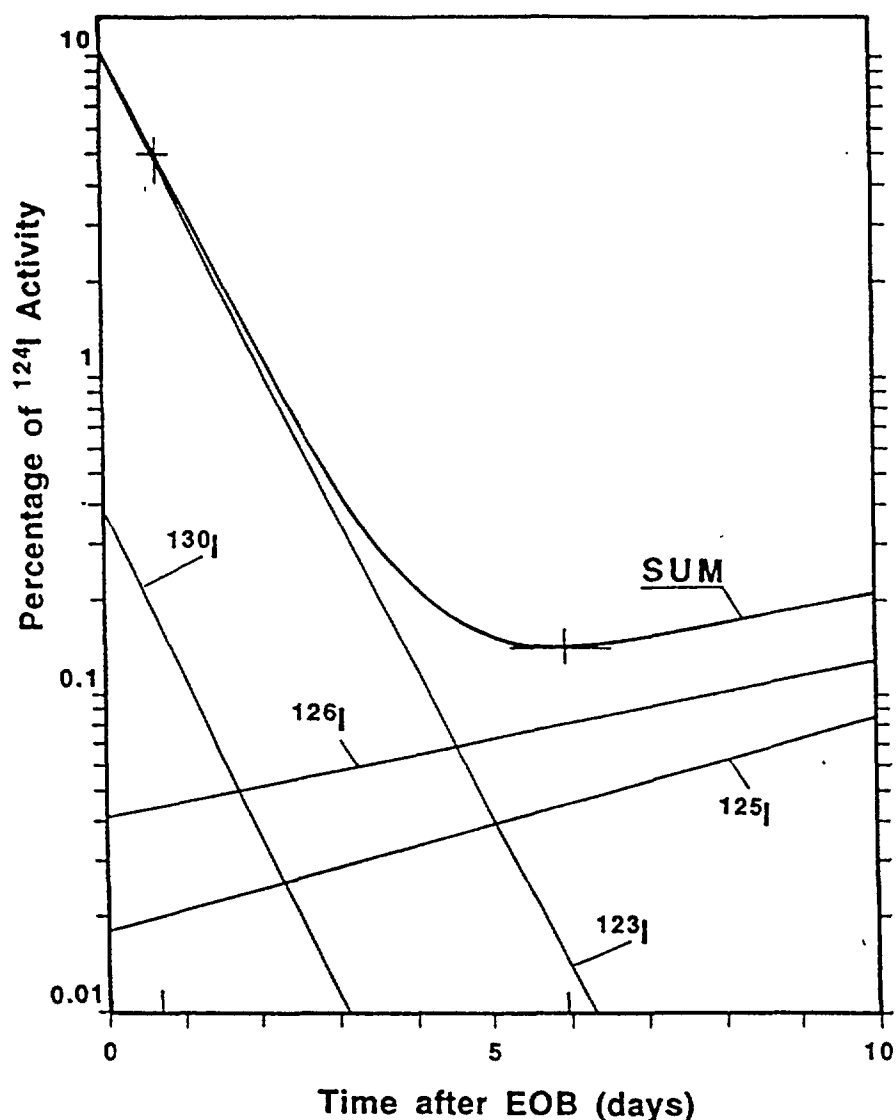


Fig. 3: Impurities in ^{124}I produced via the nuclear reaction $^{124}\text{Te}(p,n)^{124}\text{I}$ using 99.8 % enriched $^{124}\text{TeO}_2$ and 12.6 MeV protons.

of ^{124}I by the (p,n) reaction using highly enriched ^{124}Te a waiting time must be taken into account until this limit is attained.

In practice, the most important factor is that the higher yield of ^{124}I in the (d,2n) process is accompanied by high amounts of the long-lived contaminants ^{125}I and ^{126}I . In our clinical trial using [^{124}I]IUdR, however, this fact does not seem to play an important role since 95 % of IUdR are metabolized in the first hour after administration followed by rapid renal excretion (with blocked thyroid) [4,13]. In

other potential applications, the remaining ^{125}I bound to the DNA with its high amount of more than 23 emitted Auger electrons per decay [5] could produce radiobiological damage in the tumour cells. This effect was proposed for radiotherapy applications [14] but could not yet be confirmed by predicative experiments.

In the (p,n) reaction, the yield of ^{126}I is somewhat smaller than that in the (d,2n) reaction; the amount of ^{125}I drops markedly below that of ^{126}I , and ^{131}I could not be detected at all. As expected, the product with the highest radionuclide purity was obtained by using the highly enriched 99.8 % target material and the (p,n) process. The yields here of ^{124}I and ^{123}I are comparable to those obtained from the 89.6 % enriched target as the $^{124}\text{Te}(\text{p},\text{n})$ and $^{124}\text{Te}(\text{p},2\text{n})$ channels are hardly influenced by the enrichment grade alone. The results show that the amount of ^{123}I must also be considered in the (p,n) reaction with the highest enrichment grade of the target material. From excitation function measurements [11,12], this effect can be expected to be dramatically enhanced with increasing energy. Consequently, the production of ^{124}I by the $^{124}\text{Te}(\text{p},\text{n})$ process could be carried out advantageously at low energies which may again qualify this process as a domain of low-energy accelerators.

3. 5-[^{124}I]IODO-2'-DEOXYURIDINE: CELL PROLIFERATION AS APPROACH FOR PET SCANNING

Radiohalogenated pyrimidine nucleosides are useful tracers for studying the metabolic pathways of the incorporation of their precursors into the DNA and RNA for measuring cell proliferation [8] *in vivo*. Their use for diagnosis in clinical trials and especially for tumour growth prognosis in patients appears very promising. Based on tests using ^{131}I as labelling nuclide [15], the thymidine uptake for instance should be imaged with [^{124}I]IUdR (Fig. 4) and PET in patients, in order to evaluate

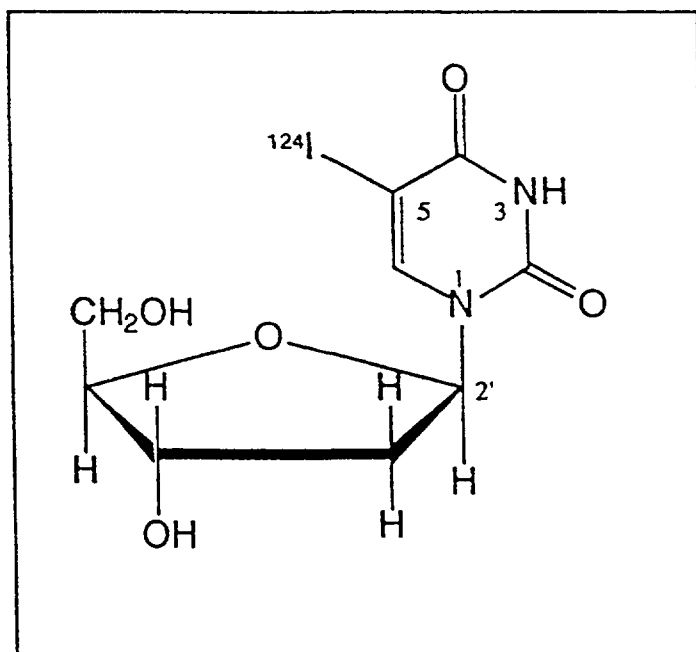


Fig. 4: 5-[¹²⁴I]iodo-2'-deoxyuridine.

the regional tumour growth potential and the grade of malignancy in a very early phase of tumor progression, and to provide spatial information for radiation treatment planning.

2'-Deoxyuridine (UdR) was labelled with ¹²⁴I by direct electrophilic substitution using Iodogen®, a mild oxidizing agent. 600 µg UdR were allowed to react in an Iodogen®-coated ReactiVial with 185 MBq (5 mCi) Na[¹²⁴I] of high specific activity (450 GBq(12 Ci)/µmol) in 100-200 µL aqueous solution for 15 min at 65°C, adjusted with 250 µL 0.2 M phosphate buffer to pH 7.2 to 7.4. The product was separated by a SEP-PAK C-18 cartridge which was pre-conditioned with ethanol. After washing the column with 30 ml water, the product was eluted with 2 ml ethanol. The solution was evaporated to dryness under nitrogen at 65°C, the substance was taken up in 8 ml physiological saline solution and put through a 0.22-µm sterile filter. The entire labelling procedure was carried out manually behind a local shielding of 5 cm lead and took about 1.5 h, including all preparations.

The radiochemical purity was determined by means of a fixed procedure by both TLC on silicagel-60 and reverse-phase HPLC on a RP-18 column. Based on about 45 syntheses, the average yield was between 45 and 65 %. The contamination grade of [^{124}I] was determined to be 2.7 % by TLC (mainly iodate) and 4.6 % by HPLC which was taken as the official value for the authorities. The chemical stability in solution (cleaving of approx. 1 % of the ^{124}I activity per day in the form of iodide under storage at 4°C) allowed a time window of two days after the end of synthesis (EOS) for application, considering the required radiochemical purity grade of 95 % [^{124}I]IUdR before administration [13].

The brain tumour patients were studied using PET and [^{124}I]IUdR in order to assess whether a specific [^{124}I]IUdR retention (k_i) in tumours and normal brain tissue can be measured, and whether the tumor- k_i is correlated with the labelling index obtained from the same patients through operation. The retention k_i was determined 24 hours after injection of [^{124}I]IUdR ("washout" strategy) in 3 low-grade meningiomas (LGG) and 12 malignant gliomas (glioblastoma, GBM; WHO IV). Plasma metabolites of IUdR ([^{124}I]IUdR, [^{124}I]iodouracil, non-bound ^{124}I) were measured using HPLC.

For the purpose of validation of brain tumour [^{124}I]IUdR uptake, the day after completion of the PET study the patients received unlabelled bromodeoxyuridine (BrUdR) as intravenous infusion, 4 hours before tumor resection. This compound like IUdR was incorporated into dividing cells. The tumour labelling index values LI, obtained from the tissue specimens which indicate the percentage of dividing tumour cells, were determined, thus giving a measure for tumour cell proliferation.

The retention of [^{124}I]IUdR was relatively homogeneous in LGG. In Fig. 5 GBM displays a marked variation of regional IUdR uptake, suggesting a wide range of proliferative activity in these tumors. In 15 brain tumour patients a significant correlation between [^{124}I]IUdR tumour retention (PET) and the labelling index LI

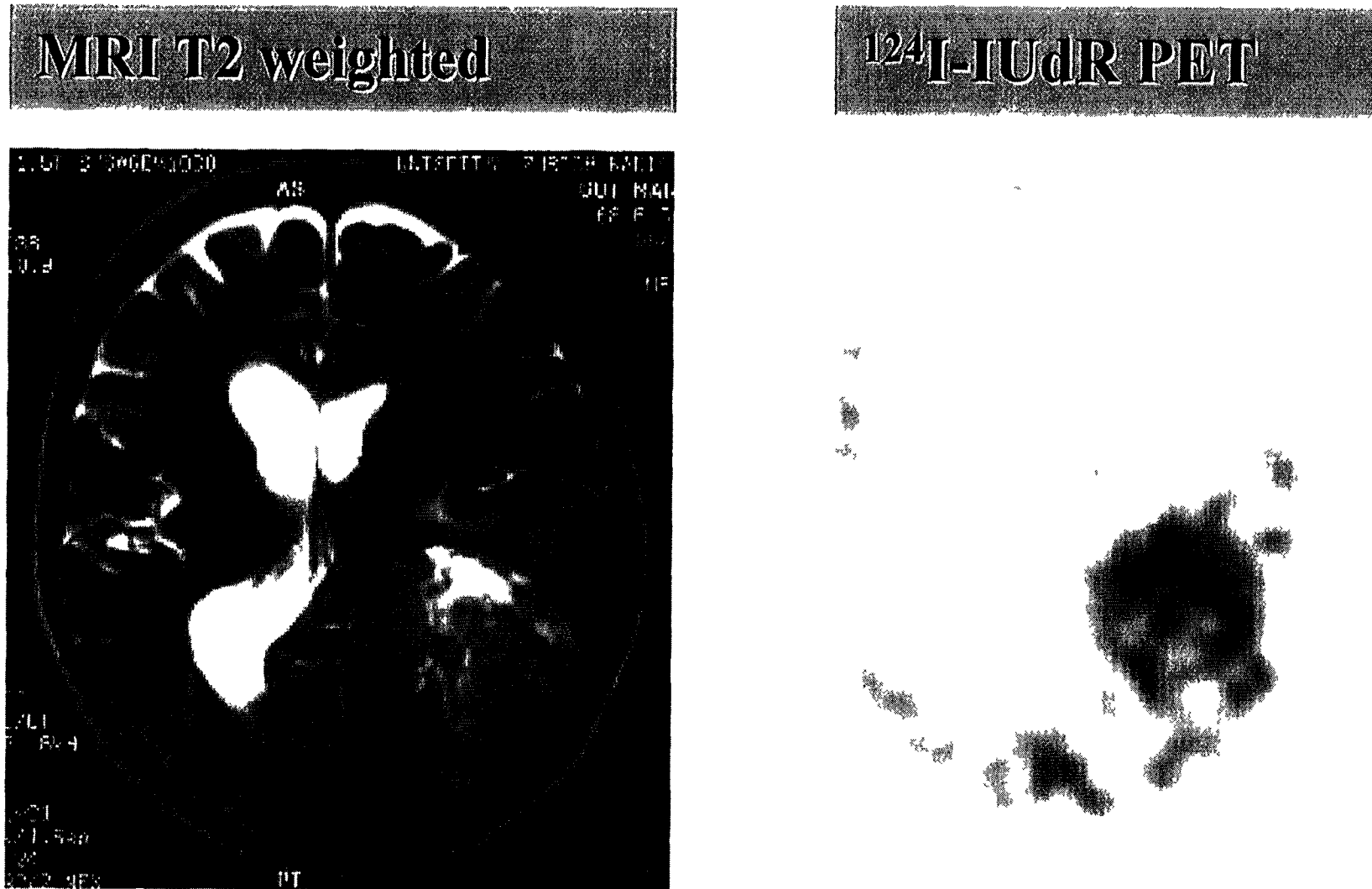


Fig. 5: MRI and ^{124}I -IUdR PET scans of a Glioblastoma multiforme. The PET scan was taken 24 h after application and demonstrates the cell proliferation.

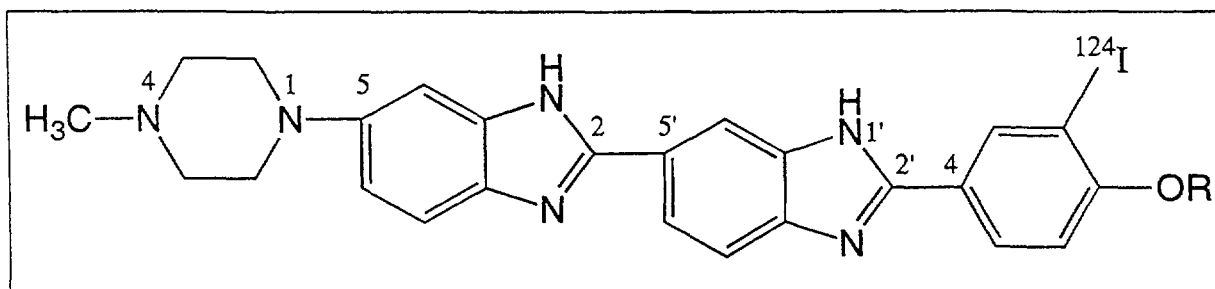


Fig. 6: [^{124}I]IodoHoechst 33258 ($\text{R} = \text{H}$), and [^{124}I]IodoHoechst 33342 ($\text{R} = \text{C}_2\text{H}_5$).

(obtained through subsequent tumour resection) was established (K_i : LGG 4.4 ± 0.9 , GBM $11.7 \pm 9.7 \mu\text{l/g/min}$. LI: 3.0 ± 2.5 , GBM $7.1 \pm 4.3\%$. Spearman rank (K_i vs. LI): $\rho = 0.927$, $\rho = 0.005$). These preliminary data clearly demonstrated that PET with [^{124}I]IUdR assesses tumour cell proliferation non-invasively.

The following approaches are planned to be performed with the [^{124}I]IUdR-PET system: the [^{124}I]IUdR retention in the tumour region extends beyond the margins of GBM determined by contrast enhanced magnetic resonance tomography (MRI). This effect indicates the presence of proliferating cells in the tumour vicinity and is under current investigation [4]. In addition, PET scans in the presence of the 5'-butyryl ester of IUdR, an IUdR deiodination inhibitor [16], will be performed.

4. [^{124}I]IODO-HOECHST 33258: TOWARDS THERAPY WITH AUGER ELECTRONS

Hoechst 33258 and 33342 are bis-benzimidazoles with high affinity to the A-T base pairs of DNA (Fig. 6) [17]. Both compounds attach very rapidly and stably to the small groove of DNA, via hydrogen bridges at the nitrogen atoms in the positions 1 and 1' of the benzimidazol rings (and possibly in position 1 of the piperazine ring) [18]. They are used as fluorescence stains in cytology, and this approach may introduce a new class of DNA-active drugs.

When labelled with a short-range radioactive or activatable nuclide and transported into the cell nucleus, both compounds create double-strand breaks in the DNA which affect cell proliferation [19]. By this approach, Auger electron therapy of small tumours and micrometastases is discussed.

Both compounds were labelled with ^{124}I by direct electrophilic substitution of the unlabelled Hoechst compounds with Chloramine-T. The procedure was adopted from earlier experience using long-lived ^{125}I , with some minor improvements [20]. 2 mg H 33258 or 33342, respectively, were dissolved in 0.5 ml water and adjusted to pH 7.6 with phosphate buffer. It reacts at room temperature for 90 min with $\text{Na}[^{124}\text{I}]\text{I}$ and 2 "Iodo-beads®" (immobilized Chloramine-T on polystyrene beads). After finishing the reaction, the non-bound iodine was removed by a Sephadex A 25 column. The quality control was performed by both TLC (Polygram CEL 300 (eluent: $\text{MeOH}/\text{H}_2\text{O}$ 80/20, v/v)) and HPLC (RP 18 column, 0.01 M triethylammonium hydrogencarbonate/THF 2/1, v/v, adjusted to pH 6.2 using CO_2). The yields were 70 % for H 33258 and 30 % for H 33342, respectively. The final solution contains less than 1 % free iodine. The stability tests showed that the product remained unchanged at 4°C for more than 1 week. The solution was adjusted to 1 $\mu\text{Ci}/\mu\text{l}$ and used in this form to produce double-strand breaks in *in vitro* cell culture experiments.

In a series of experiments, the activity of H 33258 was investigated on Du-145 (human prostata carcinoma line), HTB-40 (human adenocarcinoma line) cell cultures, and for comparison in V-79 hamster fibroblasts. In order to distinguish between the activity of Auger electron emitters and non-Auger electron emitters, H 33258 was labelled with ^{124}I , ^{125}I and ^{131}I . Auger electrons are emitted by ^{125}I and ^{124}I , but not by ^{131}I . The results are shown in Fig. 7 (the control diagram was taken with unlabelled H 33258).

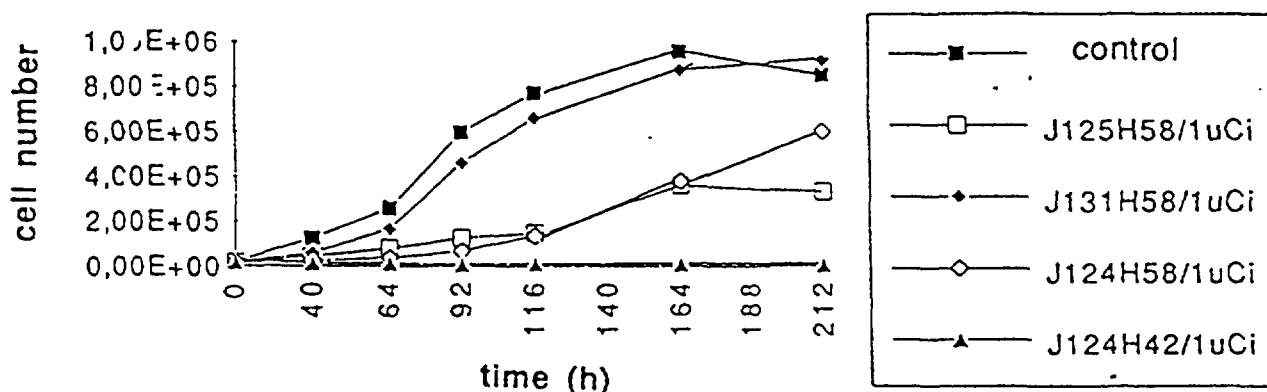


Fig. 7: Radiobiological activity of [^{124}I , ^{125}I , ^{131}I]IodoHoechst 33258, measured by the number of surviving cells in the cell line HTB-40, vs. time. The control diagram shows the results with unlabelled Hoechst 33258. The experiments started with 6'000 cells.

The experiment showed that ^{124}I has radiobiological activity if coupled to the DNA. To our knowledge, this experiment constituted the first evidence for the radiotoxicity of ^{124}I at all. The experiment further showed that the iodinated form of H 33258 is also bound to the DNA. The radiotoxicity of ^{124}I proved to be similar to that of ^{125}I but not to that of ^{131}I . This strongly indicates that actually the Auger electrons are responsible for the radiotoxicity of ^{124}I . In particular, the radiotoxicity was verified on human cell lines and not only on the hamster fibroblasts.

This experiment has to be quantified in order to be able to make statements on the grade of radiotoxicity. This approach should help to set up novel test procedures leading to *in vivo* radionuclide therapy .

5. OTHER APPLICATIONS FOR ^{124}I

Boron neutron capture therapy (BNCT) becomes a useful clinical modality for treating solid tumours for which current therapies are of only marginal utility, as

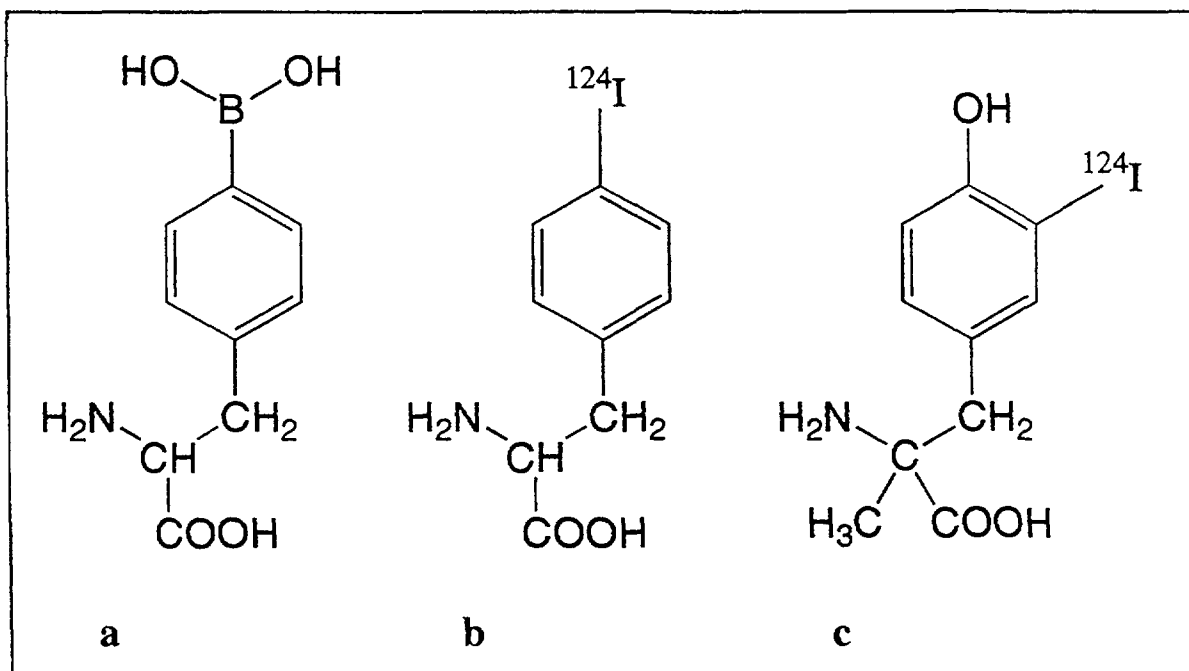


Fig. 8: (a) *p*-Boronophenylalanine, (b) *p*-[¹²⁴I]iodophenylalanine, (c) DL-3-[¹²⁴I]iodo- α -methyltyrosine.

soon as effective boron-containing compounds are designed, synthesized, and evaluated. One of the two existing compounds currently used clinically is *p*-dihydroxyboryl phenylalanine (*p*-boronophenylalanine, BPA). In order to study the pharmacokinetics of BPA in tumours and the surrounding tissues, and to draw conclusions for its dosimetry, there is a big need for appropriate radioactive substitutes for BPA (Fig. 8).

In this context, the following molecules were also labelled with ¹²⁴I, so far without further clinical or biological investigations:

- *p*-[¹²⁴I]iodophenylalanine,
- [124I]iodo- α -methyltyrosine.

All of these compounds were labelled by direct electrophilic substitution, analogously to the above-mentioned molecules.

BPA has already been labelled with 2-h ¹⁸F [21], but the cell proliferation is visualized after the "washout" effect as described above, and thus the half-life of 2

hrs for ^{18}F seems too short for an efficient PET accompaniment of BNCT. It is evident that the iodine label changes the pharmacological and physiological behaviour of a small molecule like BPA strongly, and [^{123}I]iodo- α -methyltyrosine was found to be an insufficient surrogate for BPA [22], but validation of these labelled compounds is still pending.

Uracil is the main metabolite of UdR *in vivo*. In order to study the metabolism of [^{124}I]UdR and to visualize cell proliferation mechanisms more clearly, comparison with the corresponding labelled uracil is necessary. Consequently, 5-[^{124}I]iodouracil was synthesized by direct electrophilic substitution using chloramine-T, in > 90 % yield and with > 95 % purity. Pharmacokinetic investigations are being carried out.

6. CONCLUSION

The described examples demonstrate the potential for wide-spread utilization of ^{124}I in nuclear medicine and biology. Its half-life of 4.2 h matches the kinetics of tumour metabolism and cell proliferation. Thus, ^{124}I might become a universal radionuclide in future nuclear oncology. The high production yields and purity levels of ^{124}I using the proton-induced nuclear reaction would introduce its large-scale production with the widely available "baby" cyclotrons and will efficiently increase the general availability of ^{124}I . The use of ^{124}I as therapeutical Auger-electron emitter depends on its coupling (via Hoechst 33258) to a tumour-seeking protein and on the exact study of the cell-kinetic responses. Finally, the well-known chemistry of iodine, established in the labelling of numerous compounds with 8.0-d ^{131}I , allows to conveniently apply this specialized know-how to the corresponding procedures using ^{124}I .

ACKNOWLEDGEMENTS

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USE OF A NEW TANDEM CATION/ANION EXCHANGE SYSTEM WITH CLINICAL-SCALE GENERATORS PROVIDES HIGH SPECIFIC VOLUME SOLUTIONS OF TECHNETIUM-99m AND RHENIUM-188

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XA9848004

Abstract

In this paper we describe the first application of our simple and inexpensive post-elution tandem cation/anion exchange column system which is based on generator elution with salts of weak acids such as ammonium acetate instead of saline solution to provide very high specific volume solutions of technetium-99m and rhenium-188 from clinical-scale molybdenum-99/technetium-99m generator prepared from low specific activity (n,γ) molybdenum-99, and tungsten-188/rhenium-188 generators, respectively. Initial passage of the bolus through a strong cation exchange cartridge converts the ammonium acetate to acetic acid which is essentially not ionized at the acidic pH, allowing specific subsequent amine-type (QMA SepPak™) anion exchange cartridge column trapping of the microscopic levels of the pertechnetate or perrhenate. Subsequent elution of the anion cartridge with a small volume (< 1 mL) of saline then provides high specific volume solutions of technetium-99m by concentration of the high eluant volumes obtained by elution of clinical-scale (1 Ci) generators. This new approach also works very effectively to obtain high specific volume solutions of rhenium-188 (> 500 mCi/mL) from the alumina-based tungsten-188/rhenium-188 generator.

INTRODUCTION

Nuclear reactors play an important role in providing radioisotopes for both diagnostic and therapeutic applications in nuclear medicine and radionuclide generator systems [1-3] prepared from reactor-produced parent radioisotopes are attractive to obtain the daughter products at facilities remote from the production site. The availability of radionuclide generator systems continues to represent an important source of radioisotopes for applications in both diagnostic and therapeutic radioisotopes nuclear medicine. Chromatographic-type molybdenum-99/technetium-99m generators fabricated from fission-produced molybdenum-99 are widely used to obtain high specific volume solutions of technetium-99m. For therapeutic applications, the rhenium-188 radioisotope (half-life 16.9 hours; β^-_{max} 2.12 MeV ; 15% gamma 155 keV) has many attractive properties, since it is obtained carrier-free from the reactor-produced tungsten-188 parent (half-life 69 days). The tungsten-188/rhenium-188 generator is thus an attractive candidate for use in isolated clinical sites, in less developed countries and in a centralized radiopharmacy.

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1. MATERIALS AND METHODS

General. All chemicals and reagents were analytical grade unless otherwise indicated. The > 86% enriched tungsten-186 was purchased from the ORNL Isotope Distribution Office. BioRad alumina was used as the generator adsorbant. Cation-exchange cartridges (IC-H Plus) were purchased from Alltech Associates (Deerfield, IL) and the QMA "Light" SepPak™ anion-exchange columns were obtained from Waters Corporation (Milford, MA). Generator columns were fitted with a coarse glass frit at the bottom under the alumina column. The columns were fitted with machined Teflon plugs on both top and bottom which were fitted with Luer locks for attachment to short lengths of arterial extension tubing. The molybdenum-99/technetium-99m generators (1 Ci) were purchased from Mallinckrodt Medical (St. Louis, MO).

Radionuclide Analyses. Radiouclide measurements were determined using a calibrated HpGe solid state detector (EG&G ORTEC, Oak Ridge, TN) coupled to a PC-based MCA (Nuclear Data/Canberra, Inc.). Samples of constant geometry were counted with low (< 5 %) deadtime. Data were analyzed using Nuclear Data Accu-Spec Spectroscopy software. Nuclear data were taken from Lederer and Shirley [4] and the radioisotope levels were determined by quantification of the following photo peaks: ¹⁸⁸Re, 155 keV (15%); ¹⁹²Ir, 316 keV (82.8%); ¹⁹¹Os 129 keV (25.9%), ^{99m}Tc 140 keV (89 %), ⁹⁹Mo 739.6 keV (12.1 %). The tungsten-188 levels were usually quantified by measurements of the 155 keV γ-ray from decay of the ¹⁸⁸Re daughter in the ¹⁸⁸W/¹⁸⁸Re equilibrium mixtures. Molybdenum-99 breakthrough was directly measured by analysis of the 739.6 keV photopeak. In some cases, direct measurement of the 227 keV (0.22%) and 290 keV (0.39%) γ-rays from decay of ¹⁸⁸W was possible after post elution "trapping" of the ¹⁸⁸W generator column breakthrough on an alumina (neutral) SepPak® as described earlier [5]. Routine generator elution yields were determined using a calibrated Capintec dose calibrator.

Reactor Production and Processing of Tungsten-188. Tungsten-188 was produced by irradiation of enriched tungsten-186 oxide targets in the ORNL High Flux Isotope Reactor (HFIR) at a thermal neutron flux of $2\text{--}2.5 \times 10^{15}$ neutrons per $\text{cm}^2\text{sec}^{-1}$. The targets were processed by dissolution in 1 *N* NaOH solution in the presence of hydrogen peroxide as described earlier [6-7]. The specific activity of W-188 averaged 4-5 mCi/mg W-186 for a one cycle, 24-day irradiation.

Fabrication and Performance of the Tungsten-188/Rhenium-188 Generator. Following dissolution, the sodium tungstate solution was acidified to pH 2-3 with 1 *N* HCl and adsorbed on a column of acid-washed BioRad alumina housed in a lead shield [7]. Generators were then conditioned by washing thoroughly with 100-200 mL of 0.9% saline by elution with a peristaltic pump at a flow rate of 1-2 mL/min and were then eluted with saline to determine the baseline elution yields. The generator system housed in a lead shield is illustrated in FIG. 1.

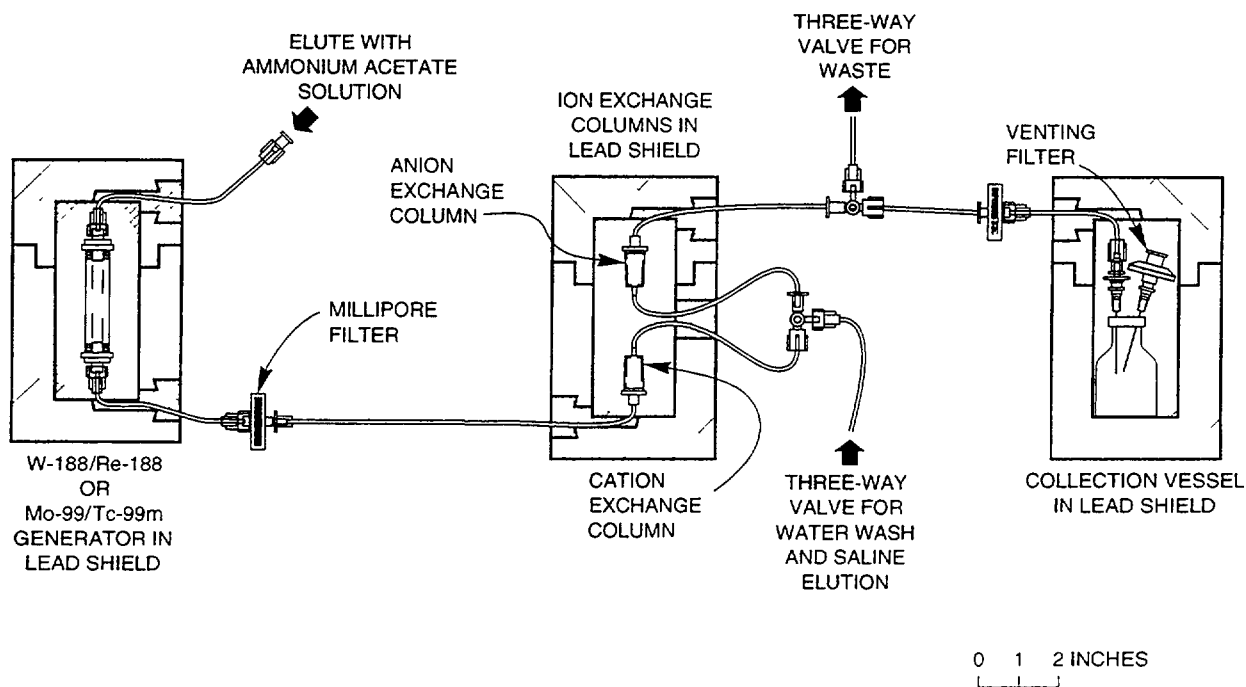


FIG 1. Schematic of the generator elution and tandem concentration system.

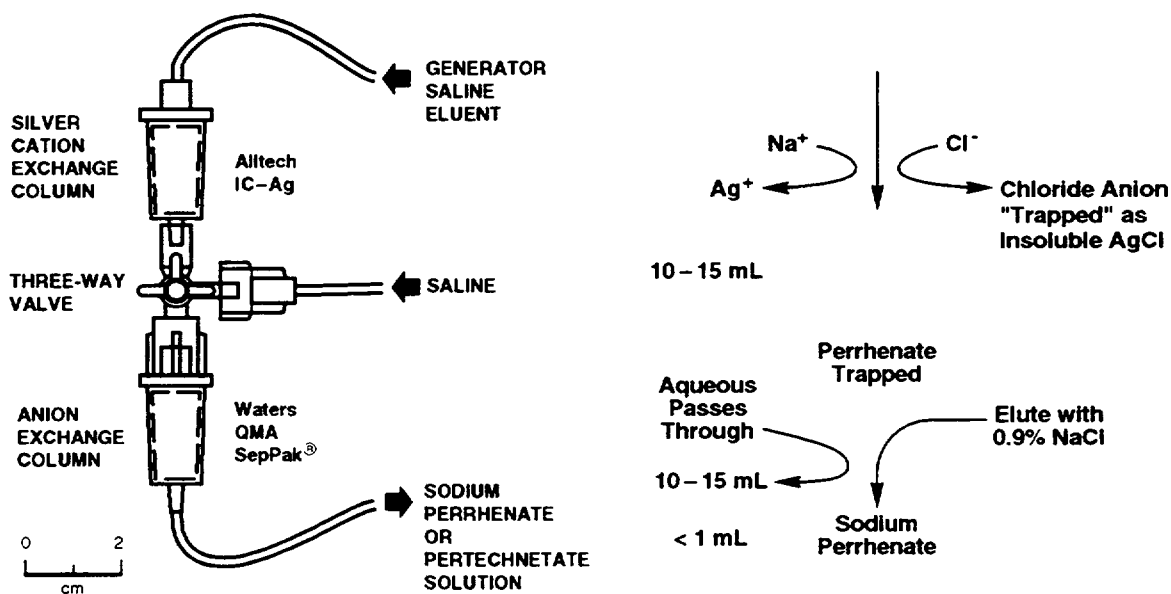


FIG 2. Detailed schematic of the tandem cation/anion tandem system used for concentration of low specific volume solutions of technetium-99m and rhenium-188 illustrating the chemical transformations on which the concept of using the salt of a weak acid for generator elution is based.

Generator Elution. Prior to elution of the generators with the ammonium acetate solutions, the generators were equilibrated with eluant by elution at a constant flow rate of 1-2 mL/min using a peristaltic pump.

Concentration of Technetium-99m and Rhenium-188 Generator Eluants Using the Tandem Cation/Anion Columns System. The typical set-up of the generator and elution and concentration systems are illustrated in FIG. 2. In order to minimize exposure, the system is used behind a leaded glass and/or Lucite shield. A short length of disposable extension tubing is attached to the lower Luer outlet connection of the generator. Inclusion of an in-line 0.22 micron Millipore filter ensures trapping of any alumina fines or other particles which may be eluted from the generator. The in-line alumina SepPak™ traps the low levels of tungsten-188 parent breakthrough [5]. The tandem cation/anion concentration system (FIG. 2) consists of a commercially available cation exchange cartridge with a capacity of 2-4 milliequivalents attached to a three-way stopcock connected at the outlet to the QMA SepPak® anion-exchange column. Another length of extension tubing then connects the outlet of the anion exchange column to the rhenium-188 collection vessel which is housed in a lead or Lucite shield. With the stopcock open the generator is eluted with 12-15 mL of 0.9% saline. The eluant collected from the QMA column contains only low levels of radioactivity and is discarded. The stopcock is then adjusted to permit elution of technetium-99m Na-pertechetate or rhenium-188 Na-perrhenate from the QMA anion trapping column with 0.9 mL of 0.9% saline.

2. CONCEPT OF THE TANDEM CATION/ANION CONCENTRATION OF SOLUTIONS OF SALTS OF WEAK ACIDS

Because tungsten-188 is produced [6-7] with relatively low specific activity in most research reactors (typically < 5 mCi/mg W-186), the large amounts of alumina required to bind the low specific activity tungsten-188 parent results in high elution volumes and low specific volume solutions of rhenium-188. Simple effective methods are thus required for concentration of the generator eluant to the high specific volumes required for radiopharmaceutical labeling and for other applications. Our development of a very new simple method using a tandem cation/anion exchange column concentration system (FIG. 1) for the efficient concentration of rhenium-188-perrhenate and technetium-99m-pertechetate makes the availability of high specific volume solutions of carrier-free rhenium-188 feasible and extends the generator shelf-life to several months. Our preliminary results [8] have demonstrated that concentration is readily feasible on an experimental scale and we now demonstrate the effectiveness of this new approach for the multiple elution of clinical-scale tungsten-188/rhenium-188 (500 mCi) and molybdenum-99/technetium-99m (1 Ci) generators.

3. CONCENTRATION OF RHENIUM-188 SOLUTIONS FROM THE TUNGSTEN-188/ RHENIUM-188 GENERATOR

For these studies, the generators were initially eluted with 0.9% saline for control elution yields, and then equilibrated with the ammonium acetate (NH_4OAc) eluant solution. Yield values were determined using a dose calibrator. Following elution of a clinical-scale tungsten-188/rhenium-188 generator (Table 1) with a salt of a weak acid such as ammonium acetate, the eluant is passed through a tandem ion exchange system consisting of an initial strong cation exchange column which transform the ammonium acetate to acetic acid, which is essentially unionized at this pH. Since the macroscopic levels of the acetate anion eluant have been transformed to an unionized form, subsequent elution through an anion column specifically traps the microscopic levels of the perrhenate anions with the principal non-radioactive volume passing through as waste. After adjustment of the intermediary three-way valve, and subsequent washing of the QMA anion column with water, the rhenium-188 perrhenate is then eluted from the anion column in < 1 mL of isotonic saline. This method provides high specific volume solutions of rhenium-188 sodium perrhenate over several weeks (Table 1).

Table 1. Average yields of rhenium-188 and technetium-99m obtained by elution of clinical-scale generators with 0.3 M ammonium acetate solution.

Tungsten-188/Rhenium-188 Generator		Molybdenum-99/Technetium-99m Generator	
0.9 % NaCl Eluant	0.3 M NH_4OAc Eluant, pH 5.5	0.9% NaCl Eluant	0.3 M NH_4OAc Containing 0.01 M NH_4NO_3 Eluant, pH 5.2
n = 3 Elutions Yield = 70.9 ± 9.9 %	n = 31 Elutions Over > 3 Month Period Yield = 62.6 ± 14.4 %	n = 8 Elutions Yield = 84.2 ± 1.2 %	n = 20 Elutions Over > 7 Day Period Yield = $69. \pm 1.2$ %

4. CONCENTRATION OF TECHNETIUM-99m SOLUTIONS FROM THE MOLYBDENUM-99/TECHNETIUM-99m GENERATOR

We have now demonstrated that this approach also works well with clinical-scale 1 Curie molybdenum-99/technetium-99m generators (Table 1). Although fission-produced molybdenum-99 is exclusively used in western developed countries, the use of low specific activity molybdenum-99 to obtain technetium-99m has been known for many years with sublimation and solvent extraction methods for use when fission-produced molybdenum-99 is not available [9]. Our new concentration method, however, allows the use of alumina-based chromatographic generator methods for separation of carrier-free technetium-99m from low specific activity molybdenum-99. Data from a

typical study are summarized in Table 1. In contrast to the ease of oxidation of reduced rhenium species to Re(VII), reduced technetium-99m species are apparently retained on the alumina column, and we have thus determined that the 0.3 M NH_4OAc generator eluant for elution of the molybdenum-99 generator must also contain low levels of a mild oxidizing agent such as 0.01 M NH_4NO_3 to maintain the reproducibly high elution yields of technetium-99m. Another advantage of our tandem concentration system is that the shelf-life of the molybdenum-99 generators can be extended indefinitely essentially until the molybdenum-99 parent has decayed. The obvious advantages of using neutron-activated molybdenum-99 include the absence of large amounts of highly radioactive waste and no need for highly enriched uranium-235.

Although elution yields are somewhat lower, this approach permits very good routine yields. Generator eluant volumes as high as 20 mL are readily concentrated to < 0.5 mL (40:1) and concentration factors are essentially unlimited, based on the size of the anion column and void volume of the tubing system, etc. By using this new method for simple eluant concentration, many reactors throughout the world can now be used for production of both tungsten-188 by double neutron capture on enriched tungsten-186 targets and molybdenum-99 by neutron activation of enriched molybdenum-98, making these generators routinely available at remote clinical sites in less developed regions to obtain the versatile rhenium-188 therapeutic radioisotope and technetium-99m for preparation of diagnostic agents.

SUMMARY AND CONCLUSIONS

The availability of our new simple and inexpensive tandem cation/anion exchange system in conjunction with use of salts of a weak acid such as 0.3 M ammonium acetate for elution of clinical-scale molybdenum-99/technetium-99m generators prepared from low specific activity (n, γ) molybdenum-99 and with tungsten-188/rhenium-188 generators is effective in providing high specific volume solutions of technetium-99m and rhenium-188. Although the yields are somewhat lower in comparison to use of the traditional 0.9 % (0.15 M) sodium chloride eluant, this new method can be used indefinitely with no apparent decrease in daughter yields with time and continued low parent breakthrough and should allow the widespread use of these generators.

ACKNOWLEDGEMENTS

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RADIOHALOGENS AND OTHER ISOTOPES

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Abstract

The development of radiopharmaceuticals for monitoring gene transfer therapy with emission tomography is expected to lead to improved management of cancer by the year 2010. There are now only a few examples and approaches to the design of radiopharmaceuticals for gene transfer therapy. This paper introduces a novel concept for the monitoring of gene therapy. We present the optimisation of the labelling of recombinant human β -NGF ligands for *in vitro* studies prior to using ^{123}I for SPET and ^{124}I for PET studies.

INTRODUCTION

The first [1] clinical experience employing gene therapy was gained in 1989. Peripheral blood leukocytes were transduced with heterologous DNA to examine the biological *in vivo* properties of tumour-infiltrating lymphocytes, and thereby optimising antitumour immunotherapy strategies. Since then numerous approaches have been undertaken to apply powerful strategy of therapeutic gene transfer to almost all kinds of human diseases. However, despite substantial progress there are still a number of key technical issues to be resolved before gene therapy can be safely and effectively applied in the clinic [2]. Major limitations still include the lack of efficient vector application procedures, efficient restrictions of the vector tropism to the tissues to be targeted as well as an insufficient long-term expression of transferred therapeutic genes.

In this context, it is of major importance to efficiently monitor the short term as well as the long term effects of technical improvements in gene therapy techniques [3,4]. Information documenting the respective vector profiles and application techniques is supposed to exert a vigorous effect on the design of subsequent, improved vector generations and application procedures. Therefore, *in vivo* molecular imaging using emission tomography may be one of the best methods to assess the expression of a gene transduced in a target tissue and to monitor *in vivo* gene transfer as a therapeutic approach to cancer, chronic viral infections and mono-genetic inherited diseases. The concepts of gene therapy have been reviewed [5].

There are now only a few examples and approaches to the design of radiopharmaceuticals for suicide gene transfer therapy. See TABLE I.

This paper summarises the concepts, current status, and an example using ligands of nerve growth factor, and prospects for this new direction of radiopharmaceutical sciences and oncology. Conventional procedures for *in vivo* monitoring of therapeutic gene transfer are based on the molecular characterisation of repetitively collected serum and tissue probes for detection of therapeutic RNA's, DNA's and proteins. These highly sensitive and specific techniques are

TABLE I. RADIOTRACERS AIMED TO GENE TRANSFER THERAPY

TRACER	GENE / VECTOR TYPE	REFERENCE
^{131}I -FIVAU, FIVRU	HSV-tk / STK retrovirus	Wiebe, et al, 1995 [6]
^{131}I -/ ^{124}I -FLAU	HSV-tk / gpSTK-A2 retrovirus	Tjuvajev, et al., 1995 [10]
^3H -5FC	HSK-tk	Haberkorn, et al., 1996 [7]
^{18}F -Acyclovir	HSK-tk / Adenovirus	Srinivasan et al., 1996 [8]
^{18}F -FHPG	HSK-tk	Goldman, et al., 1996 [13]
^{125}I -/ ^{124}I - β -NGF	Adenovirus / PLDNSN retrovirus	This Work, 1997 [15]
^{125}I -/ ^{124}I - ----MAb	Adenovirus / PLDNSN retrovirus	This Work, 1997 [24]
^{18}F -Purines	HSK-tk	Barrio, et al., 1997 [28]
^{125}I -Bombesin	Adenovirus / AdCMGRPr	Rogers et al., 1997 [29]

FIVAU = (E)-5-2-(iodovinyl)-2-deoxyuridine, R = ribofuranosyl

FLAU = Fluoro-1 β D-arabinofuranosyl-5-iodouracil

5FC = 5-Fluorocytosine

FHPG = 9-1-(1-Fluoro-3-hydroxy-2propoxyl)-methylguanidine

β -NGF = Recombinant β -Nerve Growth Factor

Bombesin = mIP-Des 14 -Bombeson-(7-13)NH₂

laborious and require a continuous control of expression strength, kinetics and localisation following gene transduction. In order to expand gene transfer to the patient setting, it is essential to understand *in vivo* cell cycle control, proliferation, apoptosis and neovascular activity, e.g. within tumours. The goal for PET is to define a convenient way for *in vivo* monitoring of the kinetics, the definition and continuous monitoring of a distinct vector tropism and distribution particularly with the initial 72 hours following *in vivo* transduction as well as over longer time periods (i.e., months to years subsequent to the initial gene transfer procedure).

Iodine-124 is the positron-emitting radionuclide of choice in the design and development of therapeutic radiopharmaceuticals. Iodine-124 has a 4.16 day half-life, and 25% on the nuclear decay events are associated with emission of a positron ($E_{\beta^+} = 2.134 \text{ MeV}$). The radioisotope has been produced [30] for PET applications [31] by using either the $^{124}\text{Te}(d,2n)^{124}\text{I}$ and the $^{124}\text{Te}(p,n)^{124}\text{I}$ nuclear reactions by irradiation of high isotopic enrichment (>97%) of ^{124}Te with 15 MeV deuterons or 12 MeV protons, respectively.

Approaches by Wiebe et al., [6], Haberkorn et al., [7], Srinivasan et al., [8], Monclus et al. [9], and Tjuvajev et al. [10,11,12] and Goldman et al [13] used a retroviral vector to transfer the suicide gene coding for the thymidine kinase of the herpes simplex virus (HSV-tk) in tumour cells. The indirect measurement of gene expression was attempted [14] through visualisation of enzyme activity of a tracer pro-drug (e.g., ganciclovir or 5-fluorocytosine) to the toxic product to follow the metabolism of the radioactive probe.

Our novel approach [15] focuses on the design of a suitable cell surface reporter system that incorporates a receptor protein and its specific ligand for the *in vivo* monitoring system of target restricted tropic vectors for selected targets of gene transfer therapy. This is exemplified by diverse asialoglycoprotein-receptor restricted, hepatotropic vector systems [16,17,18].

The cytokine nerve growth factor (NGF) comprised of 118 amino acid residues was chosen as the ligand, and a cytoplasmatically truncated mutant of the low affinity nerve growth factor receptor P75 (delta-p75) as the appropriate marker gene. The tertiary structure is comprised of three cystine disulfide residues and three Beta hairpins. The NGF is present as a dimer for biological activity [19,20]. NGF has a high affinity to at least two broad cell surface receptors [21] called p75 (K_d of 10^{-9}) and TrkA (K_d of 10^{-11}). The interaction with p75 seems to involve Lysine residues. The structure of NGF also contains two tyrosine residues that are not required for binding to p75, and therefore do not influence the biological activity when radio-iodinated [19].

There is evidence that NGF-R has a role in programmed cell death of neurons, regulation and coordination neuronal proliferation, differentiation and maintenance in the adult, the information flow between the nervous and immune system, and pain causing events.

EXPERIMENTAL

Stable LNGF-R cell lines have been generated by transduction of amphotropic PA317 packing cells with LNGF-R encoding retroviral vector pLDNSN (Boehringer Mannheim). Stable cell lines were characterised by PCR and immunochemistry. Supernatant of stable producer clone PALDNS#12 yields a titre of 7×10^5 cfu/ml, and was used for transduction of NIH 3T3 fibroblasts, and two hepatoma cell lines (HuH7 and Morris Hepatoma). Thereby, pairs of LNGF-R receptor positive and LNGF-R receptor negative (negative controls) cell lines were made available subsequent for binding studies. In addition, LNGF-R cDNA encoding recombinant adenoviral vectors were transfected which allow also the monitoring of the adenoviral gene transfer. Construction was achieved by homologous recombination of the respective cDNA with the adenoviral shuttle vector in *E. coli* [22], and subsequent plaque purification. These adenoviral vectors are currently used for LNG-R transduction of NIH 3T3 and Morris hepatoma recipient cells thereby allowing functional analysis of the adenoviral mediated gene transfer.

Initially ^{125}I was used for the optimisation of the labelling of recombinant human β -NGF ligands prior to using ^{123}I for SPET and ^{124}I for PET studies. Two methods were evaluated: lactoperoxidase, and as a superior approach using the mild oxidising agent N-bromosuccinimide (NBS). The radiochemical yields with ranged ^{125}I ranged from 85 to 95% with lactoperoxidase, and from 95 to 98% with NBS. The NBS reaction was carried-out as follows: 10 μg NGF was incubated with 0.5 mCi ^{125}I -iodide (in NaOH) and 0.3 μg NBS in a final volume of 15 μl Na-Phosphate buffer (0.13 M, pH 7.4) on ice for 5 min. The reaction was terminated by dilution with Na-phosphate buffer containing 0.5% BSA.

The NGF (2.5 μg) was incubated with ^{124}I (17 mCi/ml from UKV-Essen) and 0.08 μg NBS. The radiochemical yield was determined by tlc on silica gel 60 using a mobile phase of (acetone / n-butanol / ddH₂O / ammonia) (65/20/5/10). The radiochemical yield with ^{124}I was >90%. The product was purified with a Biogel P6 Micro BioSpin Column, that was equilibrated with Na-Phosphate

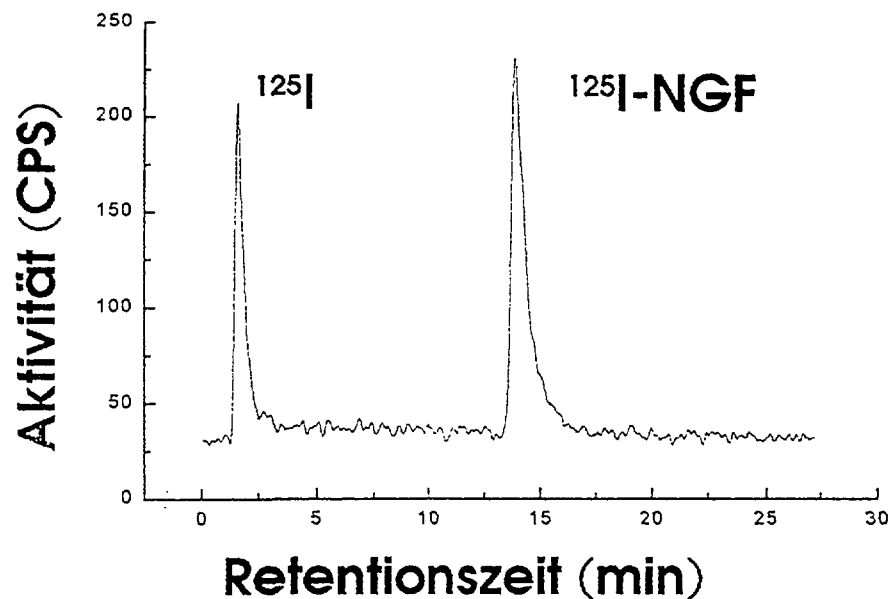


FIG. 1. RADIO-HPLC FOR PURIFICATION OF RADIO-IODINATED NGF.

buffer containing 0.5% BSA and 0.1% Protaminsulfate. Radio-HPLC was performed with a C_{18} column using a gradient of H_2O and Acetonitrile (with 0.1% mTFA). See FIG. 1

Characterisation of the retrovirally transduced cell lines has yielded NGF-R positive clones both in immunofluorescence as well as functional binding with labelled NGF. The biological NGF activity was estimated using the NGF induce neuron growth on PC12 cells to be 80% of the native NGFs. The specific binding of the labelled NGF to the PC12 cells was 70%.

A Micro-imager I (Biospace, Paris) with 30 micron resolution gave *in vitro* images confirming the radioiodinated NGF was bound to the LNGF-R surface receptor reporter protein expressed on both stable transduced NIH 3T3 as well as on Morris Hepatoma cells, but not on untransduced control cells [15]. These results provide the first experimental evidence that the chosen ligand/receptor system (LGF/LNGF-R) vector transduced target cells. This novel approach differs from applications of radiopharmaceuticals restricted for usage in suicide gene transfer therapy only (See TABLE I) in that usage of a non-functional marker gene (here: the LNGF-R gene devoid of any intrinsic receptor functions) enables an universal application of such "monitoring" vectors without interfering with the cellular functions of the vector addressed target cells.

Currently, biodistribution in rodents, and *in vivo* emission tomographic studies are planned [23,24] at DKFZ using a high resolution PC 2048-7WB PET scanner [25]. For this purpose, the Morris hepatoma animal model [26] will be used. The model closely resembles the tumour biology of human hepatocellular carcinomas in tumour growth rate, blood supply characteristics and metastases. Tumour implantation was performed by insertion of $\sim 3 \text{ mm}^3$ of Morris hepatoma tumour 3924 tissue into the sub-capsular pocket of the left lobe of the liver [27]. After 10 - 14 days the tumours were injected with LNGF-R transducing retroviral or adenoviral vectors. The PET study [24] is planned 48 hr after the transduction procedure.

CONCLUSION

The development of radiopharmaceuticals for monitoring gene transfer therapy with emission tomography is expected to lead to improved management of cancer by the year 2010. This paper summaries the progress to date.

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**PREPARATION OF $^{125}\text{IUdR}$ AND ITS EVALUATION IN
ANIMAL TUMOUR MODEL AS A POTENTIAL
THERAPEUTIC AGENT***



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Abstract

5-Iodo-2'-deoxyuridine or iodoxuridine (IUdR), an analogue of thymidine, is taken up by the proliferating cells during DNA synthesis. Radioiodinated IUdR is a potential therapeutic agent since radiohalogenated thymidine analogues are used for *in-vivo* tumour targeting and Auger electrons from radionuclides such as ^{123}I and ^{125}I are very effective in cell destruction when internalised.

$^{125}\text{IUdR}$ was prepared and studied for its suitability as an *in-vivo* tumour therapy agent. $^{125}\text{IUdR}$ was prepared both by direct iodination of 2'-deoxyuridine and iododemercuration of 5-chloromercury-2'-deoxyuridine. Radioiodination yields were between 60-80% at pH 7. Iododemercuration was preferred since with direct iodination poor yields were observed when high specific activity product was desired and also the purification procedure was lengthier. The identity of $^{125}\text{IUdR}$ was established by comparison of TLC and HPLC patterns with those of authentic IUdR. The purified $^{125}\text{IUdR}$ had radiochemical purity > 95% and was stable for 20 days at 4°C and for a week at 23°C and 37°C. Bio-uptake of $^{125}\text{IUdR}$ was studied by injecting the tracer in tumour bearing mice (Sarcoma S-180). The uptake in tumour cells was $4.28 \pm 2.7\%$ per

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gram at 3 h and $1.48 \pm 0.19\%$ at 24 h post injection. *In-vivo* deiodination of the product was observed as seen by the uptake of the activity in the thyroid. About 40% the activity from all other organs was excreted in 70 h. The optimum time for injection of the tracer for therapy was studied by observing the delay in tumour growth and survival rate in mice injected at 0,3,9 and 12 days after tumour induction. Injection of the tracer on the third day was found to be the most beneficial for retardation of tumour growth, while injection of the activity on the zeroth and ninth day had no effect.

1. INTRODUCTION

Spectacular advances have been witnessed in recent times in the fields such as molecular biology, biochemistry etc. leading to better understanding of the disease processes which, in turn, has lead to more efficacious treatment modalities. This is true in the field of oncology too, a major area of research throughout the world, where the treatment of cancer is constantly changing and moving to molecular levels. 'Targeted delivery' is becoming a reality now, thanks to the various target seeking molecules such as specific monoclonal antibodies or receptor binding peptides. In cancer treatment such target specific molecules and novel delivery systems are able to deliver chemotherapeutic drugs or radioisotopes at the specific tumour site, with decreased toxicity to other proliferating tissues (gut, bone marrow). For treatment of cancer, one of the routes that has been studied avidly is the use of an agent that will be taken up inside the cancer cells and several chemotherapeutic and antiviral agents are based on such molecules.

One such group of agents is the halogenated pyrimidine nucleosides which are used for studying the metabolic pathways of pyrimidine incorporation into the DNA of cells and for measuring cell proliferation. 5-Iodo-2'-deoxyuridine (IUdR) is a thymidine

analogue in which 5-methyl group of thymidine is replaced by iodine. Since the methyl group and iodine atom have similar Van der Waal radii, this substitution gives a compound that behaves remarkably like thymidine. Earlier studies have demonstrated substantial incorporation of radiolabeled IUdR into DNA of tumour and proliferating tissues [1]. As a thymidine analogue, IUdR is initially taken into the cell, phosphorylated and incorporated in DNA, where it is retained by the cell or its progeny. Radiolabeled IUdR, such as $^{125}\text{IUdR}$, has been used *in-vitro* for monitoring proliferation, migration and death of normal and neoplastic cells as well as for evaluation of the effectiveness of various chemotherapeutic or radiotherapeutic procedures. $^{125}\text{IUdR}$ is preferred for such studies over ^3H or ^{14}C labeled thymidine [2]. Use of radiohalogenated thymidine analogues for *in-vivo* tumour targeting in recent times and therapeutic effect achieved by Auger electron emitting radionuclides such as ^{123}I and ^{125}I within proliferating cells, have revived interest in radioiodinated IUdR as a potential therapeutic agent [3,4]. Moreover, radioisotopes such as ^{123}I and ^{131}I can facilitate tumour imaging also [5]. Auger electrons which deposit a sizeable energy at the site have been considered for tumour therapy [6]. If internalised into the DNA of the cell, such Auger electrons would be ideal for therapy. ^{125}I has been shown to emit, in all, 19 Auger and conversion electrons per decay, with energies ranging from 15 eV to 24 keV. Of these, 14 electrons have average energies of 200 eV or less. The range of these electrons in unit density matter is about 1 to 1.5 nm. Such a burst of low energy electrons results in a highly localised energy deposition of $\sim 10^9$ Rads per decay around the decay site as the molecules within 20 nm sphere are irradiated by these electrons [7]. Experiments have shown that the decay of this isotope in DNA of a mammalian cell leads to the breakage of DNA double strand [8]. *In-vitro* studies showed a steep exponential reduction in cell survival with no shoulder (absence of repair), resulting in high relative biological effectiveness (RBE) values of 7 to 8 and

oxygen enhancement ratio of ~ 1.4 [9]. In contrast, ^{125}I decay within cellular cytoplasm, at plasma membrane or outside the cell, produces no extra lethal effect and resulting cell culture curves resemble those observed with X rays [10].

IUdR derivatives such as radioiodinated 5-(2-iodovinyl)2'-fluoro-2-deoxyuridine (IVFRU) have been shown to be metabolically trapped in tumour cells and has been used as a non-invasive tool to monitor the gene expression [11] in human gene therapy which is gaining importance [12]. Nuclear Medicine is expected to play an important role as a non-invasive imaging system and gene targeted radiotherapy is a new approach emerging for treatment of cancer wherein gene therapy and targeted radiotherapy are merged [13]. Radioiodinated IVFRU and similar derivatives [14,15] are agents for such studies and the current work would help in developing such radioiodinated nucleoside analogues.

2. MATERIALS AND METHODS

Na^{125}I (specific activity 555-629 MBq/ μg) was obtained from Dupont Inc., USA. 2'-Deoxyuridine (UdR), 5-iodo-2'-deoxyuridine (IUdR), iodogen, chloramine T, chemicals for DNA isolation such as Nonidit-P40, DNAase free RNAase, ethidium bromide, sodium dodecylsulphate (SDS), trichloroacetic acid etc. were purchased from Sigma Chemicals Co., U.S.A. Buffer salts, mercuric acetate, sodium chloride, palladous chloride, sodium metabisulphite etc. were obtained from Sarabhai Chemicals Co., India. Silica gel 254 and pre-coated TLC plates were from E. Merck. Electrophoresis paper (Whatman No.3) was from Whatman International Ltd, U.K., Dowex-2 was from Dow Chemicals Co., USA, Sephadex G-10 was from Pharmacia Co., Sweden. HPLC system and the Bondapak C-18 column used were from M/s Waters (India) Ltd.

Phosphate buffers (0.5 and 0.05 M) pH 2 and 7 were freshly prepared. PBS (0.05 M Phosphate buffer, 1% NaCl, pH 7), citrate buffer (0.05 M, pH 6), TKM-1 buffer (10 mM each of Tris, KCl, MgCl₂ and EDTA, pH 7), TKM-2 buffer (TKM-1 buffer with 0.4 M NaCl) and TBA buffer (10 mM each of Tris, boric acid, EDTA) were also prepared. 2 mg/mL solution of IUdR was prepared in 0.05 M Phosphate buffer, pH 7. Glass tubes were coated with 50 µg iodogen (1mg/mL solution in CHCl₃), dried and stored at -20° C.

2.1. Preparation of ¹²⁵IUdR

2.1.1. Direct Radioiodination of 2'-deoxyuridine (UdR)

Direct iodination of UdR was carried out using iodogen as oxidant. 30 µL of 0.5 M phosphate buffer, 10µL (50 µg) of UdR solution and appropriate amount of Na¹²⁵I were added to an iodogen coated tube and mixed. The radioiodination reaction was studied by varying the reaction parameters such as pH (2 and 7), temperature (25°C, 37°C and 65°C), reaction time (5 and 15 min.) and specific activities (1.9 and 19 MBq/µg).

Purification was attempted by Dowex-2 anion exchange resin as free iodide was envisaged to be the main impurity and by gel filtration over a Sephadex G-10 column (30×2 cm) using 0.05 M citrate buffer (pH 6) as eluent. The yield of the reaction as well as the radiochemical purity of the fractions were determined by paper electrophoresis using Whatman chromatography paper No.3 in 0.05 M phosphate buffer for 1.5 h at 10 V/cm. The identity of the purified ¹²⁵IUdR was established by comparison of migration in silica TLC (85% CHCl₃ + 15% CH₃OH solvent system), with an authentic sample of IUdR.

2.1.2. Indirect Radioiodination

2.1.2.1. Preparation of 5-chloromercury-2'deoxyuridine

5-chloromercury-2'deoxyuridine was synthesised as reported [16]. In short, equimolar quantities of UdR and mercuric acetate were dissolved in water. Controlled heating of the reaction mixture was carried out at 50°C with stirring for 3 h. After slight cooling, solid sodium chloride was added in portions till the solution became clear. The solution was refrigerated overnight when precipitation was observed. The precipitate was washed with water : ethanol (1:1) and air-dried. The compound was characterised by 300 MHz ^1H NMR spectroscopy which was recorded on a Varian VXR 300 S spectrometer.

2.1.2.2. Radioiodination by iododemercuration

The ClHgUdR derivative was dissolved in distilled water by slight heating at 40°C to get a concentration of 1 mg/mL. 10 μL (10 μg) was used for radioiodination. The reaction was carried out at pH 6.0 using chloramine-T as the oxidising agent. Na^{125}I (19 to 185 MBq) was used and the reaction was carried out for 1 minute. 100 μg sodium metabisulphite was added to terminate the reaction. The radioiodinated product was diluted to 1 mL and free iodide was separated by adding a small amount of Dowex 2 resin. The reaction yield and radiochemical purity were determined by paper electrophoresis. The purified iodinated product was evaluated by using TLC as described earlier. Quality control was carried out by injecting the sample in a HPLC system using Bondapak C-18 column and eluted with a mixture of methanol: water (60:40) at a flow rate of 1 mL/min.

2.2. Stability studies

The radioiodinated IUdR was diluted to 3.7 MBq/mL with PBS pH 7.5, aliquoted and stored at 4°C, ambient temperature and at 37°C. The radiochemical purity of the aliquots stored at different temperature was determined over a period of 3 weeks using paper electrophoresis as well as TLC.

2.3. *In-vivo* studies in tumour bearing animals

Sarcoma S-180 tumour cells were injected intraperitoneally (10^6 cells/mouse) in 6 to 8 weeks old Swiss mice. The animals were maintained till the tumour development was apparent. 2 μ Ci (~70 KBq) of 125 IUdR was injected intraperitoneally into each animal. Time dependent pharmacokinetic studies were carried out at 3, 24, 48 and 70 h. post injection. Ascitic fluid was drained out to the extent possible prior to sacrificing the animals. Activity distribution in different organs was determined by excising the organs and counting them in a flat geometry NaI(Tl) counter. For determining the activity associated with the tumour cells, the cells from the ascitic fluid were separated and washed with PBS, centrifuged and counted for radioactivity. The washing and centrifugation steps were repeated till the supernatant was free of any radioactivity.

2.3.1. DNA isolation

Procedures from molecular cloning protocols [17] were followed for the isolation of DNA molecules from the tumour cells. About 1 g of the cells from the ascitic fluid was suspended in 5 mL TKM-1 buffer. 125 μ L of Nonidet-P-40 was added and mixed by inversion, centrifuged at 2200 rpm for 10 min. Supernatant was poured off and the nuclear pellet was washed with 5 mL TKM-1 buffer and centrifuged as before. The pellet was re-suspended in high salt TKM-2 buffer, 125 μ L of 10% SDS was added, mixed and incubated for 10 min at 55°C. 0.3 mL of 6 M NaCl solution was added and centrifuged at

15000 rpm for 5 min. The supernatant containing DNA was saved and two volumes of 100% chilled ethanol was added and mixed. White thread like precipitate of DNA was obtained which was centrifuged and washed with prechilled ethanol and finally with PBS. The DNA precipitate was resuspended in 1 mL PBS with 10 mg/mL of DNAase free RNAase and incubated at 37°C for 3 h. 9 M KOH solution was added to the resuspended DNA precipitate to obtain the final concentration of KOH as 1M. The suspension was further incubated for 2 h at 37°C. RNA hydrolysate was extracted twice with chloroform : phenol (1:1 v/v). DNA in aqueous phase was precipitated with five volumes of prechilled 10 % trichloroacetic acid. The DNA pellet was washed and counted for radioactivity.

DNA samples were loaded on agarose gel electrophoresis. 0.8% Agarose was prepared by dissolving it in TBE buffer with heating. Ethidium bromide (0.5 µg/mL) was added to visualise DNA. Xylenecynol and bromophenol blue were used as tracking dyes.

2.3.2. Efficacy studies of locoregional administration of $^{125}\text{IUdR}$

Twenty five animals of the same strain, weight and age were grouped into five sets. All were inoculated intraperitoneally with Sarcoma S-180 cells at the same time. One group was kept as control and the rest received $^{125}\text{IUdR}$ intraperitoneally (70 kBq/animal) on zeroth (I_0), third (I_3), ninth (I_9) and twelfth (I_{12}) day after inoculation. All the animals were maintained in the same environment and keenly observed for tumour growth, changes in the physical activities and time of survival. The abdominal circumference was taken as a measure of tumour growth. The period for which the animals survived after induction of tumour was noted for all the animals.

3. RESULTS AND DISCUSSION

3.1. Preparation of 125 IUdR

3.1.1. Direct radioiodination of UdR

Direct iodination of UdR has been reported using several oxidising agents such as chloramine-T, iodobeads, nitric acid etc. under acidic conditions [18]. Table I gives the results of the present studies at different reaction conditions of temperature, time and pH. Analysis of the fractions by electrophoresis often resulted in three peaks, one corresponding to free iodide which moved towards the anode, the second staying at the point of spotting and a third fraction which also migrated towards anode to a lesser extent (Fig. 1). The species that stayed at the point of spotting in electrophoresis, moved with R_f of 0.53, on TLC and was found to be identical to that of iodoxuridine. Contrary to the

TABLE I: RESULTS OF DIRECT RADIOIODINATION OF 2'-DEOXYURIDINE UNDER DIFFERENT REACTION CONDITIONS

Reaction conditions			% Activity with the peaks after paper electrophoresis		
pH	Time (min)	Temperature (°C)	point of spotting	charged species	free iodide
7	5	65	80	10	7
2	5	65	2-3	—	85
7	15	65	72	11	11
2	15	65	6	—	85
7	5	37	47	14	25
7	15	37	59	21	11
7	15	25	38	2	35
2	15	25	4	—	85

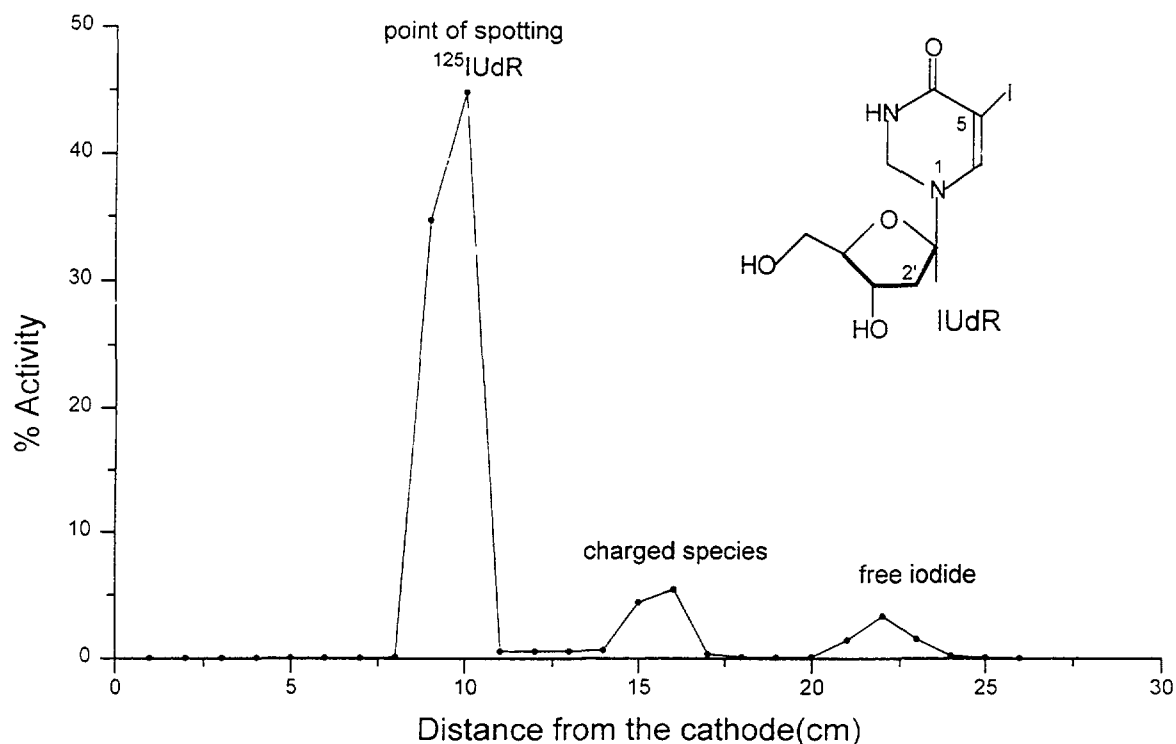


Fig.1 : Paper electrophoresis pattern of the radioiodinated deoxyuridine ($^{125}\text{IUdR}$)

reaction mixture; Sample reaction mixture - direct iodination at 1.8-1.9 MBq/ μg

reported results, it is seen that at pH 2, the yield was very poor irrespective of the reaction temperature, advocating the use of pH 7 for the reaction. At pH 7, the yield was low (~38%) when the reaction was carried out at room temperature. The yield improved on heating the reaction mixture to 37°C or 65°C. However, increasing the reaction time from 5 to 15 min resulted in inferior quality product. When the reaction time was prolonged, the proportion of the unwanted charged species increased. Hence, direct iodination for further experiments were carried out at 65°C for 5 min. When radioiodination was carried out using smaller amounts of uridine to obtain high specific activity product, the yield decreased drastically and large fractions of activity were found to be present as the unusable charged species and free iodide. A certain minimum amount of UdR (~50 μg) is perhaps required for the reaction to proceed as also suggested by earlier workers [18].

TABLE -II : BIODISTRIBUTION OF $^{125}\text{IUdR}$ IN TUMOUR BEARING MICE

Organ	% Injected dose/organ, mean \pm SD, n=3		
	3 h p.i.	24 h p.i.	48 h p.i.
Ascites fluid- total	16.48 \pm 5.4	6.16 \pm 0.23	5.73 \pm 0.76
per gram	2.79 \pm 0.35	1.03 \pm 0.27	0.77 \pm 0.2
Tumour cells*-per gram	4.28 \pm 2.7	1.48 \pm 0.19	2.47 \pm 0.7
Blood	7.37 \pm 0.57	2.83 \pm 0.56	1.22 \pm 0.17
Liver	2.65 \pm 0.49	1.33 \pm 0.28	0.78 \pm 0.06
Intestines	3.36 \pm 0.75	1.67 \pm 0.25	0.86 \pm 0.10
Kidneys	0.91 \pm 0.1	0.39 \pm 0.08	0.26 \pm 0.03
Stomach	10.55 \pm 1.5	4.2 \pm 1.08	2.03 \pm 0.79
Heart	0.22 \pm 0.01	0.07 \pm 0.02	0.04 \pm 0.00
Lungs	0.78 \pm 0.16	0.22 \pm 0.06	0.15 \pm 0.02
Bone [#]	5.07 \pm 0.4	2.3 \pm 0.6	1.8 \pm 0.24
Muscles [#]	15.7 \pm 3.8	6.85 \pm 2.3	2.7 \pm 1.23
Spleen	0.31 \pm 0.11	0.07 \pm 0.02	0.04 \pm 0.01
Thyroid	7.0 \pm 0.97	12.89 \pm 0.37	13.0 \pm 0.1

* tumour cells collected from the ascitic fluid were centrifuged and washed. As there was substantial variation in amount of ascitic fluid collected, its composition w.r.t. cell density etc., it was deemed best to express the activity in tumour cells as injected dose/g.

bone and muscle activity was estimated by assuming bone as 10% of body weight, muscle to be 40 % of body weight, and using femur and thigh muscles as samples.

TABLE III : EFFICACY OF $^{125}\text{IUdR}$ INJECTION : MAXIMUM ABDOMINAL CIRCUMFERENCE (cm) AS A MEASURE OF TUMOUR SIZE

Day [@]	Control	I₀ group	I₃ group	I₉ group	I₁₂ group
Zero	7.4 ± 0.2	#	#	#	#
Third	7.5 ± 0.2	7.4 ± 0.2	#	#	#
Nine	8.3 ± 0.4	7.7 ± 0.2	7.7 ± 0.2	#	#
Twelve	8.8 ± 0.5	7.5 ± 0.1	7.9 ± 0.2	7.7 ± 0.3	#
Sixteen	9.4 ± 0.4	8.5 ± 0.3	8.1 ± 0.3	7.7 ± 0.3	8.8 ± 0.3
Twenty	10.8*	8.9 ± 0.5 *	9.0 ± 0.3	8.3 *	*

[@] number of days between induction of tumour and injection of $^{125}\text{IUdR}$

n = 5 in each group, # - circumference same as in the control group, but here 'n' varies depending on the total animals that are untreated at that time.

* all animals died between 15 to 20 days from control group and I₁₂ group; I₀ group-two died in 17-18 days, I₉ group- four died between 15-18 days, I₃ group-all survived up to 23 days.

Hence, for higher specific activities, a large amount of Na^{125}I (in the right proportions as required) should be used with higher amount (~ 50 µg) of UdR.

Ion exchange using Dowex-2 resin could adsorb most of the free iodide but only part of the charged, damaged radiolabelled product could be removed by this method. On gel filtration over Sephadex G-10, the unwanted species was eluted first (10-12 mL) followed by $^{125}\text{IUdR}$ (26-29 mL) and free iodide was eluted at the end (43-44 mL). $^{125}\text{IUdR}$ peak fractions had 90-92% radiochemical purity as estimated by paper electrophoresis.

3.1.2. Radioiodination by iododemercuration

A very low concentration ($\sim 10^{-7}$ M) of IUdR can damage mammalian tissue culture cells [19] and hence it is essential to prepare very high specific activity tracer. Hence, indirect radioiodination method was resorted to obtain a product with high specific activity by using a minimum quantity of IUdR. Radiolabelling by demetallation is common [20] and $^{125}\text{IUdR}$ can be synthesised by demercuration [21] or destannylation of suitable derivatives [22]. 5-Chloromercuri-2'-deoxyuridine (ClHgUdR) was synthesised. The yield of the ClHgUdR was $\sim 60\%$. The product was characterised by ^1H NMR. (D_2O , δ ppm) : 2.39 (2H, broad triplet, $J = 6.4$ Hz) $\text{C2}'\text{-H}_2$, 3.71-3.86 (2H, multiplet) $\text{C5}'\text{-H}_2$, 4.05 (1H, dd, $J = 3.6$ Hz, 8.0 Hz) $\text{C3}'\text{-H}$, 4.45 (1H, dd, $J = 4.7$ Hz, 8.0 Hz) $\text{C4}'\text{-H}$, 6.28 (1H, t, $J = 6.6$ Hz) $\text{C1}'\text{-H}$, 7.71 (1H, s) C6-H .

The radioiodination yield ranged between 70 to 75% when Na^{125}I activity used was between 18 to 185 MBq. Dowex-2 purification alone was sufficient to remove the unreacted iodide and the radiochemical purity of the purified product was 93 to 98%. On TLC, 90 % of the activity was associated with the peak at R_f 0.53 which was identical with the migration of cold, authentic IUdR. The purified $^{125}\text{IUdR}$ gave a single radioactive peak on HPLC with a retention time of 4.5 min in methanol:water (60:40) solvent system at a flow rate of 1 mL/min establishing the purity of the product. This product was used for animal experiments.

3.2. Stability studies

$^{125}\text{IUdR}$ prepared by both the methods were stable for at least three weeks at 4°C . The radiochemical purity decreased marginally from 94 to 90% when stored for 20 days at 4°C . Even the fraction which was stored at ambient temperature (25°C) and at 37°C had radiochemical purity $>90\%$ after 12 and 9 days, respectively.

3.3. *In-vivo* studies with $^{125}\text{IUdR}$

IUdR, if injected intravenously, is expected to be ineffective due to its high *in-vivo* instability. Moreover its uptake by actively proliferating normal cell systems (gut, bone marrow) would result in consequent toxicity to normal tissues. By locoregional administration of the tracer, these problems could be reduced [23].

3.3.1. *Biodistribution*

The uptake of $^{125}\text{IUdR}$ at 3, 24 and 48 h p.i in different organs is shown in Table II. Activity in blood was found to decrease considerably within 48 h, while thyroid uptake increased from 7 to 13% over the same period. Uptake of activity by bone was observed. While 4.28 ± 2.7 % of the activity was seen with the tumour cells at 3 h p.i. the reduced activity ($\sim 2\%$) remained associated with the tumour cells upto 48 h p.i. Activity in ascites has less significance since all the ascitic fluid could not be taken out and also since the injection was intraperitoneal, this will only depict the activity that has been transported away from the site. Isolated DNA, as identified by the green fluorescence of marker xylenecynol, remained at point of spotting on agarose gel electrophoresis. Even after 48 h p.i., $86 \pm 4\%$ ($n = 5$) of tumour cell radioactivity was found with DNA, indicating that once inside the cell, $^{125}\text{IUdR}$ is retained by DNA.

3.3.2. *Efficacy studies of locoregional administration of $^{125}\text{IUdR}$*

Effect of single dose injection at different stages of tumour growth is shown in Table III. The animals injected with activity on the same day as the injection of the Sarcoma cells, had slightly better survival than the control. However, this is theoretical since the onset of tumour cannot be predicted on the first day. However, even a single dose of 70 kBq/animal was effective in animals treated on the third day, as the growth of tumour was delayed and all the animals of I_3 group survived for a longer period than the

control animals. Even in I₉ group, tumour regression seemed encouraging although the survival rates were not. Toxic dose determination needs validation through a separate set of experiments. Nearly all animals from I₁₂ group died at the same time with the control group animals except one which died a day earlier. The results from I₁₂ indicate that ¹²⁵IUdR therapy may be ineffective and may even be toxic at fully grown stage of tumour.

In conclusion, ¹²⁵IUdR is a promising therapeutic agent when injected locoregionally. High specific activity product prepared by the iododemetallation is preferable to reduce the amount of IUdR needed for injection, which otherwise will be toxic to the healthy cells. The therapeutic effect of ¹²⁵IUdR on the Sarcoma S-180 tumour is encouraging and further studies on dose variations are under way. The effects of ¹²⁵IUdR on other tumour models, especially that of the brain are reported to be encouraging [23,24] and will therefore constitute a subject of future study.

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RADIOPHARMACEUTICAL POTENTIAL OF I-131 LABELLED CHLORDIAZEPOXIDE



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Abstract

1,4 Benzodiazepine derivatives have been largely used in Medicine since 1960. Chlordiazepoxide (7-Chloro-2-(methyl amino)-5-phenyl-3H-1,4 benzodiazepine 4-oxide) has been mostly used and the oldest one after diazepam.

The aim of this study is labeling of chlordiazepoxide (CDZ) with I-131 and investigation of radiopharmaceutical potential as a benzodiazepine receptor agent. Iodogen has been used as iodination agent. Labeling yields have been determined by ITLC (Instant Thin Layer Chromatography) and Paper Electrophoresis. pH and iodogen amount effects to labeling yields have been examined to obtain optimum reaction conditions. Labeling yield was 90% for 1 mg chlordiazepoxide, 1 mg iodogen, 1 pH, 15-20 minutes at room temperature.

Radiopharmaceutical potential of I-131 labeled chlordiazepoxide was searched in vivo experiments carried out on rabbits by using Sophy DX Gamma Camera. I-131 labeled chlordiazepoxide of specific activity 690 MBq/mmol has been injected ear vein of rabbit and static perspectives were taken by pointed time intervals. Brain activity has increased after the injection, reached to maximum in 20th minutes and has not decreased during the imaging time (90 minutes).

Obtained results have shown that I-131 labeled chlordiazepoxide can be used as a benzodiazepine receptor radiopharmaceutical for SPECT studies of brain.

*Also: Center for Drug Research Development and Pharmacokinetic Applications.

INTRODUCTION

Increasing in the use of SPECT and PET in clinical diagnosis depend on developing and presentation of new radiopharmaceuticals to clinicians. Radiolabeling of some ligands like acetylcholine, dopamine, serotonin, benzodiazepines and other ligands may provide more knowledge about brain chemistry.

Kung reported in 1990 "Indeed a number of successful receptor binding agents for a various receptor binding agents for various receptors have been developed. Potential CNS receptor imaging agents labeled with radioactive iodine for SPECT imaging have been reported. However, the clinical potential for these agents for measuring changes of receptor concentration in humans remains to be investigated" [1].

According to this consideration development of new radiopharmaceuticals are necessary. An ideal brain radiopharmaceutical should contain below properties:

The agent should be labeled with a short half-life radionuclide and can be prepared in a short time.

The agent should be prepared with high specific activity.

The agent should freely cross blood brain barrier.

The agent should distribute itself proportionally to the cCBF (regional cerebral blood flow).

The agent should fix itself long enough time in brain. This requires nearly 20-40 minutes.

The redistribution should be weak or not be occurred.

The agent should have a high brain /blood ratio.

The agent should be neutral.

The agent should be lipophilic.

The agent should be kinetically stable and has to remain undissociated at physiological pH.

The agent should be below < 500 dalton molecular weight .

The agent should be inexpensive.

The purpose of this work is to label of chlordiazepoxide that is a benzodiazepine receptor agent might be an efficiently brain radiopharmaceutical. 1.4 Benzodiazepine derivatives are in use in clinical works widely since 1960. They have antianxiolytic and anticonvulsant effects. There are a set of benzodiazepine derivative drugs have already been used. Chlordiazepoxide is one of them. Fig. 1 shows molecular structure of chlordiazepoxide. Chlordiazepoxide has been synthesized by Steinback and coworkers in Roche Laboratory in 1955 [2]. Food and Drug Administration has approved it with Librium name [3].

Iomazenil and flumazenil are the most common benzodiazepine antagonist compounds [4]. Iomazenil has been labeled with ^{123}I [5]. On the other hand, diazepam and flunitrazepam are the agonist compounds. Flunitrazepam has been labeled with C-11[6]. Saji and coworkers have synthesized ^{125}I labeled benzodiazepine and they obtained 2'- ^{125}I -diazepam [7]. Diazepam has also been labeled with ^{131}I by iodogen method [8,9].

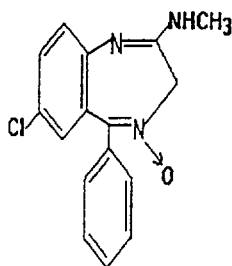


Figure-1 Molecular Structure of Chlordiazepoxide.

EXPERIMENTAL

Chlordiazepoxide was obtained from Center for Drug Research Development and Pharmacokinetic Applications. Na^{131}I was taken from Department of Nuclear Medicine. Iodogen was purchased from Sigma Co. All other chemicals were purchased from Merck and they were reactive grade.

Equipment

ITLC (Instant Thin Layer Chromatography) Conditions:

ITLC Support Material: Merck 0.1 mm Cellulose F

Developing Mediums:

ITLC1:[N-Buthanol-Water-Acetic Acid (4/2/1)].

ITLC2:[Isopropyl Alcohol-n-Buthanol-0.2NAmmonium hydroxide (2/1/1)].

ITLC Time: 2.5 Hours.

Every ITLC sheet was covered by cello-band after its development and was cut into 0.5 cm wideness. Then those were counted by using a NaI(Tl) detector in a multi-channel analyzer after signed 364 keV γ -pick of ^{131}I .

ITLC chromatograms were obtained from these figures by plotting counts versus distance. R_f values and labeling efficiencies were gotten from these figures. The dependence of the labeling yield on the iodogen amount, pH and reaction time were experimented.

Electrophoresis Conditions: Electrophoresis was done with a Gelman Electrophoresis Chamber supply using cellulose acetate strips. Cathode and anode poles and application points were indicated on cellulose acetate strips and these strips were moistened by buffer solution [n-Buthanol-Water-Acetic Acid (4/2/1)]. They were placed in electrophoresis chamber after the samples set on the strips. Standing time and applied voltage for two hours and 250 volts. Developed strips were dried and cut into one cm pieces. They were counted by a well-type NaI(Tl) scintillation detector of multichannel analyzer after marking 364 keV γ photons of ^{131}I . Electrophoresis diagrams were obtained from these counts.

Table -I R_f values of Na^{131}I and $^{131}\text{ICDZ}$ in different developing medium.

ITLC1:[n-Buthanol-Water-Acetic Acid (4/2/1)].

ITLC2:[Isopropyl Alcohol-n-Buthanol-0.2NAmmonium hydroxide (2/1/1)].

R_f values		
	ITLC 1	ITLC 2
Na^{131}I	0.19-0.38	0.20-0.40
$^{131}\text{ICDZ}$	0.86-1.00	0.80-1.00

Preparing Iodogen Coated Tubes: Milligram amount of iodogen was dissolved in CH_2Cl_2 and transferred to closed tubes. CH_2Cl_2 was evaporated by air flow and iodogen was deposited on the walls of glass tubes as a thin film. These tubes were stored at 0°C until use.

Labeling Procedure: One milligram chlordiazepoxide was dissolved in $50\ \mu\text{l}$ 50 % HCl solution (v/v) and volume was adjusted to one ml. Chlordiazepoxide solution was added to iodogen coated reaction tube, then approximately 37-74 MBq (1-2 mCi) carrier-free Na^{131}I was added. The mixture was separated from the tube at the end of the reaction and 0.2 N Na_2SO_3 solution ($100\ \mu\text{l}$) was added to reduce non-incorporated iodine. Labeling efficiencies were determined by ITLC and paper electrophoresis. R_f values are shown in table-1.

Labeling efficiency was 90%.

Preparation of iodinated chlordiazepoxide in inactive conditions: Chlordiazepoxide was iodinated in inactive conditions using iodogen method. Product was precipitated by phosphate buffer then 0.2 N Na_2SO_3 solution was added. Precipitate was allowed in refrigerated temperature for one day the dried after centrifuged. This sample was used to take GC-MS spectrum.

Influence of Different Parameters: The dependence of the labeling yield on the different parameters like iodogen amount and pH were investigated. pH of chlordiazepoxide solution was adjusted to 3, 5, 7 by NH_4OH before labeling to determine pH effect. Iodogen films were prepared with 0.1 mg, 0.5 mg and 1 mg iodogen to determine iodogen amount.

Scintigraphic Studies on Rabbits: ^{131}I labeled chlordiazepoxide has been sterilized by passing through a $0.22\ \mu\text{m}$ membrane filter. Then it has been injected from ear vein of male rabbit. Static images have been taken by Sophy DX Gamma Camera.

RESULTS AND DISCUSSION

The dependence of the yield of $^{131}\text{ICDZ}$ on different labeling parameters is shown in fig. 2 and fig. 3. According to these results optimum reaction conditions are pointed out in table-II.

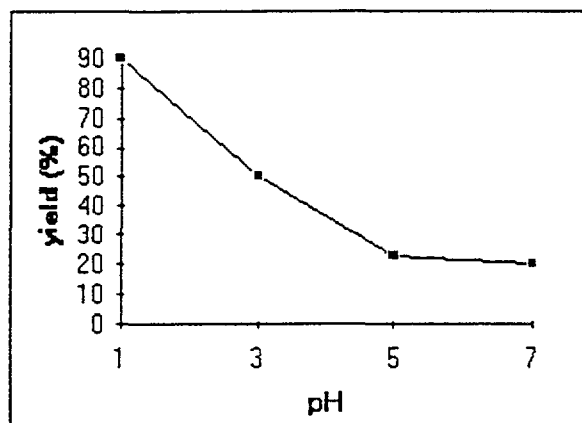


Figure-2 pH effect to labeling yield of $^{131}\text{ICDZ}$

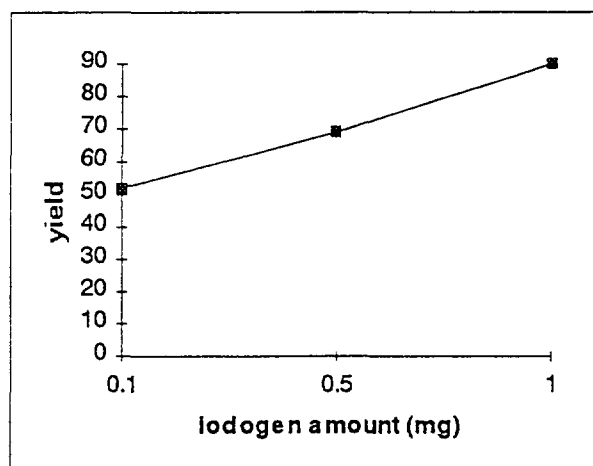


Figure-3 Iodogen amount effect to labeling yield of $^{131}\text{ICDZ}$.

Table -II Optimum conditions for labeling of chlordiazepoxide with I-131.

CDZ amount	1 mg
iodogen amount	1 mg
pH	1
reaction time	15-20 min.
Stability of $^{131}\text{ICDZ}$	19-20 hours

Table-III: Some of the molecular fragments peaks of chlordiazepoxide and iodo chlordiazepoxide Mass Spectra.

fragments	m/z
$M^+ - O$	282
$M^+ - (O - A)$	252
$M^+ - (O A - Cl)$	218
$M^+ - (O A - Cl) - B$	140
$M^+ - (O A - B)$	175
M^+	298
$B + I$	203

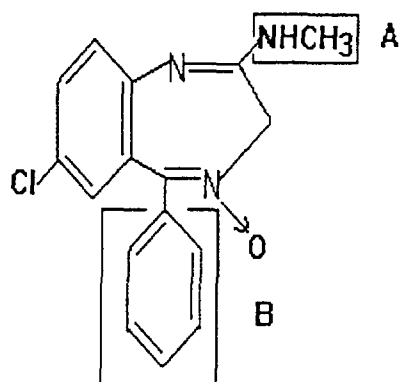


Figure 4: Molecular fragments of chlordiazepoxide.

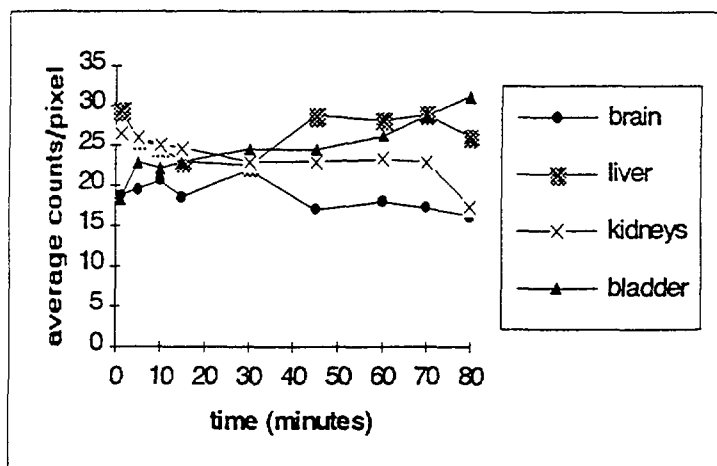


Figure-5: Biodistribution of injected radioactivity for some selected organs.

According to GC-MS spectrum iodination take place by electrophilic substitution on the aromatic ring. As a result of GC-MS spectrum A and B fragments separate from molecule (fig. 4). Table-III shows main fragments of mass spectra of ICDZ. Although molecular ion peak of chlordiazepoxide is 298, we couldn't see iodinated molecular peak at 424 in GC-MS. The reason might be too heavy mass of iodinated chlordiazepoxide. For this reason we couldn't see this peak but other iodinated fragments were seen.

In fig. 5, the biodistribution in counts per pixel of injected radioactivity for some selected organs are given for chlordiazepoxide. The highest accumulation occurs in liver and kidneys 10 minutes after injection. Within 1 hour, excretion occurs from kidneys and bladder. Brain reaches the highest activity in 30 minutes and elimination is slow during the 90 minutes.

Verhoeff and coworkers labeled iomazenil with ^{131}I and they studied benzodiazepine receptor density in human cerebellum and cerebral cortex by SPECT. In this work, they repeated the same experiment after receptors were saturated with inactive flumazenil and activity distributions in organs were determined. Highest activity has been seen in bladder, then brain, kidneys and heart for unsaturated cases. We have seen similar distribution for ^{131}I labeled chlordiazepoxide in rabbits [10].

Kuhl and coworkers obtained SPECT images of human brain with ^{123}I labeled (iodobenzovesamicol). Highest activity was seen in liver, lungs and brain as count per pixel. Activity was decreased for all organs in four hours then remained constant. In ^{131}I labeled chlordiazepoxide brain activity was increased for 20 minutes then didn't change so much up to 90 minutes [11].

As conclusion, obtained results show that chlordiazepoxide can be used as benzodiazepine receptor radiopharmaceutical. Iodogen is a fast and efficient method to label chlordiazepoxide. Labeled product is stable during the working period at room temperature. It has some advantages like that to be obtainable easily and cheap as a brain radiopharmaceutical.

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RADIOPHARMACEUTICAL POTENTIAL OF I-131 LABELLED DIAZEPAM



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Abstract

In this study, diazepam is a derivative of the 1.4 benzodiazepine family that the most widely used drug as anticonvulsant agent has been labeled with I-131, as a new radiopharmaceutical and its radiopharmaceutical potential has been determined.

Labeling of diazepam has been performed by iodogen method and optimum labeling conditions have been determined.

Optimum reaction conditions are 1 mg for iodogen amount; 1-5 mg for diazepam amount; 15-20 minutes for reaction time and room temperature for reaction temperature. Specific activity of labeled compound was 0.15 Ci/mmol level.

N-octanol/water ratio was found 1.9 for ^{131}IDZ (^{131}I labeled diazepam). In vivo experiments have been carried out to determine radiopharmaceutical potentials of labeled compound. Biodistribution studies on rats showed that ^{131}IDZ have accumulated in kidneys, liver, lungs and brain tissues.

Scintigraphic results taken with gamma camera on rabbits agree with biodistribution results of rats.

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INTRODUCTION

Since usage of radioisotopically labeled radiopharmaceuticals increase day by day, importance of the other disciplines that is related with this area have increased. Certainly developing of the Nuclear Medicine depends on the Radiopharmacy and Radiochemistry that most are the most related disciplines with this scientific area.

Since their introduction to human use as antianxiolytic and anticonvulsant in the 1950's, benzodiazepines have been the most widely prescribed drugs. They have antianxiolytic and anticonvulsant effects. Diazepam is the most used of them [1]. Fig.1 shows molecular structure of diazepam.

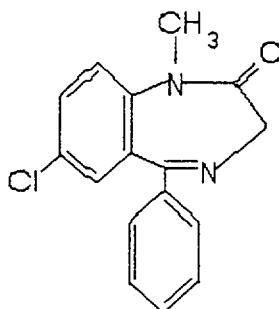


Figure-1: Molecular structure of diazepam.

Innis and coworkers reported that ^{125}I labeled Ro16-0154 has 10 times more affinity compared with fluoride derivative [2]. ^{123}I labeled Ro16-0154 have been proposed as a good benzodiazepine receptor imaging agent for SPECT. Additional iodinated derivative has higher lipophilicity. Octanol/water ratio is two times higher.

There are some reports about benzodiazepine receptor imaging agents in use for SPECT or PET [2-6]. Comparing two techniques can be useful. Iida and coworkers have been prepared 2' iododiazepam with ^{125}I by chemical synthesis and they have attached ^{11}C to N-1 position, ^{125}I to 2' position of the molecule. They showed that this ligand has 9 times more affinity comparison to diazepam [3].

Generally ^3H -diazepam and ^3H -flunitrazepam have been used as radioligands for benzodiazepine receptor studies. However working with ^3H -labeled compounds are confusing and time consuming. Liquid scintillation counting equipment is necessary for radioactivity measurements. Besides ^{125}I labeled ligands can be measured easily and more quantitatively by NaI(Tl) scintillation equipment. Furthermore ^{123}I -diazepam ($2'^{123}\text{IDZ}$) can be used for benzodiazepine receptor studies in Nuclear Medicine. It has been shown that both ^{125}I -Diazepam and ^3H -Diazepam bond to same receptors. According to these results ^{125}I -Diazepam can be used for benzodiazepine receptor studies. Saji and coworkers prepared ^{125}I labeled diazepam by chemical synthesis and reported that 2' position of diazepam in phenyl ring is the best position for iodine labeling. ^{125}I labeled benzodiazepines have higher receptor affinity and they are appropriate for SPECT studies [4] and they proposed that ^{125}I labeled diazepam can be an ideal receptor imaging radiopharmaceutical. Maziere and coworkers labeled flunitrazepam and diazepam with ^{11}C [5].

In this study, diazepam (7-chloro-1,3-dihydro-1-methyl-5-phenyl-phenyl-2H-1,4-Benzodiazepine-2-one) was labeled with iodogen method and its radiopharmaceutical potential was experimented on rabbits and rats.

EXPERIMENTAL

Diazepam was purchased commercially. Na^{131}I was obtained from Department of Nuclear Medicine. Iodogen was purchased from Sigma Co. All other chemicals were purchased from Merck and they were reactive grade.

Equipment: Labeling yields were determined by ITLC (Instant Thin Layer Chromatography) and electrophoresis methods. Procedure was the same with Unak and coworker's study [7]. R_f values were shown in table-I.

Preparing Iodogen Coated Tubes: Iodogen coated tubes were prepared as described earlier [6,7].

Labeling Procedure: Diazepam was labeled described earlier by Yurt et al. applying Iodogen method [6]. Two different solvent systems were used to solve diazepam. Solvent

1. Propylene glycol mixture (propylene glycol 40%, sodium benzoate 5%, ethyl alcohol 1.5%, v/v) Solvent 2. 50% HCl (v/v). Specific activity of labeled compound was 0.15 Ci/mmol level for solvent-1.

Preparation of Iodinated Diazepam in Inactive Conditions: Diazepam was iodinated in inactive conditions using iodogen method. The same procedure was applied with inactively iodinated chlorodiazopoxide [7]. This sample was used to take GC-MS and ^1H NMR spectra.

Measurement of the Octanol/Water Partition Coefficient: Lipophilicity was determined according to a previously reported method [8]. A 50 μl aliquot of radioiodinated sample was mixed with 3 ml each of 1-octanol and 0.1 M phosphate buffer (pH 7.4) in a test tube. The tube was vortexed (3x1 min), incubated for 1 hr at room temperature, and then centrifuged for 5 minutes. The 0.5 ml aliquots of each phase were removed and counted in a well-type NaI scintillation counter.

Scintigraphic Study in Rabbits: ^{131}I labeled diazepam with 160 mCi/mmol specific activity was sterilized by passing through a 0.22 μm membrane filter. Then it was injected from ear vein of male rabbits. Static perspectives were taken by Sophy DX 124x124 Gamma Camera.

Biodistribution Study in Rats: ^{131}I labeled diazepam was passed through from 0.22 μm membrane filter and it was administered intraperitoneally to male rats. Three rats were used for each point of the experiment. Injections were administered intraperitoneally. The rats were killed by decapitation after ether narcotization in an ether atmosphere and their organs were removed and weighted at different times. Their activities were counted by a well-type NaI(Tl) scintillation detector of multi-channel analyzer after marking 364 keV γ photons of ^{131}I . Total injected activities were supposed relatively 100 and percentage activities of organs per gram were calculated.

RESULTS AND DISCUSSION

Seventy-nine per cent yield were obtained in control experiments for strongly acidic conditions. It is known that iodide oxidizes in strongly acidic medium [9]. Iodide might enter a substitution reaction at this condition. On the other hand, if diazepam is waited for

Table -I R_f values of ¹³¹IDZ and ¹³¹IDHydrolysis product in different developing medium.

ITLC1:[n-Buthanol-Water-Acetic Acid (4/2/1)].

ITLC2:[Isopropyl Alcohol-n-Buthanol-0.2NAmmonium hydroxide (2/1/1)].

Developing medium	ITLC1	ITLC2
	R _f	R _f
¹³¹ IDHydrolysis product	0.33 - 0.50	0.43 - 0.57
¹³¹ IDZ	0.91 - 1.00	0.88 - 1.00

Table-II Some of the molecular fragments peaksof iododiazepam at Mass Spectrum.

fragments	m/z
M ⁺ + H ⁺	412
(M ⁺ + H ⁺) -CH ₃	396
M ⁺ - C=O	383
Agrubu(C ₁₃ H ₈ ClI)	326
A ⁺ + C=O	354
A ⁺ + CH ₃	341
A ⁺ - Cl	291
Ph - I	203

Table-III Per cent of injected activity per gram for several organs of rats.

Time (min.)	Brain	Liver	Lung	Kidney	Heart	Blood	Brain/Blood
10	0.04 (0.01)*	0.07 (0.02)	0.09 (0.03)	0.12 (0.08)	0.07 (0.07)	0.12 (0.05)	0.32 (0.04)
20	0.06 (0.01)	0.13 (0.01)	0.13 (0.01)	0.42 (0.14)	0.11 (0.01)	0.16 (0.01)	0.36 (0.01)
40	0.15 (0.01)	0.50 (0.09)	0.46 (0.02)	0.91 (0.06)	0.68 (0.16)	0.41 (0.07)	0.37 (0.07)
60	0.19 (0.06)	0.63 (0.10)	0.53 (0.12)	1.40 (0.26)	0.40 (0.08)	0.45 (0.01)	0.42 (0.12)
120	0.11 (0.01)	0.27 (0.06)	0.23 (0.10)	0.55 (0.19)	0.46 (0.35)	0.24 (0.10)	0.55 (0.24)

* Standard deviation

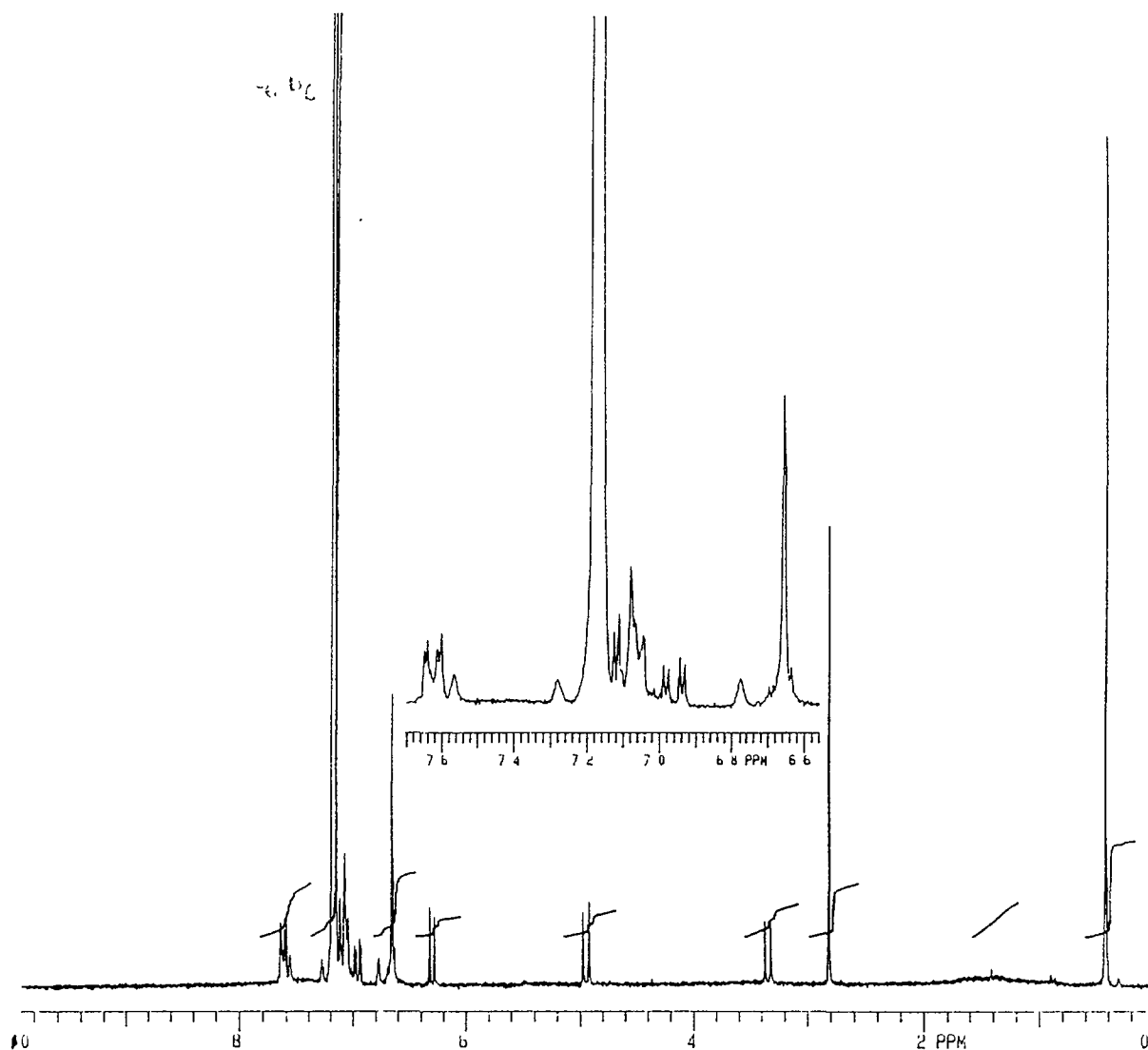


Figure-2: ^1H NMR Spectrum iodo derivative of diazepam.

a few days in acid solution, hydrolyses occur. For this reason two different products have been obtained for control studies (table-1). In the case of using of iodogen method 84% yield has been obtained for solvent-2 and 64% for solvent-1. Although the yield is less, solvent-1 was preferred to protect hydrolysis of diazepam. Optimum reaction conditions are 1 mg for iodogen amount; 1-5 mg for diazepam amount; 15-20 minutes for reaction time and room temperature for reaction temperature. On the other hand this product was stable at refrigerated temperature for a few days.

^1H NMR spectrum of iodo derivative of diazepam in C_6D_6 is shown in fig. 2. Molecular fragments of GC-MS are given in fig.3 and table-II. δ Values are 2.8 ppm for

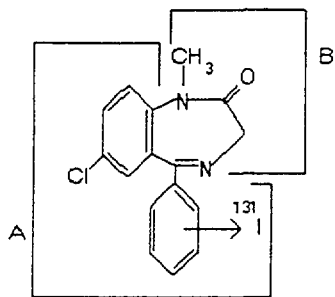


Figure-3: Molecular fragments of GC-MS.

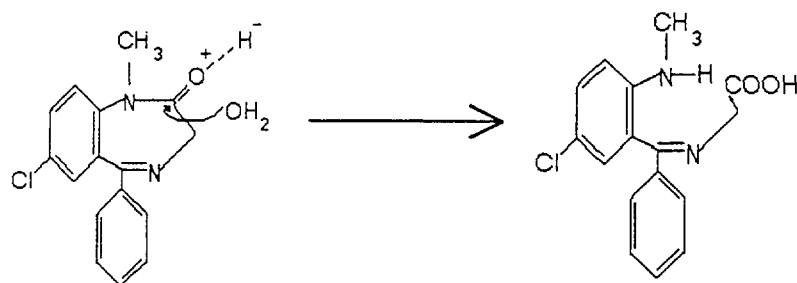


Figure-4: Hydrolysis mechanism of diazepam in acidic medium.

N-CH₃ protons, 4.97 for two doublets, 3.37 for 3rd position aliphatic C-H protons, 6.32-7.1 for phenyl ring protons. ¹H NMR results indicate that iodine may substitute with phenyl ring hydrogen by electrophilic substitution. GC-MS spectrum shows that diazepam hydrolysis in acidic medium as shown in fig. 4. Table-II shows the molecular fragments according to A fragmentation.

Fig. 5 shows the biodistribution of radioactivity for several organs in rabbits. Counts per pixel/background of brain were 1.9 after five minutes from the injection then this value remained as 1.9. The highest accumulation occurs in liver within 30 minutes after injection. Then a very rapid decline takes place. Elimination is slower for other organs like brain and kidneys.

Table-III shows percentage of injected activity per gram for several organs of rats. Uptake in heart and lungs is higher than brain after ten minutes from the injection. This result agrees with Iida and collaborates [3]. Maximum brain uptake was reached at 60th minutes. Innis and coworkers were obtained maximum brain uptake at 70th minutes at

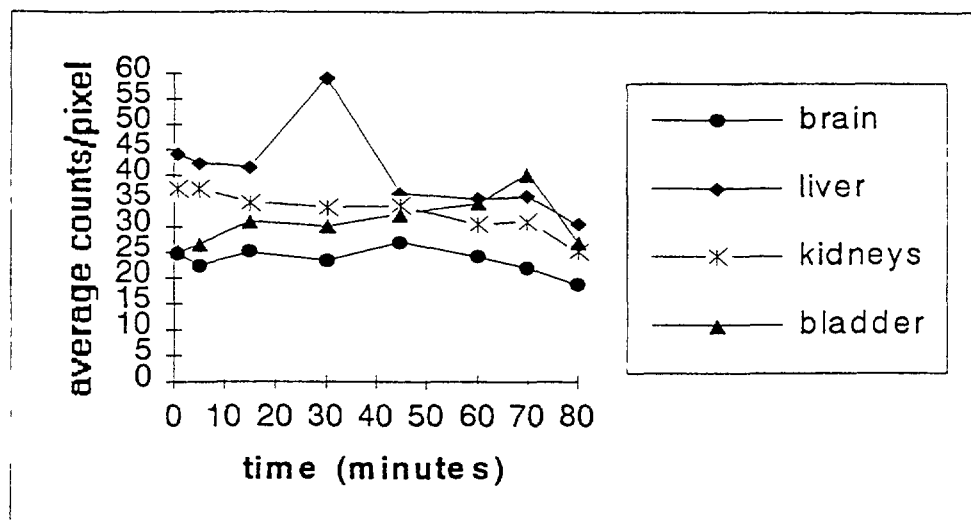


Figure-5 Biodistribution of radioactivity for several organs in rabbits.

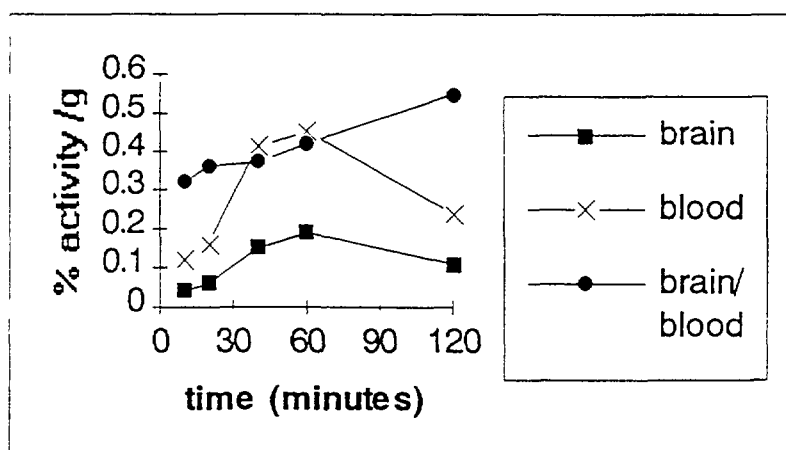


Figure- 6 Blood and brain radioactivities and brain/blood ratio in rats.

monkeys with ^{123}I -Ro-16-0154 [2]. Another study carried out with rats with ^{123}I Ro 16-0154. Maximum uptake was obtained 10 minutes after intravenous injection [10]. As a result of intravenous administration migration of radiopharmaceutical becomes faster. Percentage of injected activity in kidneys was 1.4 after one hour and 0.55 after two hours. This means that $2/3$ of activity clears from kidneys in one hour. Clearance rate is $1/2$ in lungs and it is higher in kidneys than lungs. Clearance rate is not very high for other organs ($<1/2$). If distribution depends on blood perfusion high uptake organs should be clear rapidly. It has been shown that uptake in ^{11}C -2'Iododiazepam doesn't depend on only

blood perfusion. Clearance rate is not also very high in this work. Obtained results agree with Iida [3]. For this reason we can say that distribution doesn't depend on only blood perfusion. Activity decrease in blood is faster than brain. This result agrees with Beer and coworkers [10]. The elimination from brain is considerably slower and reaches 0.19% per g organ after 1 hour and 0.11 at two hours. Highest activity is seen in liver (0.63) after kidneys. This rate decreases to 0.27 after two hours. Diazepam desmethylates in liver and elimination is slow. Decreasing rate is low because of the demetabolization in liver.

Figure 6 shows blood and brain radioactivities and brain/blood ratio in rats. The highest value in the blood and the brain is reached within 60 minutes after injection. The decrease of radioactivity in the blood is faster than in the brain. A good uptake in brain indicates that this radiopharmaceutical might be a good SPECT agent. Increasing brain/blood ratio by time shows ^{131}I -iododiazepam is cleared rapidly from blood and brain uptake is high. This result agrees with Iida [3] and Saji [4].

Lipophilicity value was found 1.9. It is known that brain uptake is fast and high related with the high n-octanol/water ratio [4]. On the other hand, Kung and coworkers reported that lipid soluble and neutral compounds can freely cross the blood-brain barrier [11].

As a result, I-131 labeled diazepam shows a potential and can be used to obtain SPECT images of brain.

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⁶⁷Ga(NODASA): A NEW POTENTIAL BIFUNCTIONAL RADIOLIGAND FOR COUPLING TO PEPTIDES

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Abstract

A new bifunctional chelator NODASA (1,4,7-triazacyclononane-1-succinic acid-4,7-diacetic acid) has been synthesised and its Ga(III) complex was crystallographically characterized by X-ray diffraction. The complex showed to be stable in serum and in acidic conditions and its stability constant was determined using a competition method with an auxiliary ligand. The conjugation of Ga(NODASA) to a model aminoacidamide proved the feasibility of a prelabelling approach.

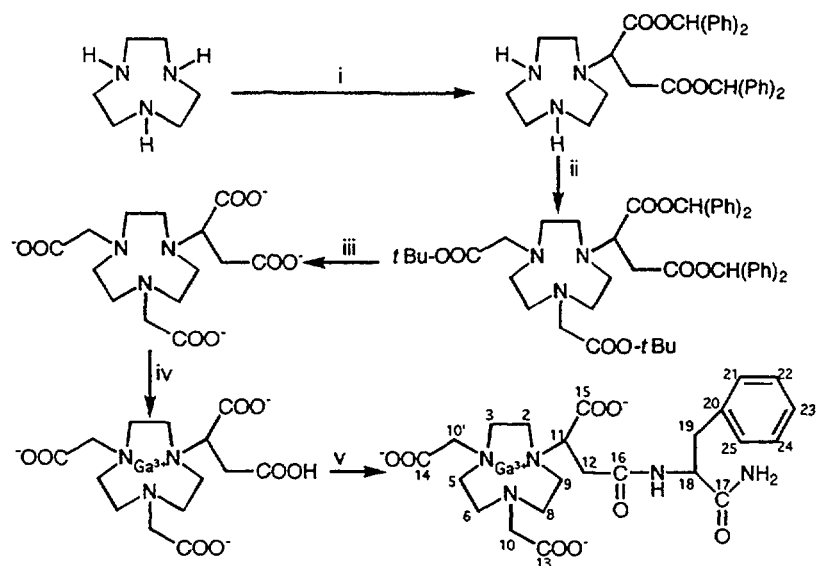
Aiming for the selective delivery of radionuclides to tumors the chemistry of bifunctional chelators had a considerable development over the last ten years.^{1,2} Many of these chelators were designed for coupling to monoclonal antibodies³⁻⁵ directed to tumor-associated antigens. The successful introduction of peptides^{6,7} that are bound by tumor membrane receptors (for instances somatostatin receptor positive tumours) originated a search for other bioactive peptides and for new chelators. As these peptides show very fast blood clearance and diffusion into tissues the use of short lived metallic positron emitters becomes feasible. In this context ⁶⁸Ga is of special interest. Due to the fact that the last one is generator produced and its short half-life ($t_{1/2}=68$ min) is compatible with the rate of localization of small targeting molecules PET studies can be done in institutions with PET cameras available but no cyclotrons.

For our purpose a bifunctional macrocyclic chelator is needed which is comprised of a gallium(III) immobilizing moiety and a short free carboxylate arm for coupling to the N-terminal end of bioactive peptides. We aim at using this bifunctional chelator in a preconjugation $^{67,68}\text{Ga}$ -labelling approach of somatostatin analogues.

Therefore we synthesised a new N-functionalised macrocyclic ligand (1,4,7-triazacyclononane-1-succinic acid-4,7-diacetic acid; NODASA) in 3 steps by alkylation of 1,4,7-triazacyclononane (Scheme 1): with 1 equivalent of bis(diphenylmethyl) D,L-bromosuccinate in chloroform (i); with 2 equivalents of *tert*-butyl bromoacetate in acetonitrile in the presence of K_2CO_3 (ii) followed by deprotection with 6 M HCl (iii). The overall yield of the synthesis of NODASA was slightly over 50%. The ligand had satisfactory elemental analysis, ^1H , ^{13}C NMR and mass spectra. Stepwise deprotonation constants for the ligand defined as $K_{\text{H}_n\text{L}} = [\text{H}_{n-1}\text{L}][\text{H}^+]/[\text{H}_n\text{L}]$ ($n=1-5$) were determined by potentiometric titration with Me_4NOH at 25.0°C and 0.5 M KNO_3 as the background electrolyte. The program TITFIT⁸ was used for calculation and the $\text{p}K_{\text{a}}$ values were found to be: $\text{p}K_{\text{HL}^3}=11.71$, $\text{p}K_{\text{H}_2\text{L}^2}=5.94$, $\text{p}K_{\text{H}_3\text{L}^1}=4.27$, $\text{p}K_{\text{H}_4\text{L}}=3.22$ and $\text{p}K_{\text{H}_5\text{L}^+}=1.95$.

Crystals of $\text{Ga}(\text{NODASA})^\#$ were obtained from an aqueous solution of the ligand and $\text{Ga}(\text{NO}_3)_3$ in equimolar amounts at pH 3 and 70°C for 1 hour (Scheme 1-iv), followed by slow evaporation of the solvent. Characterization by X-ray diffraction showed that the complex is electrically neutral and the Ga(III) ion is fully chelated in a slightly distorted octahedral environment by the three amine nitrogens and by three carboxylate oxygens (Figure 1). The β -carboxylate of the succinate arm remains protonated and does not participate in the complexation. The three nitrogen atoms of the triazacyclononane define a plane in a facial arrangement and three of the pendant

[#] Crystal data: $\text{Ga}(\text{C}_{14}\text{H}_{20}\text{N}_3\text{O}_8) \cdot 3\text{H}_2\text{O}$, monoclinic, space group $P2_1/n$ ($a=7.6077(6)$, $b=20.573(3)$, $c=12.186(2)$ Å, $\beta=97.726(9)^\circ$), $Z=4$, $F(000)=1000$, $\mu=2.56\text{ mm}^{-1}$, $\text{Cu-K}\alpha=1.54180$ Å, $T=293\text{ K}$, $\theta_{\text{max}}=77.50^\circ$, $\omega/2\theta$ scan technique, 3561 independent reflections, 3011 used in refinement, 284 parameters refined, final $R=5.08$, final $R_w=0.0626$, Chebychev polynomial weighting.



Scheme 1 - Synthesis of the chelate Ga(NODASA) and coupling to D-phenylalanineamide.

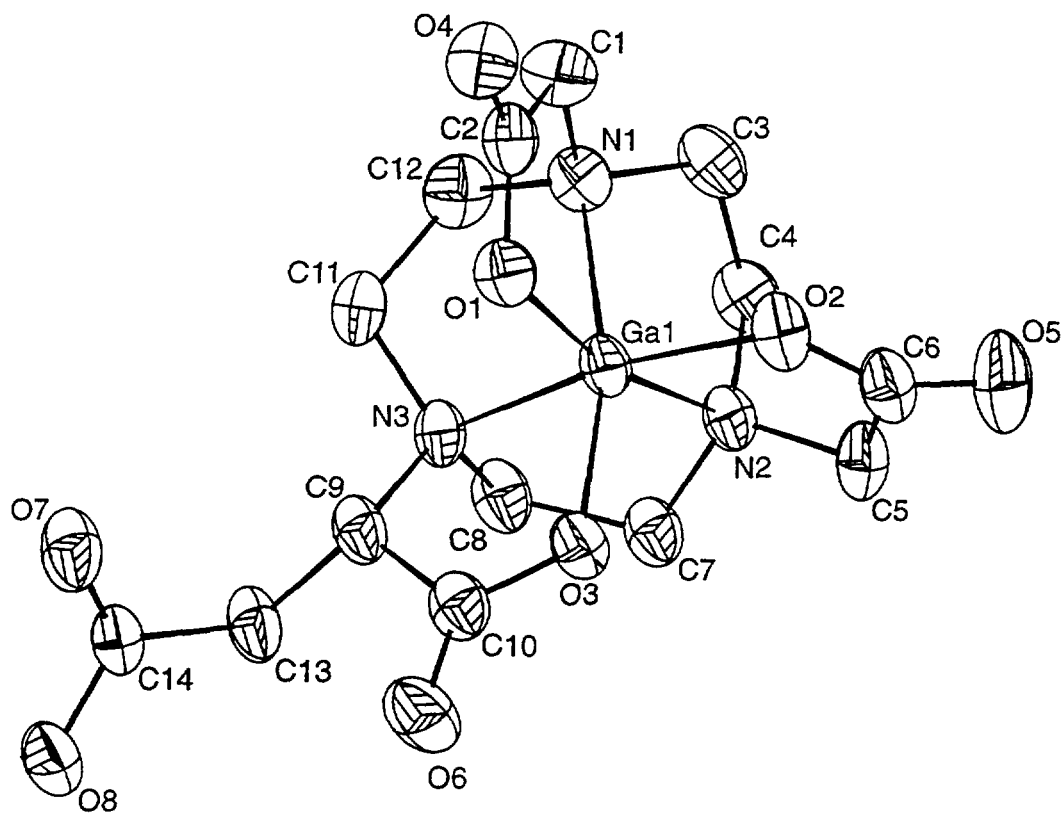


Figure 1. - ORTEP drawing of the Ga(NODASA) complex. Ga-N(1) 2.101(3), Ga-N(2) 2.098(3), Ga-O(1) 1.937(3), Ga-O(3) 1.942(3) Å; O(1)-Ga-O(3) 94.7(1), O(2)-Ga-N(3) 165.6(1), O(2)-Ga-N(2) 82.5(1), N(2)-Ga-N(3) 84.1(1)°.

carboxylate oxygens constitute another one. These two planes are almost coplanar forming a dihedral angle of 1.75° . The *trans* N-Ga-O bond angles average 165.43° which is less than the ideal 180° due to the small "bite" angle of the adjacent 5-ring chelates. This leads to a relative twist of the N_3 and O_3 planes by 14.6° away from a symmetrically staggered conformation (octahedron), similar to the parent complex Ga(NOTA) (NOTA = 1,4,7-triazacyclononane-1,4,7-triacetic acid) where this angle is 13° .⁹ The variation in the individual Ga-O and Ga-N bond lengths is very small (Ga-O averages $1.934(3)$ Å; Ga-N averages $2.104(3)$ Å) showing overall high similarity to the related complex Ga(NOTA).¹⁰

Figure 2 shows the ^1H NMR spectrum of Ga(NODASA) in D_2O . The multiplet centered at 3.75 ppm, due to the non-magnetically equivalent acetate hydrogens, is the most dramatic effect in the ^1H NMR spectrum[§] of the chelate in relation to the free ligand, which exhibit a singlet at 3.50 ppm. The sharp multiplets of the ethylenic protons might show the existence of slow intramolecular processes of interconversion between the $(\lambda\lambda\lambda)$ and $(\delta\delta\delta)$ conformations¹¹ of the ring backbone arising from the high rigidity of a system with strong coordination to a metal ion of small size that fits well into the cavity of the ligand.

Although the structural results give some important indications about the binding of the macrocycle to Ga(III), the thermodynamic stability of this chelate is very important for successful applications *in vivo*. In the present case, as the kinetics of formation of Ga(III) complexes with macrocyclic ligands is very slow, the standard potentiometric methods are not useful for the determination of the stability constants of its NODASA complex. In competition methods the position of equilibrium in a metal-ligand system is influenced by means of a third component (another metal or another ligand). Radioactive metallic ions are very suitable to be used in competition experiments once the activity of their complexes can be monitored by HPLC or TLC.

[§] The assignment was done with the aid of two-dimensional methods.

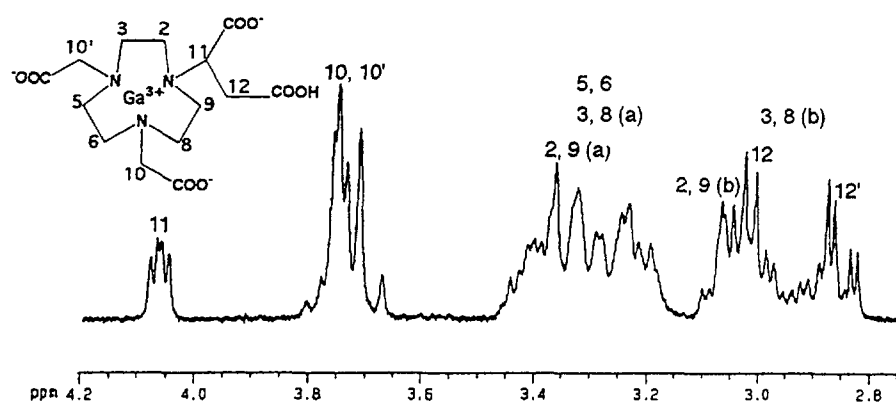


Figure 2 - ^1H NMR spectrum (400MHz) of Ga(NODASA) in D_2O , 7 mM, $\text{pD}=3.6$ and $T=22^\circ\text{C}$.

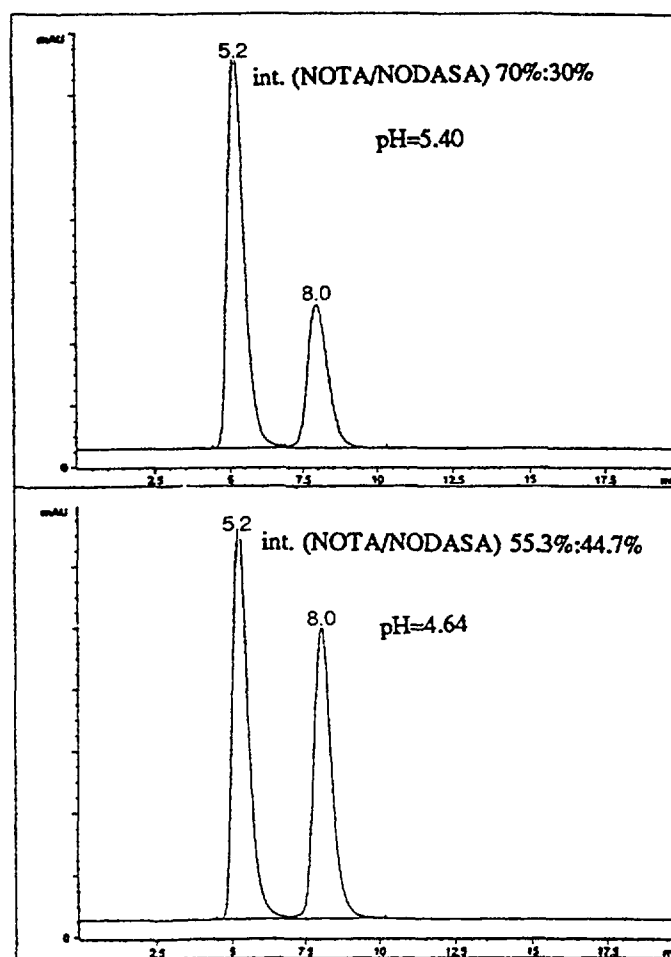


Figure 3 - Radiochromatograms of $^{67}\text{Ga}(\text{NOTA})$ and $^{67}\text{Ga}(\text{NODASA})$.

Using ^{67}Ga as a radiotracer and NOTA as the auxiliary competing ligand it has been possible to estimate the conditional stability constant for the complex $\text{Ga}(\text{NODASA})$ at different pH values. The equilibration has been followed for eleven days and aliquots of the samples have been analysed by HPLC with radiodetector. The Figure 3 shows the radiochromatograms of $^{67}\text{Ga}(\text{NOTA})$ and $^{67}\text{Ga}(\text{NODASA})$ (with retention times of 5.2 min and 8.0 min respectively) at pH 4.64 and 5.40 using an HAMILTON PRP-X200 column and eluting with 20 mM ammonium acetate buffer pH 5.0 (flow 0.5 ml.min $^{-1}$).

The conditional stability constants are defined as

$$K_{\text{cond}} = \alpha K$$

where K is the thermodynamic stability constant and

$$\alpha^{-1} = (1 + [\text{H}^+]/K_1 + [\text{H}^+]^2/K_1K_2 + \dots)$$

(K_1, K_2, \dots represent the stepwise protonation constants of the ligand). With the knowledge of the protonation constants of the two ligands and of the thermodynamic stability constant of the complex $\text{Ga}(\text{NOTA})$ ¹⁰ we can calculate the thermodynamic stability constant of the complex under study and the value found was $\log K_{\text{Ga}(\text{NODASA})} = 30.9(0.2)$ compared to 30.98 of $\text{Ga}(\text{NOTA})$.¹²

An even more important indicator for *in vivo* applications is the direct kinetic measurement under the application conditions, i.e., measurements of the rate of exchange of $\text{Ga}(\text{III})$ in blood serum. For this experiment $^{67}\text{Ga}(\text{III})$ is first incubated with about 50 times excess of NODASA at pH 6.2 in 0.5 M ammonium acetate buffer for 25 min at 90°C in order to incorporate the metal ion. Then the complex is mixed with blood serum and the exchange kinetics with transferrin are measured at 37°C. This was done by taking aliquots of the serum, kept at 37°C in an environment containing 5% of CO_2 , separating them by gel filtration (G50 Sephadex), eluting with PBS buffer, which allows the separation of the low molecular complex from $\text{Ga}(\text{III})$ -transferrin ($\log K = 23.7$)¹³ and measuring the activity in both fractions with use of radiometric detection.¹⁴ The results clearly showed that $^{67}\text{Ga}(\text{NODASA})$ virtually does not transfer any ^{67}Ga to transferrin over a period of at least 5 days, fulfilling hence the criteria for *in vivo* application.

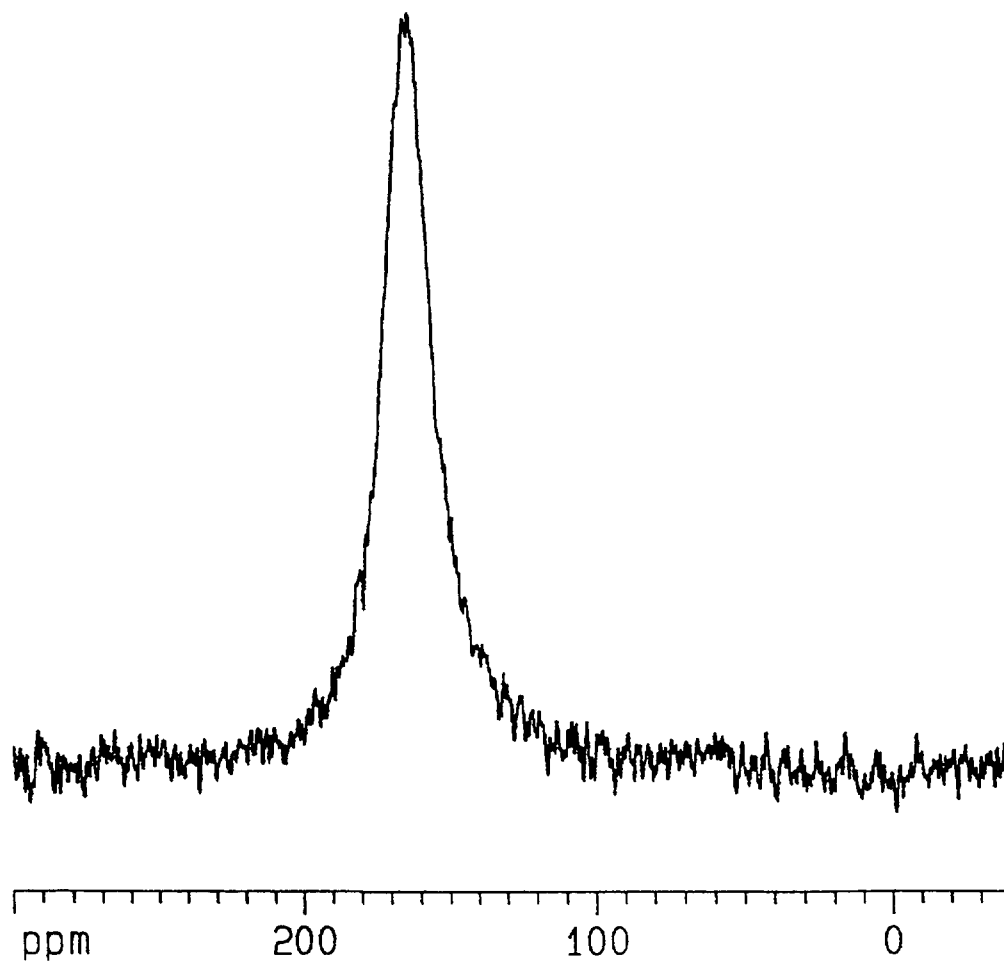


Figure 4 - ^{69}Ga NMR spectrum of Ga(NODASA) in aqueous solution (Bruker Biospec 70/20, magnetic field strength 7 Tesla, corresponding to a ^{69}Ga resonance frequency at 72.0 MHz).

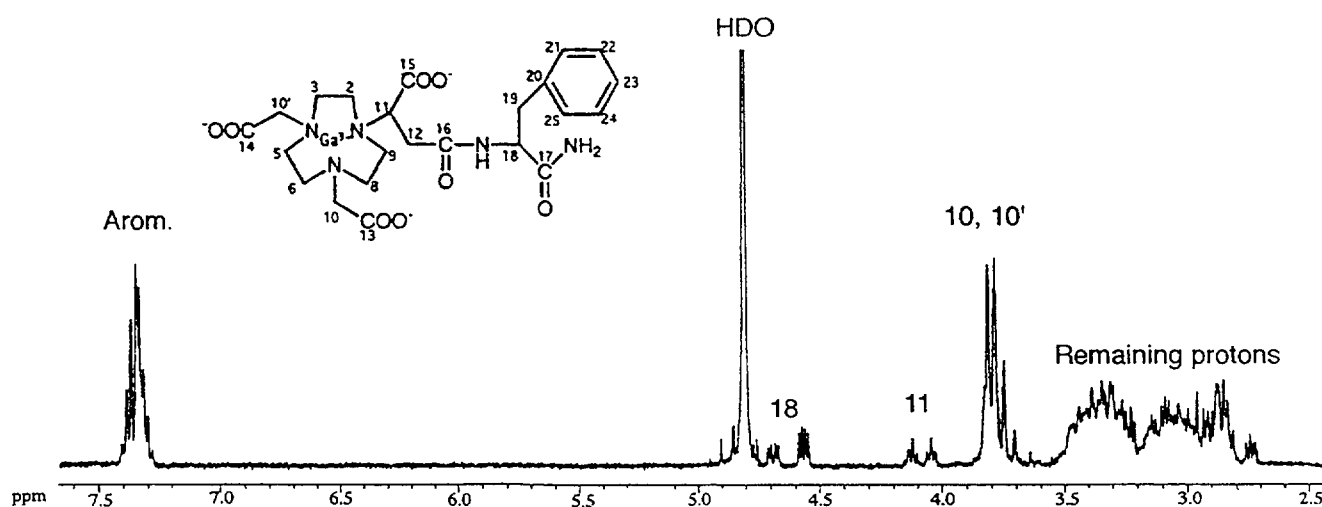


Figure 5 - ^1H NMR spectrum (400 MHz) of the conjugate in D_2O , 20 mM, $T=22^\circ\text{C}$.

The kinetic stability of Ga(NODASA) with respect to the acid-catalysed dissociation has been demonstrated with the aid of the ^{67}Ga complex, kept in 0.1 M glycine-HCl buffer, pH 2, at 37°C. Aliquots of this solution were analysed by HPLC. After 10 days the complex was still 100% undissociated. Moreover ^{69}Ga NMR spectroscopy showed that even after several weeks at pH 0.6 in HCl the complex Ga(NODASA) was still intact, only its characteristic peak at 165 ppm could be seen and no free $\text{Ga}(\text{H}_2\text{O})_6^{3+}$ was detectable (Figure 4) proving the high stability of the chelate with respect to the acid-catalysed dissociation.

The fact that the β -carboxylate remains free meanwhile the other three carboxylates are involved in 5-membered chelate rings, upon coordination to the metal ion, offers a very interesting possibility to couple the chelate to a biomolecule. As a model peptide we coupled D-phenylalanineamide to Ga(NODASA) (Scheme 1-v, in DMSO/DMF, 2:1) using HATU (O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate).¹⁵ as the coupling reagent and DIPEA (N-ethyl-diisopropylamine) as base, with high yield. In Figure 5 the ^1H NMR spectrum of the conjugate in D_2O it is shown. R_f (SiO_2 , isopropanol/ $\text{NH}_3(\text{aq})$, 7:3 = 0.40); m/z (ESI^+): 574.4 (MH^+ , 15), 594.1 (MNa^+ , 100); ^{13}C NMR 100 MHz (D_2O) δ (ppm): 32.0 (C_{12}), 38.3 (C_{19}), 44.0 ($\text{C}_{2,9}$), 53.0-52.4 ($\text{C}_{3,5,6,8}$), 54.4 and 54.8 (C_{18}), 61.5 ($\text{C}_{10,10'}$), 64.5 and 65.0 (C_{11}), 128.4-128.9 (C_{21-25}), 136.8 (C_{20}), 169.4 (C_{17}), 171.5 ($\text{C}_{13,14}$), 172.4 (C_{15}), 174.0 (C_{16}).

HATU allows coupling of carboxylate functions to primary amines within minutes rendering even the coupling of $^{68}\text{Ga}(\text{NODASA})$ to peptides feasible. Conclusion: $^{67,68}\text{Ga}(\text{NODASA})$ can be used in a pre-labelling approach followed by conjugation to a biomolecule. This approach is currently being followed using somatostatin analogues.

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INVITED LECTURE

THE DEVELOPMENT AND USE OF RADIONUCLIDE GENERATORS IN NUCLEAR MEDICINE — RECENT ADVANCES AND FUTURE PERSPECTIVES

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Abstract

Although the trend in radionuclide generator research has declined (FIG. 1), radionuclide generator systems continue to play an important role in nuclear medicine [1-3]. Technetium-99 m obtained from the molybdenum-99/technetium-99 m generator system is used in over 80 per cent of all diagnostic clinical studies and there is increasing interest and use of therapeutic radioisotopes obtained from generator systems. This paper focuses on a discussion of the major current areas of radionuclide generator research, and the expected areas of future research and applications.

1. The Molybdenum-99/Technetium-99 m Generator

The easiest and preferred route for obtaining technetium-99 m for radiopharmaceutical "kit" labeling is *via* elution of the chromatographic-type molybdenum-99/technetium-99 m generator. Because technetium-99 m is expected to continue to be the primary radioisotope used for imaging in diagnostic clinical nuclear medicine, the availability of molybdenum-99 is crucial to the radiopharmaceutical and clinical communities. In the more developed countries, fission-produced molybdenum-99 for is used for fabrication of the chromatographic-type molybdenum-99/technetium-99 m generator [4]. Although until recently only one site provided most of the fission-produced molybdenum-99 for generator fabrication in North America, there are currently several new sites throughout the world producing this important radioisotope (Table 1). With these greatly increased availability of production and processing facilities, it is expected that the international demands for molybdenum-99 can be readily met.

Although the chromatographic-type generator using fission-produced molybdenum-99 would be the method of choice for obtaining technetium-99 m , sublimation [5] and solvent extraction [6] techniques are still used in many parts of the world to obtain batches of technetium-99 m where molybdenum-99 produced by neutron irradiation of enriched molybdenum-98 (n , gamma route) is available as an alternative to fission-produced molybdenum-99. Solution

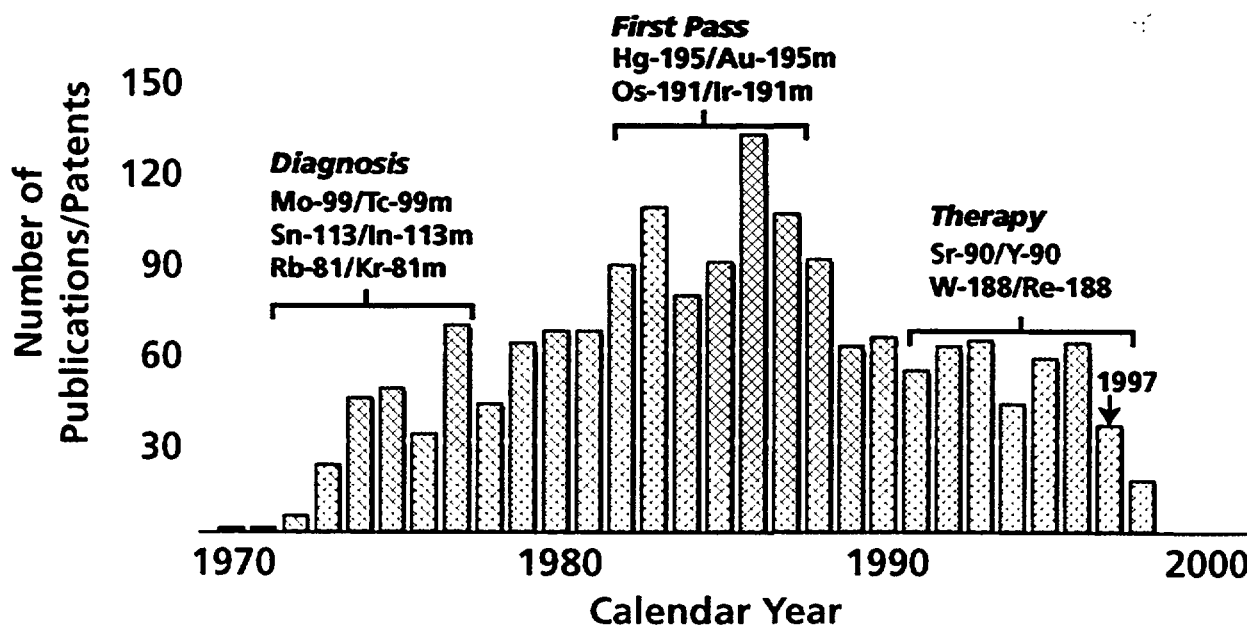


FIG. 1. Comparison of radionuclide generator publications from 1970 to 1998 (*Data from the U.S. Energy Science and Technology Database*).

Table 1. Availability of Fission-produced Molybdenum-99 - 1998 [§]

Country for Production	Institution/Reactor	Approximate Curies Produced Per Week - 1998	Approximate Maximal Production Capacity Per Week
Canada	MDS-Nordion - NRU Reactor	60,000	> 120,000
The Netherlands	Mallinckrodt - Petten HFR Reactor	11,000	18,000
Belgium	IRE, Fleureus - Various European Reactors	3,500
South Africa	AEC , Ltd. - Pelindaba	400	2,400

[§] Data obtained from manufacturers, February 1998, which represent actual production Curies and not calibrated Curies.

methods using physiologically-compatible solutions are the safest and easiest methods for obtaining technetium-99m, since radiopharmaceutical "kit" labeling is solution-based. Although solution-based generators are thus the method of choice, traditional chromatographic-type generators using alumina loaded with (n,y) molybdenum-99 have been impractical for obtaining technetium-99m for "kit" labeling because of the low specific volume technetium-99m solutions.

These dilute solutions result from the high volumes of eluant which are required to elute the bolus from the large generators required because of the low specific activity of the molybdenum-99. Although the use of (n, γ) molybdenum-99 has not been widely discussed in the recent literature, two approaches have been recently developed which may now make clinical use of such generators practical.

The use of the gel-type generator for low specific activity molybdenum-99 is based on the preparation of a zirconium gel by processing the irradiated molybdenum oxide target with zirconium oxide [7-9]. A similar strategy has been used successfully for the preparation of the gel-type tungsten-188/rhenium-188 generator. These generators are unique since the parent radioisotope is uniformly distributed throughout the gel matrix rather than being adsorbed on the top of the column bed as with the traditional chromatographic-type generators. Technetium-99m yields from this type of generator are reasonable.

Another alternative involves the post-column concentration of the low specific volume solutions of technetium-99m which are obtained from the chromatographic-type alumina generators loaded with low specific activity (n, γ) molybdenum-99. Two systems have been recently reported which use post-elution tandem cation/anion exchange system for technetium-99m concentration. Using these approaches, the high volumes required for generator elution do not represent a problem, since the solutions are subsequently easily and rapidly concentrated.

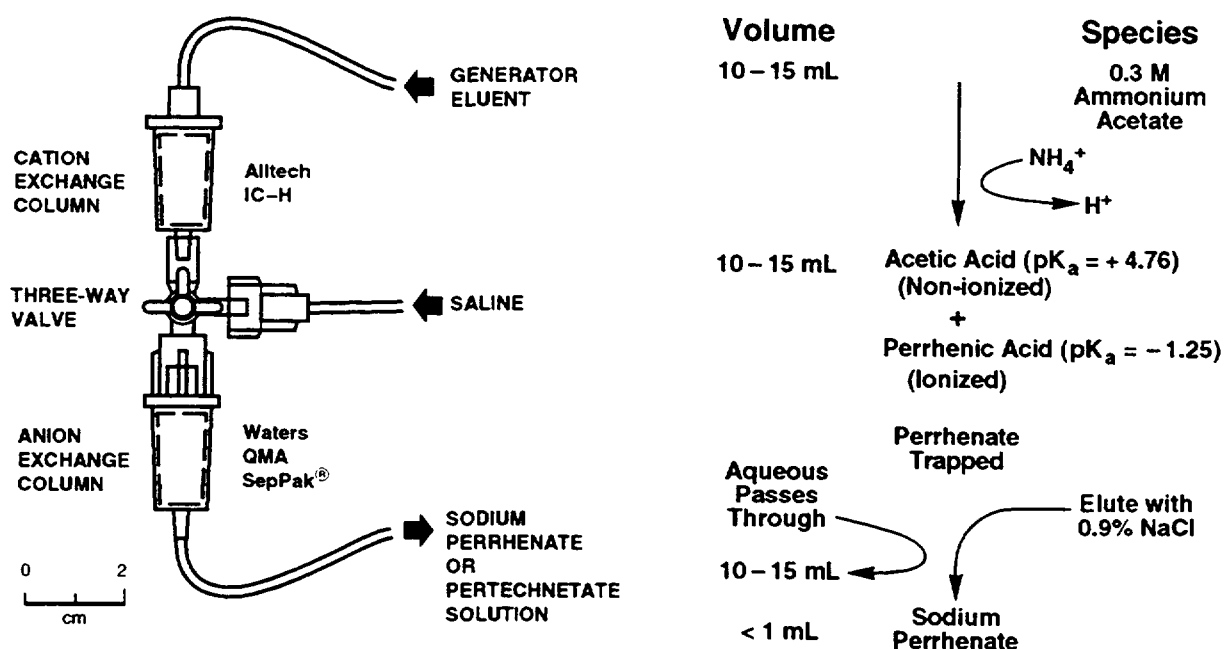


FIG. 2. Illustration of the tandem silver-cation/anion tandem system used for the concentration of low specific volume solutions of rhenium-188 and technetium-99m.

The approaches are based on the selective anion trapping of the microscopic levels of the perrhenate anion following removal of the macroscopic levels of the anion of the generator salt eluant.

One method uses initial elution of the generator saline bolus through a strong cation resin cartridge which is impregnated with silver cations to trap all of the chloride anion as the insoluble silver chloride salt [10]. The cation column is attached in tandem with an amine-type anion column by connection with a three-way stopcock. (FIG. 2). As the silver is trapped on the silver-cation column, the bolus solution then passes through the anion-exchange column. Since all of the chloride anion has been removed, the pertechnetate anion is specifically trapped on the anion column, with the bolus volume then passing through as waste. After adjusting the stopcock, the anion column can then be washed with water and the technetium-99m-pertechentate subsequently eluted with a small volume of saline. Another advantage of this approach is that the useful shelf-life of the molybdenum-99/technetium-99m generator can be extended.

Another more recent approach is the unique use of salts of weak acids rather than physiological saline for elution of molybdenum-99/technetium-99m generators [11-13]. This technique is described in detail in an accompanying paper [14] and is based on the use of ammonium acetate, for instance, rather than sodium chloride solution, during fabrication and elution of the generator prepared from low specific activity (n,y) molybdenum-99. The post-elution tandem system in this case consists of a strong cation ion column connected *via* a three-way stopcock with an amine-type QMA SepPak™ anion exchange column [14]. Passage of the generator bolus eluant through the cation column converts the ammonium acetate to a solution of acetic acid, which is not ionized at this pH, allowing selective trapping of the pertechnetate anion on the amine-based anion column as described above. This approach also works very efficiently for concentration of rhenium-188 solutions obtained from the tungsten-188/rhenium-188 generator (*vide infra*).

These two simple methods will now allow use of chromatographic-type alumina generators fabricated with low specific activity molybdenum-99. In this manner, many research reactors throughout the world can be used to produce the molybdenum-99 by neutron irradiation of enriched molybdenum-98 targets. Major advantages for the use of (n,y) molybdenum-99 compared to fission-produced molybdenum-99 include the use of enriched molybdenum-98 targets rather than the expense and complex regulations associated with the use of highly enriched uranium (HEU) targets. In addition, the complex issues associated with the handling

and storage of highly radioactive waste produced from fission of HEU is precluded. Finally, multiple research reactors throughout the world can be used for the (n, γ) molybdenum-99 production route without expensive capital improvements. Factors which must still be evaluated to determine the usefulness of this approach for the broad use of low specific activity molybdenum-99 include insuring that the large target volumes which are required for irradiation of large targets are available, and that the large amounts of enriched molybdenum-98 which will be required will be routinely available.

3. Radionuclide Generators As Convenient Source of Therapeutic Radioisotopes

The use of therapeutic radioisotopes is one of the greatest growth areas in nuclear medicine and oncology, and also more recently, in interventional cardiology for the inhibition of arterial restenosis. Two radionuclide generators (Table 2) which are of current major interest provide yttrium-90 (strontium-90/yttrium-90) [15] and rhenium-188 (tungsten-188/rhenium-188) [16-19]. In oncology, in addition to the treatment of primary tumors with radiolabeled antibodies and antibody fragments, the use of radiolabeled somatostatin analogues and other small "targeting" molecules is an important area of clinical research [20]. Palliative treatment of bone pain with a variety of agents targeted for cortical localization and radiolabeled with different therapeutic radioisotopes provides an important cost effective alternative for treatment of patients with advanced metastatic disease to the skeleton which can dramatically improve their quality of life [21].

Table 2. Examples of Key Radionuclide Generators of Current Interest Which Provide Therapeutic Daughter Radioisotopes

Parent	Daughter	Examples of Clinical Applications	Comment
Strontium-90	Yttrium-90	Tumor Therapy, Bone Pain Palliation, Intravascular Brachytherapy	Advantage - long-lived and readily available parent; disadvantage - bone seekers
Tungsten-188	Rhenium-188	Tumor Therapy, Bone Pain Palliation, Intravascular Brachytherapy	Advantage - efficient generator with long shelf-life, disadvantage - limited high-flux reactors for W-188 production
Dysprosium-166	Holmium-166	Tumor Therapy, Bone Pain Palliation, "In Vivo" Concept	Advantage - useful high energy beta, disadvantage - routine separation difficult
Actinium-225	Bismuth-213	Alpha Particle-Mediated Therapy - Myelogenous Leukemia	Advantage - alpha therapy very effective, disadvantage - limited range, specific applications

An important new therapeutic strategy in the realm of interventional cardiology is the use of beta and gamma-emitting radioisotopes for vascular brachytherapy for the inhibition of coronary restenosis after balloon angioplasty (PTCA) [22-24]. Most of the radioisotopes for this application are reactor-produced and generator-derived beta-emitting radioisotopes such as rhenium-188 and yttrium-90, are expected to have important applications. In addition, the use of equilibrium mixtures of strontium-90 and yttrium-90 provide a convenient long-lived source for this application and in a sense represent a unique example of an "*in vivo*" generator as described below (*vide infra*). Since the very small volumes (140-200 μL) for angioplasty balloon inflation require very high specific volume solutions of rhenium-188 (80-100 mCi/mL), the availability of the simple, effective methods described earlier are very important for concentration of the rhenium-188 bolus obtained from the tungsten-188/rhenium-188 generator, similar to those methods described earlier for concentration of technetium-99m from the molybdenum-99/technetium-99m generator (*vide ante*).

3. The "*In Vivo*" Generator - A Unique Application of the Generator Concept

Although not a new concept, the use of the *in vivo* generator is gaining increased attention. In this unique approach, usually following removal of the daughter, the parent radioisotope is attached to a tissue-specific targeting molecule. After administration and localization at the target site, the decay of the parent continually produces the therapeutic daughter radioisotope. If the "recoil" during decay of the parent does not destroy the parent/daughter-carrier association and the daughter-carrier bond is not impaired, this is an interesting and potentially important approach for targeted therapy [25]. Although there are several candidates (Table 3), recent research has demonstrated the feasibility of this approach with the dysprosium-166/holmium-166 pair [24].

4. Generators Providing Alpha Emitters - Growing Interest for Cancer Therapy

Because of short range, independence from dose rate and oxygen tension, and in particular the high linear energy of transfer (LET), alpha-emitting radioisotopes (Table 4) are of continuing interest for therapeutic applications. Because of the extremely localized deposition of high energy from alpha particles, the possibility of using alpha-emitting radioisotopes for cancer therapy is of great interest [27]. In particular, those alpha-emitters which have the appropriate radionuclide properties and are available from generator systems are of prime interest because of availability and expected cost effectiveness. Intensive research at several centers is currently focused on the bismuth-213 alpha emitter ($T_{1/2} = 45.6$ min.) which is obtained by decay of the actinium-225 parent ($T_{1/2} = 10$ days) [28] available from thorium-229, which is produced through the decay chain from uranium-233.

Table 3. Examples of Parent-Daughter Pairs for Use with the *In Vivo* Generator Concept [23]

Parent	Half-Life	Daughter	Half-Life
Dysprosium-166	81.6 hours	Holmium-166	26.4 hours
Lead-212	10.6 hours	Bismuth-212	60.6 minutes
Nickel-66	2.52 hours	Copper-66	5.1 minutes
Palladium-112	21.64 hours	Silver-112	3.13 hours

Table 4. Examples of Radionuclide Generators Providing Alpha-Emitting Daughters

Parent	Half-Life	Daughter	Half-Life	Daughter	Half-Life
Thorium-228 → Radium-224	3.7 days	Lead-212	10.6 hours	Bismuth-212	60.6 Minutes
Uranium-223 → Thorium-229	7,340 years	Actinium-225	10.0 days	Bismuth-213	45.6 minutes

5. Molecular Nuclear Medicine - Targeting the Nucleus with Therapeutic Radioisotopes Available from Radionuclide Generator Systems - A New Challenge

An important new challenge for nuclear medicine is the possibility of targeting radioisotopes to the cell nucleus. As with all radiopharmaceutical agents, effective targeting is the major challenge. While techniques for stable attachment of most radioisotopes for both diagnosis and therapy are well established, the identification of the labeled carrier molecules which effectively target is still the major challenge, especially for therapeutic agents, to deliver the maximal dose to the target tissue while minimizing adsorbed dose to non-target tissues. This strategy involves the attachment of specific radioisotopes with "tailored" radionuclidic properties to carrier molecules which effectively localize in the nuclei of targeted cells. In this manner, instead of using high energy beta emitters, low energy Auger emitters could be used for therapeutic applications, such as rhodium-103m ($T_{1/2}$ 65.1 m), which is available from the ruthenium-103/rhodium-103m generator fabricated from reactor-produced ruthenium-103 ($T_{1/2}$ 39 d).

SUMMARY AND CONCLUSIONS

Although research and development with radionuclide generators is not currently as intense compared to 2-3 decades earlier, the need for high levels of generator-derived

radionuclides is reflected in the resurgence of interest in the use of therapeutic radioisotopes from generator systems. The increasing applications and use of therapeutic radioisotopes in clinical nuclear medicine practice requires increasing access to generator-derived beta- and alpha-emitting radioisotopes, including yttrium-90, rhenium-188 and bismuth-213. As applications and use of these and other generator-produced radioisotopes increase, it would be expected that more efficient and automated generators will become available for routine widespread use.

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ANTI-CANCER DRUG POTENTIAL OF RADIOIODINATED GLUCURONIDE CONJUGATE OF β -NAPHTHOL

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Abstract

High radiotoxic effects associated with Auger electrons of iodine-125 on the living systems have been reported in the literature. The extreme radiotoxicity of Auger electrons experimentally observed occurs when Auger emitter radionuclide is incorporated into the structure of DNA or taken place very close to it. The microdosimetric considerations showed that each decay of iodine-125 results one dsb on the DNA helix. Of course, this leads to die the cell.

On the other hand, it is also known from the literature that the normal and cancer cells have some times high level activities of some kind of hydrolytic enzymes. These enzymatic activities can lead to the selective accumulation of some specific toxic aglycones into the nucleus. For example, the toxic aglycones of glucuronide compounds such as aniline-mustard, β -naphthol, 8-hydroxy-quinoline, etc. have the selective incorporation potential into the nucleus of living cells having high level of β -glucuronidase activity, e.g. RIF and spindle-shaped sarcoma tumors. Starting from this enzymatic activities and consequently the selective accumulation of some kind of glucuronide compounds, iodine-125 as an effective Auger electron emitter, and iodine-131 as a scintigraphic agent will be able to be selectively incorporated into the nucleus of cancer cells having high level of β -glucuronidase activity.

In this study, β -naphthyl-glucuronide was extracted from the urine of a rabbit treated with β -naphthol, and the purified product was radioiodinated using iodogen method. The preliminary scintigraphic tests showed that this compound was rapidly accumulated in kidneys reaching up to the maximum level about 10 min after the injection, and very quickly transferred into the bladder.

1. INTRODUCTION

The elevated activity of β -glucuronidase as an hydrolytic enzyme in some kind of tumors has been reported in the literature[1-3] This enzyme is able to deglucuronidate the glucuronide conjugates on the cell membrane. So, its aglycone portion can selectively be transported into the cancer cells. On the other hand, the glucuronidation metabolism is known well as a major detoxification pathway with the formation of water-soluble glucuronide conjugates of toxic materials in the living systems. Consequently, the glucuronide conjugates have potential applications in cancer research.

In 1984 Bicker[4] published a short report on the use of 8-hydroxy-quinolyl-glucuronide for a new direction of cancer chemotherapy. According to the data given by Bicker, 8-HOQ-Glu was accumulated 3-4 times more in spindle-shaped sarcoma tumors induced by methyl-nitrosourea in the neck of male Hauben rats respecting to surrounding normal tissues. Bicker also reported that β -naphthyl-glucuronide (β -Naph-Glu) was accumulated approximately 10 times more in the same tumors of rats treated with the glucose just before the administration of β -Naph-Glu. Practically any incorporation was observed for rats non-treated with glucose.

On the other hand, it is well known from the literature[5, 6] that iodine-125 radionuclide incorporated into the living cells is able to cause extreme radiotoxic effects. This radiotoxicity of iodine-125 as an effective Auger electron emitter is maximum when it is incorporated into the structure of DNA or found very close to it. The radiotoxicity level of iodine-125 is, at least, 10 times greater than that of β or/and γ emitter radionuclides such as iodine-131, carbon-14, tritium, etc.. This effect is attributed to the local absorption of Auger electrons[7-9]. Iodine-125 decays by electron capture (EC) with a probability of 100 %, and then decays further by internal conversion (IC) with a probability of 93 % to the stable tellurium-125 ground state. Thus, two successive Auger cascades occur per each decay of iodine-125. As a specific Auger electron emitter, iodine-125 emits averagely 21 Auger electrons in condensed matter per decay, and their energies are between 10 eV and 34 keV. The ranges of these Auger electrons are very short as being as nm scale in the condensed matters. The local absorption of these Auger electrons results high energy deposition

in the decay vicinity of iodine-125[10-12]. When iodine-125 decays in the nucleus of a living cell, high dose absorption by DNA results to die the cell. For this reason, iodine-125 has large potential applications in cancer research, too.

Obviously for such applications, iodine-125 must be selectively incorporated into the nucleus of cancer cells rather than the normal cells. Starting from the β -glucuronidase activity of some kind of tumors indicated above, the glucuronide conjugates can be used as selective carriers for being incorporated iodine-125 radionuclide into the nucleus of cancer cells. In the case of the use an effective toxic aglycone in the structure of the glucuronide conjugate, its cytotoxicity and the radiotoxicity of iodine-125 will be combined. So, the anti-cancer drug potential of the glucuronide conjugates bearing iodine-125 will considerably be very effective.

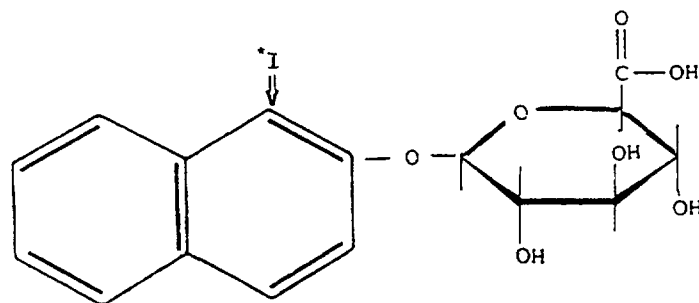
In addition, in the case of the use of iodine-131 for radioiodination procedures, the glucuronide conjugates will be able to be used as specific imaging agents of tumors having high level of β -glucuronidase activity. For this reason, some glucuronide compounds such as 8-hydroxy-quinolyl-glucuronide (8-HOQ-Glu), phenyl-N-glucuronide (Ph-N-Glu) and p-amino-phenyl-N-glucuronide (p-NH₂-Ph-N-Glu) have been earlier radioiodinated with iodine-131, and tested their metabolic pathways in the living system of rabbits[13].

In this study, the radioiodinated β -Naph-Glu has been subjected for evaluation of its diagnostic and therapeutic potential in cancer research.

2. EXPERIMENTAL

2.1. Metabolic Production of β -Naphthyl-Glucuronide :

β -Naph-Glu is not a commercial product. For this reason. it should be synthesized for its using in cancer research studies; but unfortunately, its chemical synthesis and purification procedure require hard chemical works. Nevertheless, as is outlined in the introduction section, the glucuronidation metabolism is known well as a major detoxification pathway in the body with the formation of water-soluble glucuronide conjugates of toxic materials. Of course, β -naphthol as a non-water soluble toxic compound should rapidly be eliminated by the excretion system as its



Scheme-1 : β -Naphthyl-Glucuronide (β -Naph-Glu).

glucuronide conjugate. This means that β -Naph-Glu may be extracted from the urine of a laboratory animal treated with β -naphthol. In earlier studies 8-HOQ-Glu was successfully extracted from the rabbit urine treated with 8-hydroxy-quinoline by Robinson et al.[14] and Ünak et al.[13, 15]. Similarly, in this study β -Naph-Glu was obtained as a metabolic product extracting from the urine of a rabbit treated with β -naphthol (1g/kg). Urine was collected by a urinary catheter. The metabolic product were purified by successive recrystallizations. The spectroscopic examinations of the purified product verified its formal structure given in Scheme-1.

2.2. Radioiodination Procedure :

The final product was radioiodinated applying the iodogen method. As is known well from the literature that 1,3,4,6-tetrachloro-3 α ,6 α -diphenylglucuril (trade mark iodogen) is an oxidative agent for radioiodination of different kind of organic compounds[16, 18]. The method has been applied under the same conditions described earlier by Ünak et al.[15, 19]. According to this description, about 10 mg iodogen was dissolved in 1.5 mL CH_2Cl_2 in a tube of about 1.5 cm diameter containing small broken glass pieces for augmentation of contact surface. Following the complete evaporation of CH_2Cl_2 , iodogen coated the wall of the tube and the glass pieces with a thin film. A few mg of purified compound dissolved in about 1.5 mL water was added to this iodogen coated tube, and then about 7.4×10^7 Bq (2 mCi) of Na^{131}I or Na^{125}I was added. This reaction mixture was kept at room temperature without stirring for about 20 min. At the end of this time, this reaction mixture taken by a syringe was filtered off using a milipore. The rest solution in the iodogen tube was washed with water of about 0.5 mL and added to the initial filtered solution.

2.3. Preliminary *in vivo* Tests of Radioiodinated Product :

Radioiodinated β -Naph-Glu with iodine-131 was tested on rabbits to determine the dynamic metabolism of this compound using a gamma-camera. For these tests, the radioiodinated product was intravenously injected via ear vein and whole body scintigraphies were immediately started to monitor after the injections.

3. RESULTS AND CONCLUSION

The chemical structure of metabolic β -Naph-Glu was studied using ^1H , ^{13}C -NMR, UV, and IR spectroscopy techniques. According to these spectroscopic analyses, their structures shown in Schemes 1 was clearly verified.

The radioiodination technique applied by the use of iodogen method, was resulted very high radioiodination yield, and thin layer radiochromatographic analyses showed that the labeling yield was as high as >95 %. High radioiodination yield was also verified by the scintigraphic test on rabbits (Fig.-1). Any accumulation of radioactive iodide was not observed in the thyroid gland. As is known well, iodogen is an oxidative agent, and is able to oxidize the I^- to ^+I for realization its electrophilic substitution to a benzenoid ring. Consequently, this was a reasonable result for substitution of iodine to any position of an aromatic ring of naphthalene. It can be postulated that most probable bonding position to naphthalene is its 1st position as indicated in Scheme-1.

Figure-1 shows the preliminary *in vivo* test of radioiodinated β -Naph-Glu with iodine-131 in a non-tumor-bearing rabbit. It exhibits similar metabolic pathway and rapid elimination character in the body as glucuronide conjugates such as 8-HOQ-Glu, Ph-N-Glu, p-NH₂-Ph-N-Glu earlier studied by Ünak et al.[13]. As is shown in Fig.-2, iodine-131 activity was rapidly transported to kidneys and accumulated in these organs where the activity reached up to the maximum level about 10 min after its administration, and very quickly transferred into the bladder. The dynamic scintigraphic tests also showed that the radioiodine bond of this compound is sufficiently stable in *in vivo* conditions.

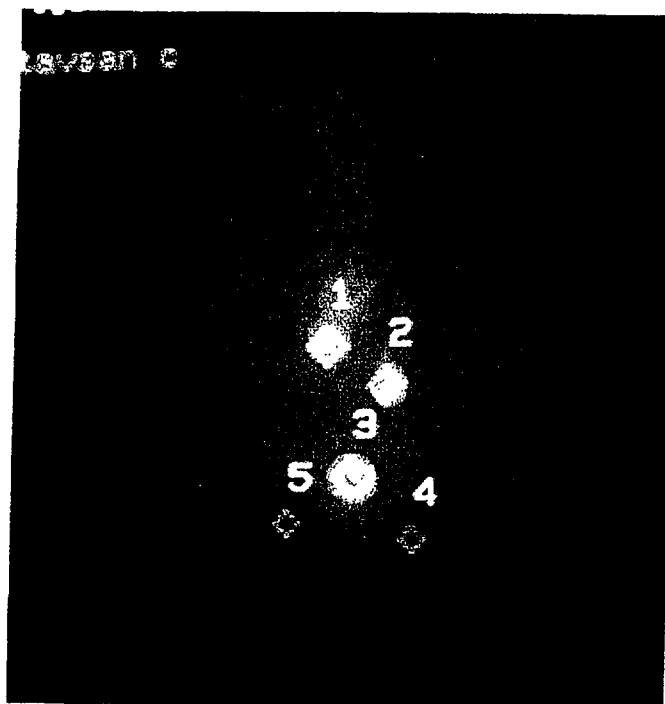


Figure-1 : Dynamic scintigram of radioiodinated β -naphthyl-glucuronide in a rabbit.

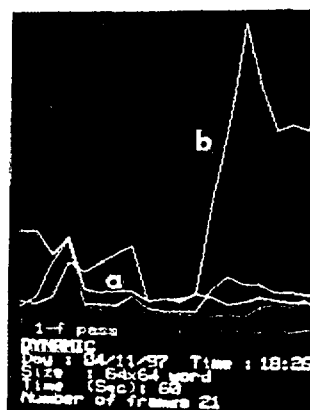


Figure-2 : Iodine-131 activity variations in different organs of rabbit.

1. Yellow-a line : Left kidney.
2. Pink line : Right kidney
3. Yellow-b line : Bladder ,
4. Red line : Right leg,
5. Bleu line : Background.

Briefly, the results obtained in this study showed that glucuronide conjugate of β -naphthol radioiodinated with iodine-125 or iodine-131 have potential applications in cancer diagnosis and therapy.

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***In Vitro* AND *In Vivo* BEHAVIOUR OF ^{111}In COMPLEXES OF
POLYAMINOCARBOXYLATE LIGANDS:
STABILITY, BIODISTRIBUTION AND EXCRETION
STUDIED BY GAMMA IMAGING**



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Abstract

With a view to potential radiopharmaceutical application, six $^{111}\text{In}^{3+}$ complexes of polyamino-carboxylate ligands, TTHA, TTHA-bis(butylamide), DTPA-bis(butylamide), DTPA-bis(glucamide), DTPA-bis(metoxylethylamide) and DTPA-bis(heptylamide), were investigated. The stability of three of them, TTHA, TTHA-bis(butylamide) and DTPA-bis(butylamide), was evaluated by measuring the exchange of $^{111}\text{In}^{3+}$ from the complexes to transferrin in blood serum, by monitoring the radioactivity of the gamma-emitting isotope. Our preliminary results show that these complexes have high stability under physiological conditions.

Biodistribution studies of the In^{3+} chelates and gamma imaging in rats, indicate that all the complexes studied display renal clearance, similar to other low molecular weight complexes (*e.g.* In-DTPA), except the DTPA-bis(heptylamide) complex, which accumulates preferentially in the liver and spleen, in agreement with its greater lipophilicity.

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1-Introduction

Nuclides of indium have found widespread use in nuclear medicine [1]. ^{111}In , an γ -emitting isotope, has nearly ideal characteristics: it decays by electron capture, emitting two usable gamma photons of 173 KeV and 247 KeV with a 184% photon yield [2]. ^{111}In has an half-live of 2.8 days that makes it suitable for follow up studies for several days [2]. It also presents suitable chemical properties for *in vivo* applications : it occurs only in the +3 oxidation state in water , and its aqueous chemistry is dominated by the property of strong Lewis acidity , binding strongly to oxygen donors ligands [3].

DTPA, is a polyaminocarboxylate ligand widely used in Nuclear Medicine diagnosis. Aiming at obtaining new chelates with different tissue selectivity, we have studied the $^{111}\text{In}^{3+}$ complexes of various bis(amide) derivatives of the polyaminocarboxylate ligands DTPA and TTHA with different charges and hydrophilic/lipophilic character: DTPA-bis(butylamide), DTPA-bis(heptylamide), DTPA-bis(metoxylethyl-amide), DTPA -bis(glucamide), TTHA and TTHA-bis(butylamide) (see Fig. 1). In fact, the factors determining biodistribution and biliary versus urinary excretion of substances injected in the blood stream, though not very clear, are chiefly influenced by three main radiotracer properties: polarity, molecular size and molecular weight, and structure [4].

Since both metal ions and free ligands have high toxicity, high thermodynamic and kinetic stability is required for chelates to be used *in vivo*. The radiopharmaceutical must remain intact during its residence time in the body. Serum transferrin, which is normally only about 30% saturated with iron [5], retains a relatively high capacity for binding other metal ions. It is widely accepted that indium(III), as an analog of iron(III), binds to circulating transferrin, and is then found in body areas of high iron uptake, namely bone marrow and inflammatory tissues [5]. So, before small molecules or

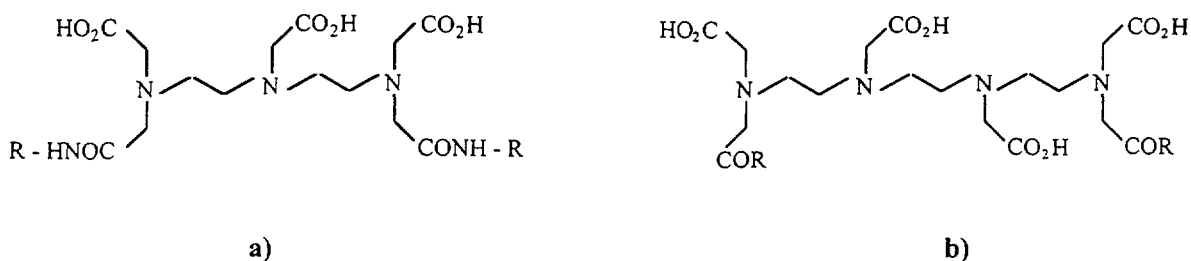


Fig. 1. Chemical structures of the ligands used in this study, where **a)** **R** = *n*-butyl (DTPA-bis(butylamide), DTPA-BuA₂), *n*-heptyl (DTPA-bis(heptylamide), DTPA-HpA₂), metoxyethyl (DTPA-bis(metoxylethylamide), DTPA-MEA₂) glucosyl (DTPA-bis(glucamide), DTPA-GlucA₂) and **b)** **R** = OH (TTHA) and NH-(*n*-butyl) (TTHA-bis(butylamide), TTHABuA₂).

biomolecules labeled with $^{111}\text{In}^{3+}$ can be considered valid radioindicators, *in vivo*, it must be shown that the chelates are thermodynamically stable or kinetically inert to the extent that loss of In^{3+} to transferrin is negligible.

2. Materials and Methods

2.1. *In vitro* Stability

The stability of three of the chelates, $\text{In}(\text{TTHA})^{3+}$, $\text{In}(\text{TTHA-BuA}_2)^-$ and $\text{In}(\text{DTPA-BuA}_2)$, has been determined by measuring the exchange of $^{111}\text{In}^{3+}$ to transferrin in blood serum as a function of time. We performed this study by gel filtration, after appropriate time intervals, of the solutions containing transferrin and the $^{111}\text{In}^{3+}$ complex, using a method described in the literature [6]. A carrier free solution of 150 μl of $^{111}\text{InCl}_3$ in 0.1 N HCl (370 Mbq/ μg In) was mixed with 2 ml of 0.05 M sodium citrate (pH 6.5). This solution was mixed with 10 μl of a ligand solution, in such a way that a 1:1 ligand-metal ratio was obtained. A 200 μl aliquot of this mixture was added to 3 ml of human serum (solution activity ≈ 1.5 mCi) and incubated for 48 h at 37° C. At appropriate time intervals, 25 μl of this mixture was subjected to gel filtration (Sephadex G-25, 1x15 cm column). The column was eluted with 0.01 M PBS buffer (pH 7.4) at a flow rate of c.a. 38 ml/h. Samples were taken after a dead volume of 4 min. Activity in the samples was detected with a well γ -counter.

The same procedure has been used with a transferrin solution (2×10^{-5} M). Before mixing this solution with the complex, we added an NaHCO_3 solution (final HCO_3^- concentration 5 mM [7]). A 200 μl aliquot of this mixture was added to 10 μl of an $^{111}\text{In}(\text{TTHA-BuA}_2)^-$ solution. At appropriate time intervals, 25 μl of this mixture was subjected to gel filtration as described above.

2.2. Images and biodistribution data

Gamma images and biological distributions for the six indium complexes were determined using 300g Wistar rats (three groups of 5 animals each and a group of 8 in the case of DTPA-BuA₂ with ca. 150 μCi of the respective ^{111}In complex injected into the femoral vein or the tail vein. The animals were anaesthetised with Ketamine (50mg) /Largactil (10:3). Image acquisition was initiated immediately after radiotracer injection. The rats laid in dorsal or ventral *decubitus* over the detector. Sequences of 180 images, of 10 seconds each, were acquired to 64x64 matrices. Blood samples were

taken at 5, 15 and 30 minutes after injection. We also acquired static data at 24, 48 and 72 h after the injection.

Three regions of interest were drawn on the image files, corresponding to the thorax, liver and left kidney. From these regions of interest, activity/time curves were obtained using home-made software.

The animals were then sacrificed and the major organs were removed, weighed, counted and the percent injected dose/gram of tissue was calculated for each tissue type.

3. Results and Discussion

3.1 *In vitro* Stability

Our preliminary results on the *in vitro* stability of three of the complexes studied in blood serum and in a transferrin solution are summarized in Table I. They indicate that, under physiological conditions, $\text{In}(\text{DTPABuA}_2)$ has low dissociation (0.30 % after 24h), comparable to $\text{In}(\text{DTPA})^{2-}$ (1,5 % after 24h [8]). This contrasts with the higher dissociation observed for $\text{In}(\text{TTHA})^{3-}$ (5.43 % after 48 h) and $\text{In}(\text{TTHABuA}_2)^-$ (1.64 % after 2 h) in blood serum, and for the later in a transferrin solution (7.54% at 24h). These results are in agreement with the published pM values of In^{3+} for the two ligands and for the transferrin complexes [$\text{pM}(\text{In}(\text{tf})) = 20.4$, $\text{pM}(\text{In}(\text{TTHA})^{3-}) = 22.88$, $\text{pM}(\text{In}(\text{TTHABuA}_2)^-) = 19.43$ and $\text{pM}(\text{In}(\text{DTPABuA}_2)) = 20.4$] [9].

Table I. Dissociation (%) of the ^{111}In -chelates in blood serum and in a transferrin (tf) solution.

Time (h)	TTHA	TTHABuA ₂		DTPABuA ₂
	blood serum	blood serum	tf solution	blood serum
2	0.30	1.64	0.37	0.04
24	2.36	-	7.54	0.30
48	5.43	3.26	-	3.26

3.2 Images and biodistribution data

Figure 2 illustrates the scintigraphic images obtained in this study, showing images at 30 minutes of a rat injected with $^{111}\text{In}(\text{TTHA})^{3-}$ and $^{111}\text{In}(\text{DTPA-HpA}_2)$. The activity/time curves, obtained from the dynamic acquisitions, generally show two different portions: an initial, generally sharper, portion and a slower second one. This type of profile indicates that those complexes undergo both renal and liver-spleen retention. The activity/time curves for thorax correspond only to blood activity. The exceptions to this behaviour are the $^{111}\text{In}(\text{DTPA-MEA}_2)$ complex, with exclusive renal

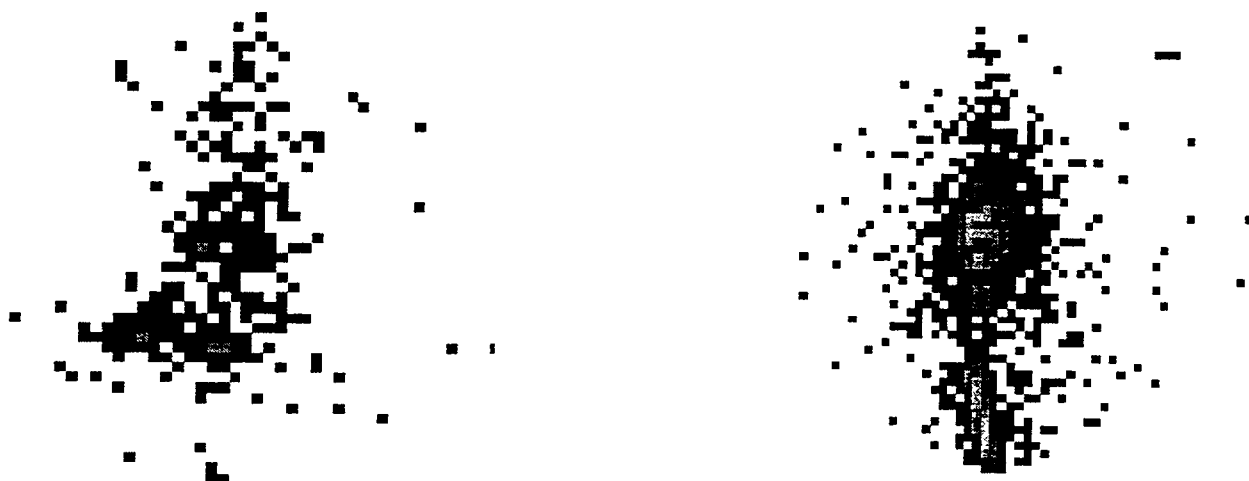


Fig. 2. Scintigraphic images at 30 minutes of a rat injected with $^{111}\text{In}(\text{TTHA})^{3-}$, (left) and with $^{111}\text{In}(\text{DTPA-HpA}_2)$ (right)

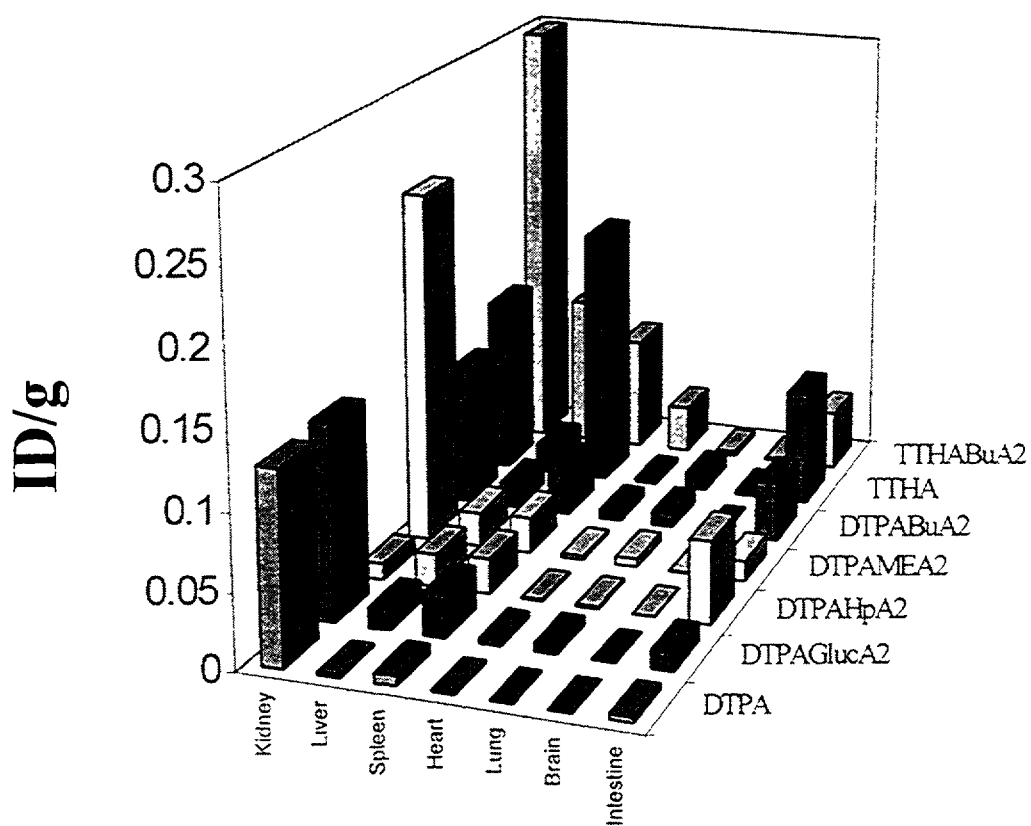


Fig. 3: Biodistribution of ^{111}In metal complexes in rat tissues at 72h after administration of the chelates.

72 hours obtained for the complexes are shown in Fig 3, and correlate well with the scintigraphic images. As shown by these data, the major activity is preferentially localized in the kidneys. This indicates a renal excretion of the chelates, which is consistent with their structure, molecular weight and hydrophilicity. An exception to this behaviour was the $^{111}\text{In}(\text{DTPA-HpA}_2)$ complex, which was found to clear with high specificity *via* the hepatobiliary system (one can estimate the amount of complex cleared through the liver by adding the activity in liver and intestine), in accordance with its greater lipophilicity. High uptake of the ^{111}In complexes of TTHA and TTHA-BuA₂ by the reticulo-endothelial system (RES) may be related with the formation of colloidal particles of indium hydroxide [10]. High radioactivity levels in blood after 30 minutes suggest that the complexes may bind to serum proteins, perhaps albumin, but further *in vitro* studies are necessary to validate this hypothesis.

None of the complexes passed through the blood-brain barrier, as expected for high molecular weight and non-lipophilic complexes. We have no evidence of bone marrow accumulation, which is seen when the indium-transferrin complex is formed [11].

In conclusion, biodistribution and gamma imaging studies of the $^{111}\text{In}^{3+}$ chelates in rats indicate that most of the complexes studied, both negatively charged and neutral, show renal clearance, similar to other low molecular weight hydrophilic complexes (*e.g.* $\text{In}(\text{DTPA})^{2-}$). However, the neutral $^{111}\text{In}(\text{DTPA-HpA}_2)$ complex accumulates preferably in the liver and spleen, according to its greater lipophilicity.

ACKNOWLEDGEMENTS

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Abstract

This paper addresses the utilization of three very distinct enzyme systems for imaging in oncology. The first of these is an enzyme encoded by a viral gene that is not present in non-infected mammalian cells. This enzyme is a nucleoside kinase that converts selected unnatural nucleosides to nucleotides in virus-infected or gene-transfected cells, but not in normal cells. The most commonly used viral kinase in gene therapy today is Herpes simplex virus type-1 thymidine kinase (HSV *tk*). The imaging applications of this gene therapy system are demonstrated using data from a murine tumour gene therapy model, with $^{123}\text{IVFRU}$ as the diagnostic radiopharmaceutical.

The second enzyme system is endogenous to mammalian cells, but is found in highest concentrations in tissues of neural crest derivation. The overall biochemical pathway of interest involves the conversion of tyrosine to either dopamine (neurotransmitter pathway), or to melanin (pigmentation pathway). In this system tyrosinase is the 'branching' enzyme, converting dopa to dopaquinone, thereby averting its conversion to dopamine. With selective agents, the tracer can be trapped in this 'melanin pathway', which is particularly active in melanomas. Data on the development of radioiodinated tyrosinase substrates, based on S-cysteaminyl phenol (SCAP), a highly specific tyrosinase substrate, are presented to illustrate this concept.

The final example is that of endogenous enzymes that are virtually ubiquitous in biobistribution. One class of enzymes, the reductases, are particularly active in the liver and their activity is amplified in tissues that are hypoxic. They are important in radiotherapy, where they can be utilized to bioreductively activate compounds that can restore the radiosensitivity of hypoxic cells. The 2-nitroimidazoles are of special interest because they are easily reducible by a number of reductases, a process that is made selective by the reversibility of reduction in the presence of cellular oxygen. The reduction intermediates react covalently with tissue nucleophiles and are metabolically trapped. Data from pre-clinical and clinical hypoxia imaging studies with $^{123}\text{IAZA}$ are used to demonstrate the imaging application of this process.

INTRODUCTION

Selective accumulation of diagnostic radiopharmaceuticals in a tissue is dependent upon a unique combination of uptake, trapping and clearance, such that there is a net accumulation of the substance in the target cells. Much attention has been given to accumulation *via* immunorecognition/binding/internalization (e.g. monoclonal antibodies and fragments, and peptides) and high affinity receptor-based (e.g. neuroreceptor ligands; hormone receptor ligands) binding as a basis for uptake-based imaging. Much less attention has been focused on enzymes, a special class of protein receptors, that bind with selective substrates for the purpose of molecular biotransformation rather than for signal transduction. One notable example of enzyme-based uptake is that of ^{18}FDG , which accumulates by virtue of its interaction with hexokinase and its subsequent accumulation as a slowly metabolized, non-diffusible phosphate ester[1,2].

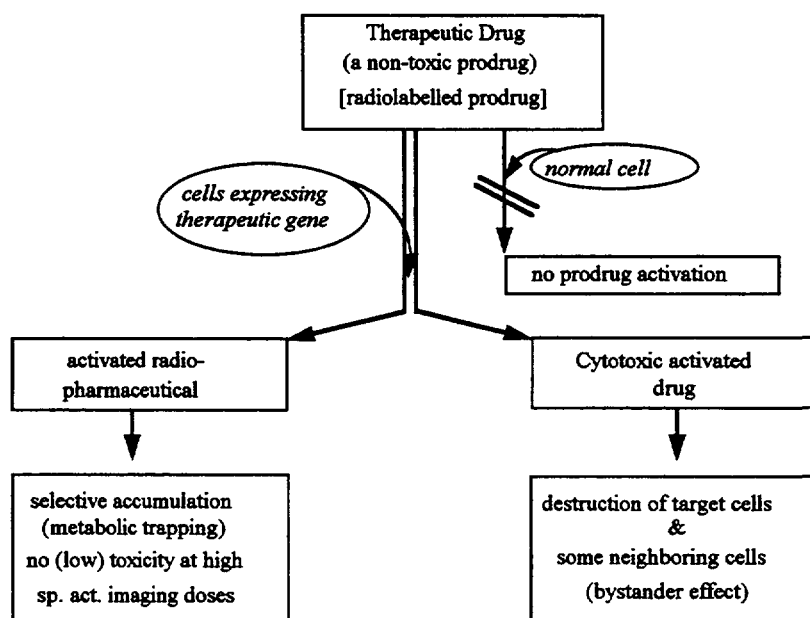
Enzymes are attractive targets for diagnostic imaging because of their ability to convert multiple copies of the substrate (tracer) per molecule of enzyme. In this way, even though the protein enzyme may be present in limited concentration, as is the case with receptor proteins (e.g. hormone and neurotransmitter receptors)[3] or immuno-recognition sites (antigens)[4], radiolabelled substrate turnover by the enzyme leads to signal intensification as long as the substrate is available, provided that the metabolic product is prohibited from leaving the cell.

Enzyme-mediated metabolic trapping is one of several mechanisms by which high accumulations of radiotracers are achieved in target tissues. Three cellular enzymes that convert highly diffusible (prodrug) radiopharmaceuticals to either covalently-bonded or poorly diffusible metabolic intermediates are being harnessed to selectively trap radiopharmaceuticals in tumours:

- viral thymidine kinase for monitoring in gene therapy of cancer,
- tyrosinase for detecting melanomas and tumours of neural crest derivation, and
- reductases for detecting or characterizing viable but hypoxic tumours.

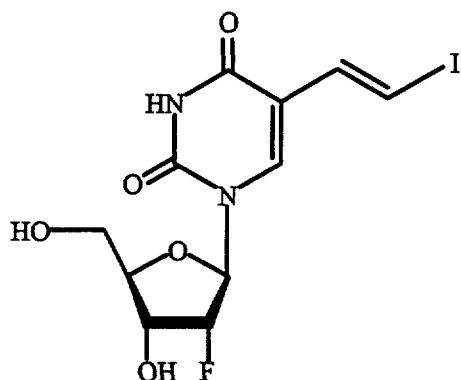
HERPESVIRUS THYMIDINE KINASES (*tk*) FOR GENE THERAPY IMAGING

Gene therapy, one of the most exciting spin-offs of basic molecular biology, offers several approaches to the treatment of cancer, including the introduction of genes that will encode for molecular targets not normally found in mammalian cells [5]. Although there are several possible therapeutic mechanisms to be invoked by the gene therapy process, one of the earliest and perhaps most clinically advanced, is the 'suicide' or 'selective prodrug activation' mechanism [6]. The 'suicide' process chosen by most investigators is that based on the insertion of a viral kinase into target tissue, which can then bioactivate a prodrug to a cytotoxic drug that kills the cell (Scheme 1 [7]). A number of clinical trials around the world are based on the introduction of the Herpes simplex virus (HSV) gene that encodes for viral thymidylate kinase (*tk*). Once the gene is expressed in the target (cancer) cells, 'suicide' therapy can be effected simply by administering a selective, systemically non-toxic antiviral drug such as ganciclovir, that is highly toxic to the cells (i.e. the transfected cells) that can bioactivate it [8-10].



Scheme 1. 'Suicide' or selective prodrug activation gene therapy: applications in imaging and therapy

As part of the radiopharmacy program at the University of Alberta, we have a long-standing project to develop radiodiagnostic and therapeutic antiviral nucleosides, particularly those with high affinity for Herpes simplex virus-encoded thymidylate kinase (HSV *tk*). A number of metabolically stable, potent antivirals such as *E*-5-(2-iodovinyl)-1-(2-deoxy-2-fluororibofuranosyl)uracil (IVFRU) have been developed for use as therapeutic agents, and when radiolabelled, for use as diagnostic agents for imaging based on focal HSV *tk* expression [11,12]. This work has direct application to monitoring gene transfection and transduction, and to gene therapy of a number of pathological conditions, including cancer, since confirmation of expression



IVFRU

of the viral gene is vital to the assessment of clinical success of the gene transfer process, for both timing of the associated drug therapy and monitoring of potential toxicity due to expression in non-target tissues. Radionuclide scintigraphy is probably the only practical means by which to detect whole-body viral gene expression.

E-(2-[¹²³I]iodovinyl)-1-(2-deoxy-2-fluororibofuranosyl)-uridine ([¹²³I]IVFRU) has been evaluated as a diagnostic probe for use in gene therapy models [11,12]. Studies *in vitro* in murine (KBALB) and human cell lines (143B) transfected with HSV (*tk*) show high uptake in

comparison to control, untransfected cells (Fig. 1) and selective accumulation *in vivo* in a murine model.

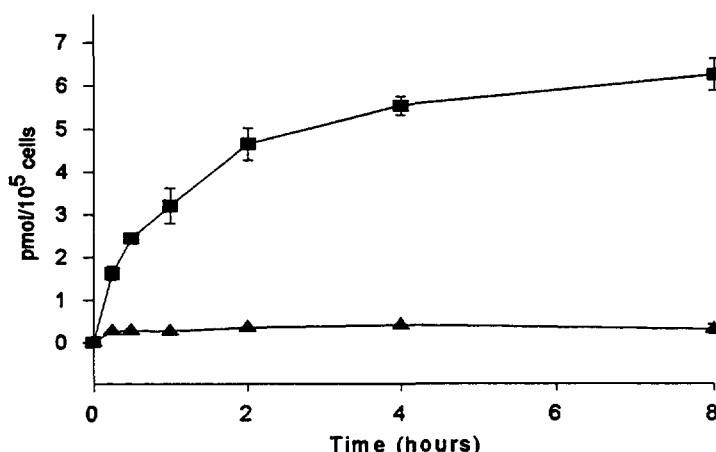
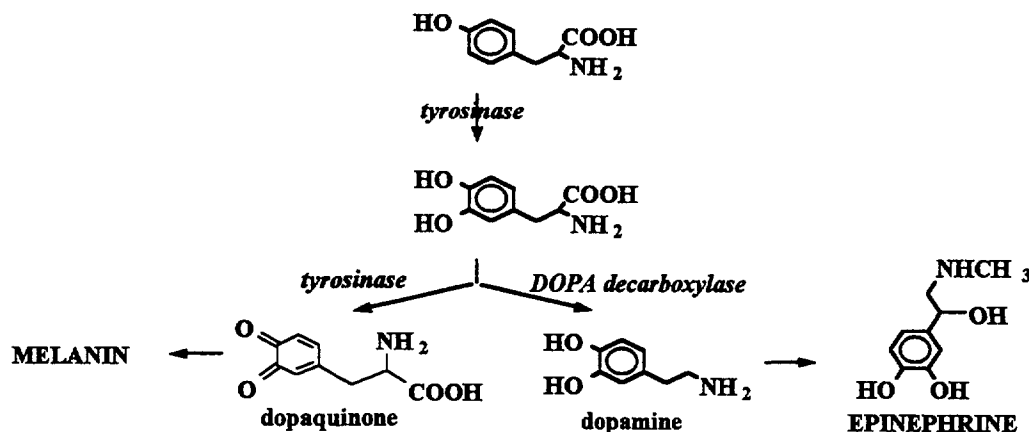


Figure 1. Uptake of ¹²⁵I[IVFRU] by KBALB-LTK (■; HSV *tk* expressing) and KBALB (▲ not-transfected) cells *in vitro* (adapted from [13]).

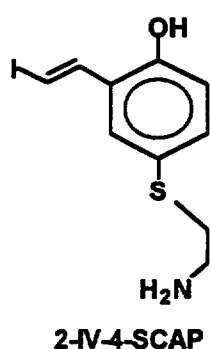
Clear delineation of implanted, transfected tumours was possible by scintigraphy. Furthermore, the effects of treatment with ganciclovir could be monitored scintigraphically with [¹²³I]IVFRU. Images taken within 4 h of injection of ¹²³I[IVFRU] in animals with gene-transduced tumours showed clear delineation of the tumour (>2% ID/g) if no ganciclovir had been administered, whereas a single dose of ganciclovir 24 h prior to administering the radiopharmaceutical essentially completely blocked (<0.5% ID/g) tracer uptake even though the tumour mass was essentially unchanged. Studies of the physiological effects of this tumour cytotoxicity are underway [11-13].

TYROSINASE

Tyrosinase is the enzyme that diverts dihydroxyphenylalanine (dopa) from the neurotransmitter pathway that leads to the production of epinephrine, to the melanin biosynthetic pathway [14] as depicted in Scheme 2 [15].



Scheme 2. A schematic representation of the conversion of tyrosine to epinephrine (neurotransmitter pathway) and to melanin (pigmentation pathway) [15].

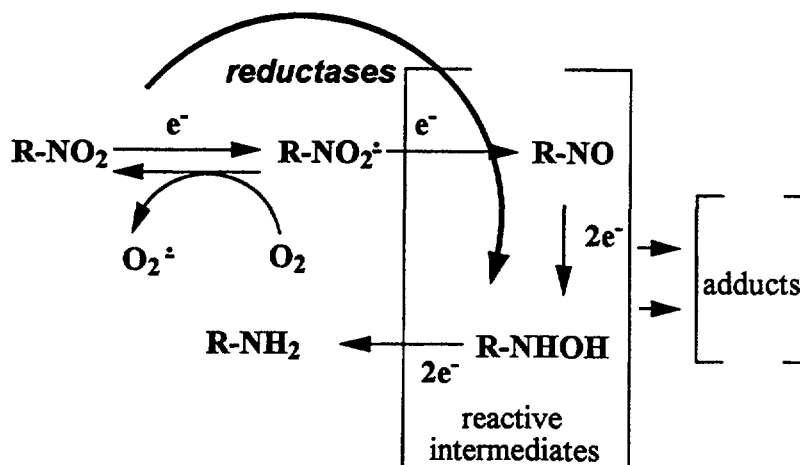


Exploitation of the melanin pathway has attracted a number of scientists interested in developing diagnostic and therapeutic agents for treating melanomas. Utilization of this enzyme, which bioactivates substrates specifically to enter the melanin pathway, however, has been of great interest since the development of a family of tyrosinase specific substrates [16-18]. A number of tyrosine analogues have been synthesized for use as antimelanoma therapeutics. Of these, several sulphur-containing analogues have been shown to be particularly good substrates for tyrosinase and to have strong cytotoxic activity against malignant melanoma cells *in vitro*. Radioiodinated derivatives of these therapeutically active aminoalkyl-S-phenols have been prepared for *in vitro* and *in vivo* assessment. 2-(2-Iodovinyl)-4-thio(2-aminoethyl)phenol (2-IV-4-SCAP) is a second generation analogue that was designed to be more stable than the active, but biologically labile 2- and 3-iodo-4-thio(2-aminoethyl)phenols from which it was elaborated [19,20].

In addition to the potential radiodiagnostic properties of these radioiodinated aminoalkyl-thiophenols, there is also interest in their radiotherapeutic activity.

REDUCTASES

Radiobiologists have studied the clinical implications of tumour hypoxia on radiotherapy outcomes for over four decades [21]. The discovery of nitroimidazole radiosensitizers to enhance the therapeutic effect of low LET radiation [22] has led to the development of radiolabelled nitroimidazoles for diagnostic imaging [23]. Although early studies with these radiotracers were focused on the diagnosis of tumour hypoxia as a prognostic agent for tumour radiotherapy, the importance of tissue oxygenation in stroke, myocardial ischaemia and metabolic disease has broadened interest in the diagnostic power of these compounds. It has been shown that hypoxic tissue can be selectively delineated with radiolabelled nitroimidazole-based radiosensitizers, via the mechanism depicted in scheme 3.



Scheme 3. A diagrammatic representation of the hypoxia-selective binding of nitro compounds.

Hypoxia-selective trapping of [^{123}I]iodoazomycin arabinoside ([^{123}I]IAZA), a radiosensitizer of hypoxic cells, occurs in viable hypoxic cells that have functional reductase enzyme activity [24]. Extensive experimental and clinical studies with [^{123}I]IAZA have demonstrated selective trapping *in vivo* in cancer [25]. The major objective of imaging with [^{123}I]IAZA is not to detect tumours or other pathologies, but to determine the presence of viable, hypoxic tissues in the pathological lesion of interest. Although several new hypoxia-imaging agents are being developed, the preponderance of clinical oncological data available today has been derived with [^{123}I]IAZA using planar and SPECT techniques.

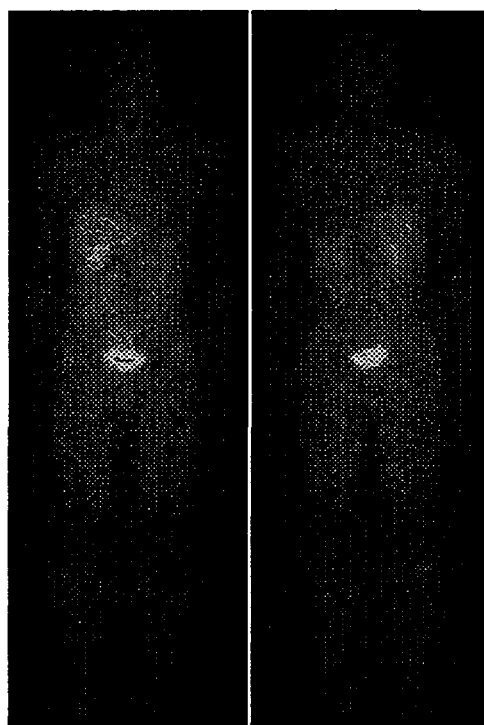
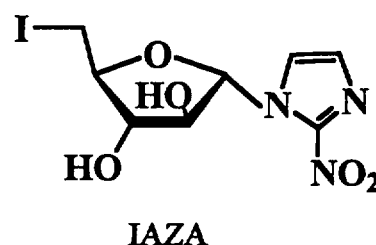


Plate 1. Planar scintigrams of a human volunteer, 3 h after i.v. injection of 208 MBq of [^{123}I]IAZA [26].

Recently, the pharmacokinetics and tracer kinetics of [^{123}I]IAZA in human volunteers were determined, thereby providing the opportunity to obtain reliable dosimetry estimates for [^{123}I]IAZA from blood level data and planar images. In addition, a kinetic model to predict the relative size of the hypoxic fraction in a given region of interest has been developed from these data. An [^{123}I]IAZA scintigram of a volunteer 4 h after injection (Plate 1) depicts that the tracer is rapidly distributed to all tissues except normal brain, and also rapidly cleared by a combination of renal and hepatobiliary processes [26].

SUMMARY

Three scintigraphic cancer diagnostic modalities that are based on enzymes specific to, or concentrated in pathological tissues have been described. Each of these three biochemical targets are exploitable in unique settings, and each can be extrapolated to the development of therapeutic agents that would utilize similar mechanisms of action. The nucleoside kinases, such as Herpes simplex type-1 thymidine kinase (HSV *tk*) have a major role in gene therapy of cancer, whereas tyrosinase is applicable to agents to be used for the diagnosis and treatment of melanomas, and the reductases can be exploited in pathways where bioreductively-activated radiodiagnostics and therapeutic agents can play a role.

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RADIOSYNTHESIS, EVALUATION AND PRECLINICAL STUDIES OF A NEW 5HT_{2A} RADIOLIGAND

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Abstract

¹²³I-5-I-R91150, a radioiodinated analogue of R91150 (a ligand (antagonist) of Janssen Research Foundation), showing high affinity and selectivity for 5HT_{2A} receptors, was developed as a potential in vivo 5HT_{2A} receptor tracer for SPECT. The applied radiochemistry, whereby the radioiodine was substituted on the 5 - position of the benzamide ring, allowed to obtain the tracer with high specific activity and high purity.

In vitro and in vivo rat studies revealed that the new tracer bound reversibly with the required high affinity ($K_d = 0.1$ nM) and high selectivity (a factor ranging from 10000 to at least 50 vis à vis other receptors) to 5HT_{2A} receptors.

In young normal subjects the major part of the ¹²³I-5-I-R91150 radioactivity in the brain is present in cortical areas. Cortical area to cerebellum activity ratio reaches an equilibrium value of about 1.8 around 90 min. till 4 hours p.i.. This binding was specific and reversible. The cortical activity reflects a distribution in the brain similar to that of the mapping of 5HT_{2A} receptors from post mortem studies.

These findings suggested that ¹²³I-5-I-R91150 allows imaging and quantitative estimation with SPECT and could be used for further clinical studies.

The radiobromine analogue was synthesised as a potential PET tracer.

1. INTRODUCTION.

5HT_{2A}-Receptors seem to play an important role in psychiatric disorders. In order to investigate the role of these receptors in pathology there is an increasing interest in obtaining a selective and high affinity radiolabelled ligand suitable for in vivo receptor binding studies. For in vitro and rodent studies ¹²⁵I labelled tracers are preferred. Labelled with ¹²³I or ⁷⁵Br a suitable ligand offers the opportunity to perform SPE(C)T or PET.

Recently 4-amino-N-[1-[3-(4-fluorophenoxy)propyl]-4-methyl-4-piperidinyl]-2-methoxybenzamide (R91150, a Janssen Research Foundation compound) derivatives have been discovered as a new class of 5HT_{2A} antagonists. The parent compound shows very high affinity and selectivity for 5HT_{2A}-receptors (K_i for inhibition of [³H]ketanserin binding to rat frontal cortex membranes is 0.2 nM ; selectivity with regard to other neurotransmitter receptor sites such as 5HT_{1A}, 5HT_{1B}, 5HT_{1D}, 5HT_{2C}, 5HT₃, α₁ and α₂ adrenergic, histamine-H₁ and dopamine-D₂ is at least a factor of 50). The non - radioactive 5-bromo-benzamide and 5-iodo-benzamide analogues showed an affinity and selectivity similar to the parent compound mentioned above [1]. So it was decided to label this compound with radioiodine and radiobromine in order to develop a new radiolabelled ligand for SPECT and PET with high affinity for 5HT_{2A} receptors. In view of the lower increase in lipophilicity (increased lipophilicity causes increased non-specific binding) we have chosen the 5-position in the benzamide group i.e. ortho to the polar amine function.

2. EXPERIMENTAL.

2.1. Radiosynthesis of [¹²³I]- or [¹²⁵I]-4-amino-N-[1-[3-(4-fluorophenoxy)propyl]-4-methyl-4-piperidinyl]-5-iodo-2-methoxybenzamide .

0.7 mg 4-amino-[1-[3-(4-fluorophenoxy)propyl]-4-methyl-4-piperidinyl]-2-methoxybenzamide.2H₂O was dissolved in 500 µl of glacial acetic acid. The

radioiodide (N.C.A. for ^{125}I and a specific activity brought to 370 TBq / mmol for ^{123}I) was added while stirring followed by the addition of 100 μl of 30% H_2O_2 . The reaction was allowed to proceed for 15 minutes at room temperature. The reaction vial was transferred to a small ice-bath. To the reaction mixture 2 ml of ice-cold H_2O and 1.8 ml of 1M Na_2SO_3 were added while stirring and the pH was brought to 7.4 by addition of 2N NaOH. This solution was passed through a Baker Bond Octadecyl 100 mg column. The column was consecutively rinsed with 10 ml of a NaOH solution of pH 10 and 10 ml of H_2O . The starting compound and radioactive tracer were recovered in 600 μl of MeOH. 300 μl acetonitrile and 500 μl of H_2O were added and the complete mixture was filtered through a 0.45 μ filter. The filtrate was injected for semi-preparative HPLC separation. The pure radioactive tracer was recovered in 8 ml eluent, approximately 20 ml of H_2O was added and the solution brought to pH 9 with 2N NaOH. Preconcentration was performed on a Baker Bond Octadecyl column as described above.

After blowing the column apparently dry, the radioactive tracer was recovered in 500 μl EtOH. For human application 5ml of isotonic saline was added and the solution sterilised by means of a 0.22 μm Millex GV filter. The mean labelling yield reached 98% . The overall radiochemical yield is about 80%. Analytical and semi-preparative HPLC control of the final product revealed a purity of at least 99%.

2.2.Radiosynthesis of N.C.A. ^{77}Br -4-amino-N-[1-[3-(4-fluorophenoxy)propyl]-4-methyl-4-piperidinyl]-5-bromo-2-methoxybenzamide .

The longer lived ^{77}Br was used as isotope for development work.

0.7 Mg 4-amino-[1-[3-(4-fluorophenoxy)propyl]-4-methyl-4-piperidinyl]-2-methoxybenzamide. $2\text{H}_2\text{O}$ was dissolved in 500 μl of glacial acetic acid. 50 μl of the radiobromide solution was added while stirring followed by the addition of 100 μl of 30 % hydrogen peroxide solution. The reaction was allowed to proceed for 15

minutes at room temperature. The reaction vial was transferred to a small ice-bath. To the reaction mixture 1.8 ml of 1M Na₂SO₃ was added while stirring and the pH was brought to 7.4 by addition of 2N NaOH. The obtained solution was passed through a Baker Bond Octadecyl 100 mg column. Purification and recovery was performed as described for the radioiodinated compound.

3.RESULTS AND DISCUSSION.

3.1.Radiochemistry.

As in theory the 3-position, ortho to both an amino and methoxy group of the benzamide group, is more activated for electrophilic substitution than the 5-position a mixture of 3 and 5 iodinated compounds could be expected. Nevertheless the electrophilic substitution occurs nearly entirely at the 5-position. This is due to steric hindrance by the freely rotating methoxy group on the 2-position. Less than 0.5% of the compound labelled at the 3-position is observed by HPLC.

The overall yield of N.C.A. radioiodinated R91150 amounts to 80% and quality control revealed a purity of at least 99%.

In case of the radiobromination reaction, after 15 minutes the radiobromide is quantitatively consumed for substitution, 80 - 85 % is found in the 5-position and 20 - 15 % in the 3-position.

The overall radiochemical yield of ⁷⁷Br-5-Br-R91150 obtained with the proposed method varied from 75 to 80 %. Analytical and semi-preparative HPLC control of the final radiobrominated product revealed a purity of at least 99 % .

3.2.In vitro and in vivo binding properties to the rat brain.

The binding of the radioiodinated compound to rat frontal cortex membranes is saturable . A linear Scatchard plot yields a K_d value of 0.11 ± 0.01 nM and a B_{max}

value of 38.0 ± 0.7 fmoles/mg. Inhibition of the binding by ketanserin and ritanserin shows that the binding is reversible.

In vivo in rat brain the frontal cortex (FC) to cerebellum (CER) and FC to Blood ratio increases to a steady value of about 10 for FC / CER and 6 for FC / Blood up from 60 minutes to 3 hours [2]. Displacement by ketanserin, which was i.v. injected 60 minutes after the administration of the radioactive compound, showed that also in the in vivo brain the binding was reversible.

^{123}I -5- I- R91150 was also demonstrated to be suitable for imaging $5\text{HT}_{2\text{A}}$ receptors in different cortical areas and the striatum of a living baboon with a dedicated SPECT [3].

3.3. Preclinical studies.

Preliminary studies of the distribution of ^{123}I -5-I-R91150 (with a specific activity of $37 \cdot 10^7$ MBq/mmol) in young normal subjects showed that an average of 2% of a bolus dose is present in the brain between 30 and 240 minutes p.i..

The major part of the radioactivity in the brain is present in cortical areas. Cortical area to cerebellum activity ratio reaches an equilibrium around 90 min. p.i. which is maintained up to 4 hours. The cortico - cerebellar ratios (relative binding indices with a mean value of 1.8) reflects a distribution in the brain (cerebral cortex > striatum >> cerebellum) similar to that expected for $5\text{HT}_{2\text{A}}$ receptors from post mortem studies [4]. This binding was specific and reversible as shown by inhibition of the uptake by risperidone, an antipsychotic which is known to have high affinity for $5\text{HT}_{2\text{A}}$ receptors [5].

As ex vivo studies and limited PET studies pointed at a decline of $5\text{HT}_{2\text{A}}$ receptors with age, we decided to carry out this type of study in a large group of male and female volunteers with the new tracer and SPECT. This is an important factor in understanding $5\text{HT}_{2\text{A}}$ receptor related pathologies compared to matched

controls in clinical studies as well as for the follow - up of pharmacological dose - drug actions for antipsychotics used for treatment of those pathologies.

For semi quantitative analysis, ratios of ligand binding in different regions of interest to the binding in the cerebellum were calculated.. 5-HT_{2A} binding was shown to decline with age but no gender difference was demonstrated. These results are in agreement with in vitro and PET findings of a decline in 5-HT_{2A} receptor binding with age. These findings also highlight the necessity of age matched controls in clinical studies.

ACKNOWLEDGEMENTS

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THERAPEUTIC RADIOPHARMACEUTICALS

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PREPARATION AND BIOLOGICAL BEHAVIOUR OF SAMARIUM-153-HYDROXYAPATITE PARTICLES FOR RADIATION SYNOVECTOMY



XA9848015

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Abstract

The preparation and labelling procedures of ^{153}Sm -hydroxyapatite (^{153}Sm -HA) are described in this paper. Hydroxyapatite (HA) was prepared and studied as a radiosynovectomy agent. HA particles were prepared from the reaction of calcium nitrate and ammonia phosphate at high pH. Samarium-153 labelling was done in two steps with citric acid. A series of experimental conditions, such as: specific activity, citric acid mass, radioactive solution volume, in-vitro stability, have been carried out. Radiolabelling efficiency was greater than 95%. In vitro studies showed high stability ($\geq 99\%$). Animal studies showed a good retention in the synovium, with a very low extra-articular leakage over 6 days after administration.

1. INTRODUCTION

Rheumatoid Arthritis (RA) is an ubiquitous incapacitating disease that places substantial demands on health care resources [1]. The characteristic disease manifestations of RA are joint pain, swelling and reduced mobility as a result of the synovial tissue inflammation. It causes destruction of joint structures with deformation and loss of function. Radiosynovectomy is a radiation therapy used over 30 years for palliation of pain and swelling [2]. It consists of intra-articular injection of beta-emitting radionuclide in colloidal or particulate form, which gets in contact with synovium. Phagocytic cells absorb some of the injected dose, which is transmitted to the synovium. If the amount of radioactivity injected is large enough the tissue will be destroyed. Regenerated tissue will be asymptomatic for 2-5

years [3] Compared with surgical synovectomy, the radiation therapy is simpler, less traumatic and the hospitalization time is shorter, the cost is lower and the duration of relief is comparable

Ideal radionuclide must be a short-lived beta-emitter with no or low gamma-ray emission Since radioactivity leakage needs time, short half-life nucleus is required Thus decay occur without leaking and extra-articular irradiation is avoid New radiosynovectomy agents are designed with desired characteristics biodegradable, high affinity with target organ and high in vivo stability [4]

2. MATERIALS AND METHODS

2.1. Radionuclide

^{153}Sm chloride was produced in RA-3 reactor (Centro Atómico Ezeiza) by irradiation of 98.7% $^{152}\text{Sm}_2\text{O}_3$ via $^{152}\text{Sm}(n,\gamma)^{153}\text{Sm}$ The target material was dissolved in diluted nitric acid to a concentration of 5 mg/ml It was then put inside a quartz ampoule and carried to dryness by heating under dry nitrogen flow. The sealed ampoule was irradiated for 36 hours, at a thermal neutron flux of $7 \cdot 10^{13} \text{ n/cm}^2\text{s}$ Irradiated target was dissolved in HCl 0.1 N to get it as chloride, with a specific activity about of 5.55-11.10 GBq (150-300 mCi) $^{153}\text{Sm}/\text{mg Sm}_2\text{O}_3$

2.2. Hydroxyapatite preparation

Hydroxyapatite particles were prepared from the reaction of calcium nitrate and ammonia phosphate at high pH [5,6], 0.33 mol of $\text{Ca}(\text{NO}_3)_2$ was dissolved in 300 ml of water The solution was adjusted at pH 12 by addition of concentrated ammonia and diluted to 600 ml A $(\text{NH}_4)_2\text{HPO}_4$ solution (0.2 mol in 500ml, similarly brought to pH 12 and diluted to 800 ml) was added, drop by drop, stirring vigorously A voluminous precipitate was formed The reaction mixture was gently boiled for 10 minutes The precipitate was allowed to settle and

the supernatant solution was separated by decantation. The precipitate was rinsed with hot water, dried at 150 °C and heated for an hour at 240 °C to remove the ammonium nitrate. By strong heating at 800 °C for an hour, the product becomes largely anhydrous and hardened.

2.3. Particles size

Particle size range was studied using light microscopy. With an eyepiece graticule, the diameter of each particle in 100 consecutive fields from each sample (magnification x10) was recorded.

A process of sieving using sieves of 200 and 400 mesh was carried out. The portion of the sample retained on sieve 200 mesh (range over 75 µm) was discharged.

2.4. Labelling

Labelling was done in two steps.

- (a) ^{153}Sm -citrate was prepared by adding sufficient citric acid monohydrate to the $^{153}\text{SmCl}_3$ solution to give a concentration of 15 mg/ml citric acid in 0,1 N HCl. The mixture was allowed to stand at room temperature for 30 minutes.
- (b) The radioactive solution (370 MBq) was added to 1ml of particulate suspension (10 mg), stirring continuously (30 min, 37 °C).

2.5. Labelling efficiency

The radioactive mixture was transferred to a centrifuge tube using 4 ml of saline to rinse, centrifuged at 1000 rpm for 5 minutes. The supernatant was then transferred to another tube. Measurements of radioactivity were made and labelling efficiency was calculated as percentage of initial activity.

2.6. In vitro stability

In vitro stability studies were performed by incubating particles in normal saline and 1% human serum albumin solution over 48 hours at 37 °C with agitation. At different times

radiolabelled particles were centrifuged at 1000 rpm for 5 min. and activity in the particles and supernatant was measured.

2.7. Animal model

Normal rabbits were used as models to evaluate in vivo stability of radiolabelled HA particles. The studies were performed in New Zealand rabbits. Male and female rabbits weighing about 4 kg were used. Prior the administration microparticles were resuspended in 2 ml of saline (or glucose 5% in order to avoid the decantation) and autoclaved for 20 min at 121 °C. Each rabbit was injected intra-articular (into the left posterior knee joint) with 0.2 ml containing 37 MBq (1 mCi) of ^{153}Sm -HA. Images were obtained with a gamma camera using a high-resolution collimator (500.000 counts were measured with a 128x128 pixels matrix). The percent-injected dose in blood, urine and different organs was calculated daily over a period of 6 days. After that the animals were killed and the tissues were counted.

3. RESULTS

The yield of the hydroxyapatite synthesis was always greater than 80%. The particle size distribution showed a range of 5 μm to 50 μm (Table I).

TABLE I. Size distribution HA-microparticles

% of microparticles	Size (μm)
18	5-15
35	15-25
26	25-35
14	35-45
5	45-55
2	55-65

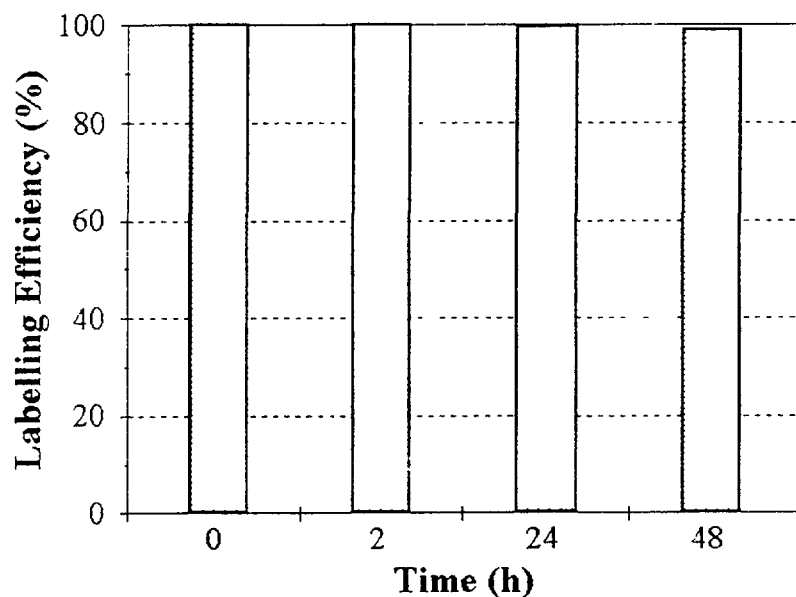


Fig.1. In vitro stability of radiolabelled HA particles

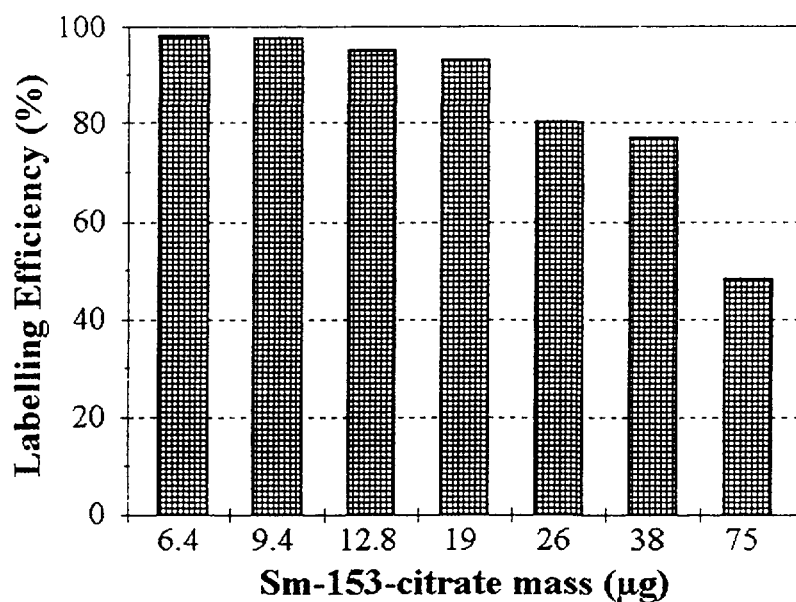


Fig.2. Effect of ^{153}Sm -citrate mass on labelling efficiency of HA particles

Labelled particles showed to be stable more than 48 h. No dissociation of activity from the particles was observed (Fig. 1). HA particles retained more than 99% from the original labelling at the studied conditions. No changes in particle size were observed.

Labelling did not depend on the presence of emulsifiers or microparticle pre-treatment. Though, it was highly dependent on complex mass as can be seen on Fig.2. The labelling

efficiency was greater than 99% when the ^{153}Sm -citrate quantity was less than 7 $\mu\text{g}/10\text{ mg}$ of particles. On the other hand, the labelling efficiency was less than 50% when ^{153}Sm -citrate mass was 75 μg .

The sedimentation velocity was lower when the particles were dispersed in glucose 5% solution. This is an important factor because if the sedimentation occurs very fast, many particles remain into the syringe and the administration is difficult.

Respect to animals' studies, no extra-articular localization of activity was detected by whole-body scans. All organs showed insignificant accumulation of ^{153}Sm activity. The principal observed fact over the whole study, was the permanence of the injected product in the joint.

4. DISCUSSION

Hydroxyapatite microparticles preparation method was reached. Reproducible method was with high yield. Size could be measured by optic microscopy. Particles size ranged from 5 to 50 μm , although it was difficult to estimate size below 5 μm .

Radiolabelling of HA particles with Sm-153 is simple to perform with high yields and radiolabelled-HA particles demonstrate high in vitro stability. Furthermore, labelling showed to be independent from ^{153}Sm specific activity but was highly dependent on ^{153}Sm -citrate mass.

The leakage of Sm-153 up to 6 days post administration in the joint was very low.

Stability experiments proved labelled particles were stable over 48 h.

Finally, biological behaviour was the expected one.

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PREPARATION OF ^{186}Re COMPLEXES OF DIMERCAPTOSUCCINIC ACID HYDROXY ETHYLIDINE DIPHOSPHONATE

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Abstract

$^{99\text{m}}\text{Tc(V)}$ -DMSA and $^{99\text{m}}\text{Tc}$ -HEDP are widely used for imaging medullary carcinoma and bone, respectively. ^{186}Re -HEDP is now well established as a therapeutic radiopharmaceutical for palliation of pain due to bone metastases. It is expected that $^{186/188}\text{Re(V)}$ -DMSA could find application for treating medullary carcinoma. In the present paper we report the work carried out for the preparation of ^{186}Re complexes of DMSA and HEDP and their bio-distribution studies in Wistar rats. ^{186}Re was prepared by irradiation of natural Re metal at a flux of 3×10^{13} neutrons/cm²/s for seven days and processed after a cooling period of four days. The specific activity of ^{186}Re formed was ~ 35 mCi/mg. Complexes with RC purity $>98\%$ could be prepared in both the cases by carefully optimizing the reaction conditions. Bio-distribution studies carried out in rats revealed that pharmacological behaviour of $^{186}\text{Re(V)}$ -DMSA was similar to that of $^{99\text{m}}\text{Tc(V)}$ -DMSA. ^{186}Re -HEDP showed a bone uptake of $\sim 30\%$ at 3 h post injection which remained almost constant for 48 h.

1. Introduction

External beam radiotherapy is an effective modality for treatment of malignant tumours as well as metastases. However, it has the disadvantage that along with malignant tissues normal tissues near the vicinity of the tumour also receive radiation

dose. In order to overcome this problem, research for the development of labelled biomolecules which are capable of selectively delivering the radioisotope and thereby the radiation dose to the target is currently undertaken in several laboratories. ^{186}Re and ^{188}Re are two of the radionuclides which are expected to find wide application for targeted radiotherapy. ^{186}Re ($t_{1/2}$ 90h, E_{β} 1.07, 0.93 MeV, E_{γ} 137 keV) and ^{188}Re ($t_{1/2}$ 17h, E_{β} 2.1 MeV, E_{γ} 155 keV) can be prepared in nuclear reactors with adequate specific activities. In addition, carrier free ^{188}Re can also be eluted from ^{188}W - ^{188}Re generator [1]. The chemical properties of Re are similar to Tc as both exist in Group VIIb of the Periodic table [2]. Hence rhenium analogues of technetium radiopharmaceuticals have been prepared and explored as therapeutic agents [3-4]. Though technetium and rhenium possess chemical analogy with each other, it is known that rhenium complexes are difficult to prepare than their technetium analogues [2]. Rhenium complexes have higher tendency to get re-oxidised back to perrhenate than do the analogous technetium complexes. This re-oxidation and consequently the presence of high radiochemical impurities in the final product is one of the major hindrance in the development of Re radiopharmaceuticals.

Unlike $^{99\text{m}}\text{Tc}$ which is available in very high specific activity from the ^{99}Mo - $^{99\text{m}}\text{Tc}$ generator, ^{186}Re is always contaminated with stable Re from the target material. The total concentration of technetium present during the preparation of $^{99\text{m}}\text{Tc}$ radiopharmaceuticals is of the order of 10^{-8} - 10^{-7} M [5] whereas the total concentration of rhenium is of the order of 10^{-3} M. Presence of large amount of carrier Re significantly alters the chemistry behind the preparation of Re complexes. Due to the above problems stringent experimental conditions are to be standardised to make rhenium radiopharmaceuticals. In the present paper, we describe the work carried out for the preparation of $^{186}\text{Re(V)}$ -dimercapto succinic acid (DMSA) and ^{186}Re -hydroxy ethylidine diphosphonate (HEDP)

$^{99m}\text{Tc(V)}$ -DMSA is a radiopharmaceutical used for the detection and follow up of patients with medullary thyroid carcinoma [6]. $^{186}\text{Re(V)}$ -DMSA would therefore have potential for targeted radiotherapy of medullary thyroid carcinoma [4,7]. Bone is the most common site of metastases in cancer patients and the most prominent symptom associated with bone metastases is pain which becomes progressively severe as the disease advances. ^{186}Re -HEDP can be used for palliation of pain resulting from metastatic bone lesions [3,8].

2. Materials and methods

Rhenium metal (Spect pure, 99.999%) used for irradiation studies was obtained from Johnson Matthey Co., U.K. Stannous chloride and DMSA was obtained from Sigma Chemical Company. HEDP used in these studies was synthesised by a reported procedure [9]. Flexible silica gel TLC plates (7.5x2.5cm, coating thickness 0.25 mm) were from J.T. Baker Chemical Company. Whatman no. 3 paper was used for paper electrophoresis. High purity germanium detector was used to determine radionuclidic impurities present along with ^{186}Re . A solid scintillation counter with NaI(Tl) crystal which is generally used for ^{99m}Tc was used for radioactivity measurements.

2.1 Production of ^{186}Re

10 mg of natural rhenium metal was irradiated in the Dhruva reactor for seven days at a flux of $3 \times 10^{13} \text{ n/cm}^2/\text{sec}$ and allowed to cool for four days. The sample was dissolved in 5 mL of 2M HNO_3 . Radionuclidic purity of ^{186}Re formed was estimated by gamma ray spectroscopy. 1.5 mL (3 mg, 16.1 μM) of rhenium solution prepared above was aliquoted in a vial and HNO_3 was evaporated to dryness by heating. 1 mL of 25% ammonia solution was added to the dry residue. Excess ammonia was removed by heating and the ammonium perrhenate residue was dissolved in 5mL of 5 M NaOH solution. Rhenium activity was extracted into 5mL of methyl ethyl ketone (MEK) and the

extraction efficiency was estimated by determining the radioactivity in equal aliquots of MEK and aqueous phase. Extraction was repeated once more with an equal volume of MEK and both the extracts were pooled together. MEK was removed by gentle heating and the residue was dissolved in 5 mL of normal saline. Radiochemical purity of ^{186}Re was determined by thin layer chromatography using acetone as solvent.

2.2 Preparation of $^{186}\text{Re(V)}$ -DMSA.

DMSA (2 mg, 11 μM) was dissolved in 0.1 mL of bicarbonate buffer (0.5M, pH 9) and 0.7 mL of normal saline. To this 0.02 mL of stannous chloride (20 mg/mL) in Conc. HCl and 0.2 mL (100 μg , 0.54 μM) of $^{186}\text{ReO}_4^-$ solution were added. The reaction mixture was purged with nitrogen and heated in a boiling water bath for 30 minutes. The reaction mixture was allowed to cool to room temperature and the pH was adjusted to 8 with 1 M NaOH solution. Since rhenium in lower oxidation states has a tendency to get converted to ReO_4^- , all the solutions used for complexation were purged with nitrogen prior to use. Also the complexes once formed were purged with nitrogen and stored till use.

Several experiments were carried out to optimize the conditions for getting maximum complexation yield. These include the effect of DMSA concentration, reaction temperature, reaction time, pH etc.

2.3 Preparation of ^{186}Re -HEDP

HEDP (50mg, 335 μM) was dissolved in 0.2 mL of bicarbonate buffer and 0.7 mL of normal saline. 0.04 mL (250 mg/mL) of stannous chloride and 0.2 mL of rhenium (100 μg , 0.54 μM) were added to the HEDP solution. The pH of the reaction mixture was about 2 and the solution was purged with nitrogen and heated in a boiling water bath for 30 min.

As discussed in the case of Re(V)-DMSA, similar optimization experiments were carried out to get maximum complexation yield in the case of Re-HEDP also.

2.4 Quality control techniques

2.4.1 Thin layer chromatography

TLC was performed using flexible silica gel plates. 5 μ L portion of the test solutions were applied at 1.5 cm from the lower end of the TLC plate. The strips were developed in acetone until the solvent reached the top of the strip. The strips were dried, cut into eight equal segments and the radioactivity was measured.

2.4.2 Paper electrophoresis

5 μ L samples were spotted on Whatman 3 chromatography paper 10-12 cm from the cathode and paper electrophoresis was carried out for 1h at 300 V in 0.02 M phosphate buffer at pH 7.5. The strips were cut into 1 cm segments and the radioactivity was measured.

2.5 Bio-distribution studies

Bio-distribution studies of $^{186}\text{Re(V)}$ -DMSA and ^{186}Re -HEDP complexes were performed in male Wistar rats weighing 300-400 g. 80-100 μCi of the complexes in ~ 0.3 mL volume was injected through penile vein and the rats were sacrificed at specific time intervals by cardiac puncture. The tissues and organs were excised and counted over a NaI(Tl) scintillation detector with flat geometry. The distribution of the activity in different organs was calculated as percent injected dose. Blood activity was calculated assuming blood volume as 5% of the total body weight.

Effect of probenecid to reduce the uptake of $^{186}\text{Re(V)}$ -DMSA by kidneys was also studied. 25mg/Kg of probenecid was injected 30 min prior to the injection of $^{186}\text{Re(V)}$ -DMSA and bio-distribution studies were carried out as above. Bio-distribution studies of $^{99\text{m}}\text{Tc(V)}$ -DMSA which was prepared by a reported method was also carried out (10).

3. Results and Discussion

3.1 ^{186}Re and $^{186}\text{ReO}_4^-$

The amount of ^{186}Re formed by irradiation for seven days followed by four days cooling was about 350-400 mCi, giving a specific activity of 35-40 mCi/mg. No other radionuclide other than ^{186}Re and ^{188}Re was detected in the gamma spectrum. It was observed that a two step extraction could remove ~95% of the Re activity into MEK. The removal of MEK was achieved by gentle heating of the extracted solution and the activity could be reconstituted in appropriate amount of saline to give the desired radioactive concentration (~15 mCi/mL in the present case). Results of the TLC and paper electrophoresis studies are given in Fig. 1 and 2, respectively. In TLC, the perrhenate activity moved to the solvent front and in paper electrophoresis, as expected, the activity moved towards anode. No activity was seen at the point of spotting in both the chromatography methods. The RC purity of the perrhenate solution was >99 % as estimated by TLC.

TLC and paper electrophoresis studies were used as quality control techniques. In TLC using acetone as solvent, the complexes remained at the point of spotting (Fig. 1). Reduced hydrolysed rhenium, if present, is expected to remain at the point of spotting and hence an additional quality control procedure was essential to estimate this RC impurity. The paper electrophoresis pattern of the complexes is shown in Fig. 2. The complexes moved towards anode with a migration rate similar to $^{186}\text{ReO}_4^-$. Reduced hydrolysed species, if formed by reduction of $^{186}\text{ReO}_4^-$, being uncharged is expected to remain at the point of spotting.

3.2. $^{186}\text{Re(V)}$ -DMSA

The paper electrophoresis studies indicate that the $^{186}\text{Re(V)}$ -DMSA complex formed is negatively charged similar to the Tc(V) -DMSA reported (10). By combining

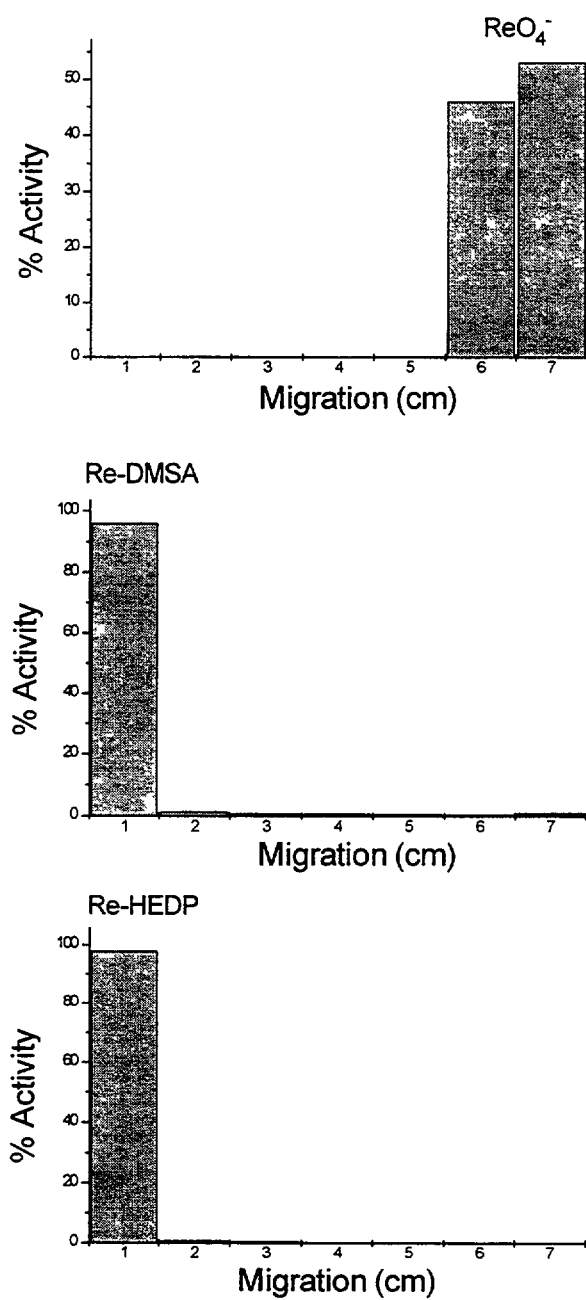


FIG. 1. Thin layer chromatography patterns of $^{186}\text{ReO}_4^-$, $^{186}\text{Re(V)}\text{-DMSA}$ and $^{186}\text{Re-HEDP}$.

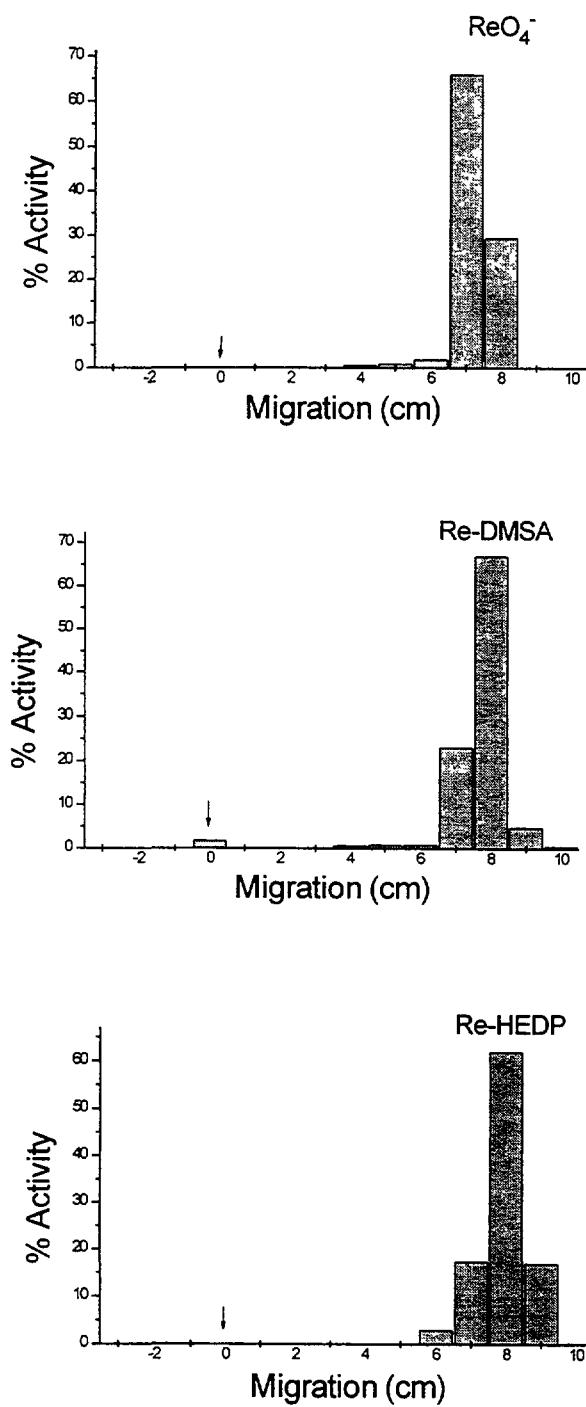


FIG.2. Paper electrophoresis patterns of $^{186}\text{ReO}_4^-$, $^{186}\text{Re(V)}\text{-DMSA}$ and $^{186}\text{Re-HEDP}$.

the results of PC/acetone and paper electrophoresis, the yields of the complex, $^{186}\text{ReO}_4^-$ and the reduced hydrolysed Re could be calculated.

Results of the studies on the effect of concentration of DMSA on complexation yield are given in Table I. It was observed from the studies that 0.5 to 2 mg of DMSA is optimum of get maximum complexation yield. is about. The optimum concentration of SnCl_2 needed for maximum complexation yield was 400 μg when 2 mg of DMSA was used for reaction and the reaction was carried out at 100°C (Fig. 3). Complexation reaction was carried out with the 2 mg of DMSA and 0.4 mg of SnCl_2 at pH 2 at room temperature and at 100°C for different time intervals. The reaction was found to be slow at room temperature and only 70% complexation yield was obtained even after 24 h of

Table1: Effect of ligand concentration on complexation yield

DMSA (mg)	0.06	0.13	0.25	0.5	1	2	-
%Yield	78	86	94	99	99	99	-
HEDP (mg)	0.15	0.3	0.6	1.5	3	10	50
% Yield	29	28	50	98	98	98	98

Table II: Effect of pH on complexation yield

pH	%Complex yield	
	DMSA	HEDP
2	97	98
4	50	97
6	13	87
8	2	88

incubation whereas the complexation yield was >97% when the reaction mixture was heated in a boiling water bath for 15 min.

Results of the effect of pH on complexation yield is given in Table II. The complexation yield was maximum at pH 2 and progressively decreased as the reaction pH was increased. Hence, the complexation reaction was carried out at pH 2. At this pH the analogous complex of technetium exists as Tc(III)-DMSA, hence it could be expected that the complex in the present case could also be Re(III)-DMSA. It is reported that complexation reaction at pH >7 results in Tc(V)-DMSA (10), however, with Re the complexation yield was only ~2% at pH 8. Hence, the complex was prepared at pH 2 and the pH of the complex formed was adjusted to 8 by addition of 1 M NaOH solution. This step was expected to possibly convert Re(III)-DMSA to Re(V)-DMSA. A change in the colour of the solution from pale yellow to pink was seen with increase in pH suggesting a different nature of the complex in acidic and alkaline medium. Though the complexation yield was poor at pH 8, the complex prepared at pH 2 and adjusted to pH 8 was found to be stable for 7 days.

3.3. ^{186}Re -HEDP

Quality control procedures used for characterisation of ^{186}Re -HEDP was the same as that used for $^{186}\text{Re(V)}$ -DMSA and the behaviour of the complex in the chromatography technique was similar to Re(V)-DMSA (Fig. 1 and 2). The effect of HEDP concentration on the complexation yield is given in Table I. The complexation yield was ~98% at HEDP concentrations greater than 1.5 mg. The effect of pH on complexation yield was studied by carrying out the reaction at different pH (Table II). It was observed that the complexation yield was maximum at pH 2 and even at pH 8 the complexation yield was ~88%. The optimum amount of stannous chloride needed was studied and it was observed that 400 μg of SnCl_2 was sufficient to give a complexation yield of ~98% when

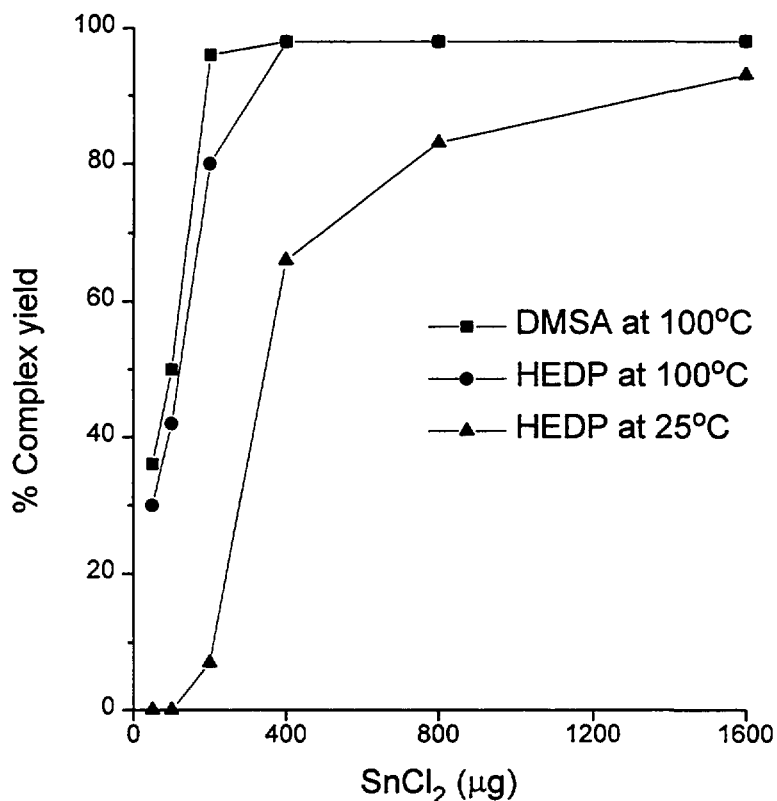


FIG.3. Effect of stannous chloride concentration on complexation yield

1.5 mg of HEDP was used (Fig. 3). However, it was observed in subsequent studies that the complexes prepared with 400 μg of SnCl₂ was unstable on storage. Hence, stability of Re-HEDP was studied at different reaction and storage conditions and it was observed that very careful optimization of the reaction conditions were essential to get a product with maximum stability. The results of the stability studies are summarised in Fig. 4. The product which was prepared with 1.5 mg of HEDP and 400 μg of SnCl₂, though showed 98% RC purity at the end of the complexation reaction, decomposed almost completely in about 48 h. When the complex was prepared with 50 mg of the ligand and 10 mg of stannous chloride, it was stable for 48 h at room temperature and for 120 h when stored at 4°C. The stability of the product was found to be less when the pH was adjusted to 8, though even at this pH better stability was seen at 4°C.

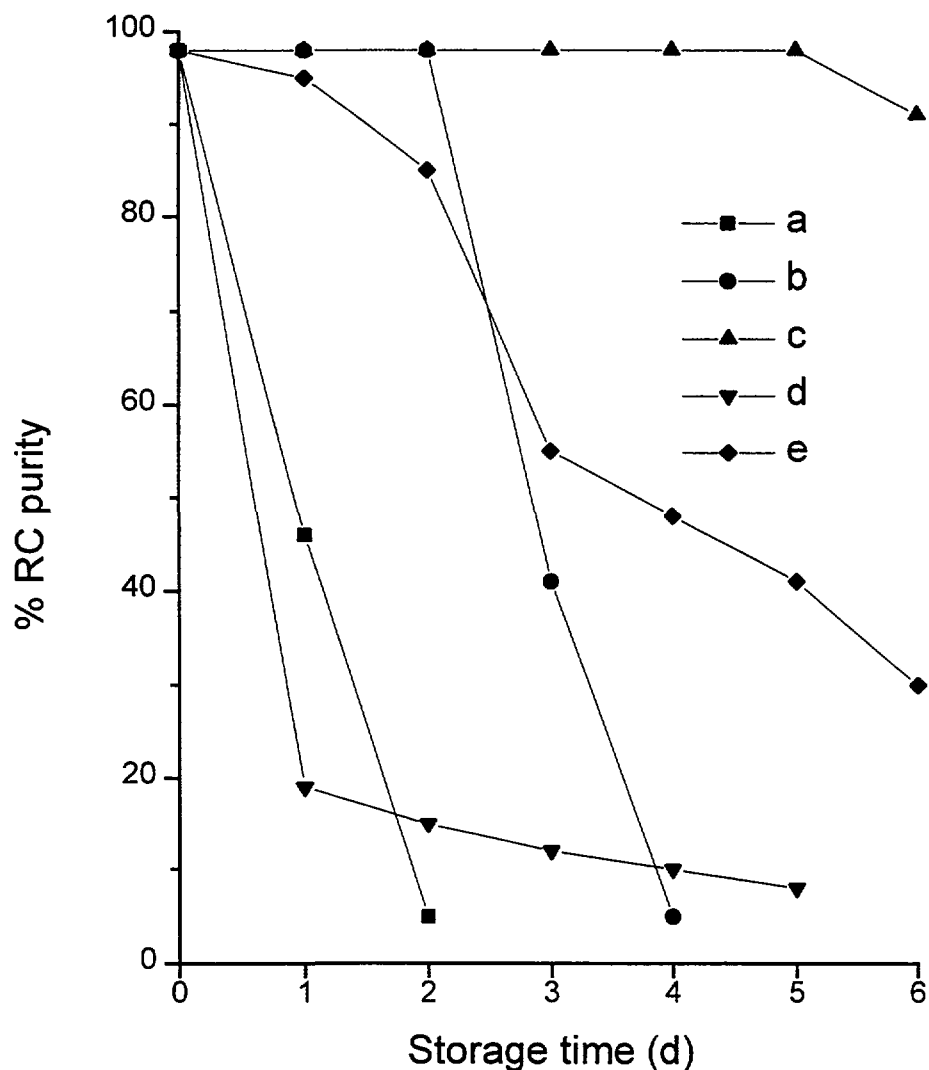


FIG.4. Radiochemical purity of ^{186}Re -HEDP prepared with

- a. 1.5 mg of HEDP and 0.4 mg of SnCl_2 at pH 2 and stored at 25°C
- b. 50 mg of HEDP and 10 mg of SnCl_2 at pH 2 and stored at 25°C
- c. 50 mg of HEDP and 10 mg of SnCl_2 at pH 2 and stored at 4°C
- d. 50 mg of HEDP and 10 mg SnCl_2 at pH 2, adjusted to pH 8 and stored at 25°C
- e. 50 mg of HEDP and 10 mg SnCl_2 at pH 2, adjusted to pH 8 and stored at 4°C

Complexation studies were also carried out with $^{186}\text{ReO}_4^-$ prepared by dissolution of the target in 2M HNO_3 and without the solvent extraction procedure. It was observed that the complexation yield at optimized reaction conditions as described above were 7% and 5% for DMSA and HEDP, respectively. Presence of oxidising agents and other impurities in the rhenium solution might be responsible for the lower yield observed.

3.4. Bio-distribution studies of $^{186}\text{Re(V)}\text{-DMSA}$ and $^{186}\text{Re-HEDP}$

The biological behaviour of $^{186}\text{Re(V)}\text{-DMSA}$, $^{99\text{m}}\text{Tc(V)}\text{-DMSA}$ and $^{186}\text{Re-HEDP}$ is given in Table III. More than 75 % of the injected activity was excreted in 24 h. No significant difference in blood clearance as well as uptake in major organs was noticed between $^{186}\text{Re(V)}\text{-DMSA}$ and $^{99\text{m}}\text{Tc(V)}\text{-DMSA}$. The near identical biological behaviour of the Re and Tc complexes of DMSA suggest that the former is indeed $^{186}\text{Re(V)}\text{-DMSA}$. Kidney uptake of $^{186}\text{Re(V)}\text{-DMSA}$ was marginally higher as compared to $^{99\text{m}}\text{Tc(V)}\text{DMSA}$. In order to reduce the kidney uptake of $^{186}\text{Re(V)}\text{-DMSA}$, probenecid was administered 30 min prior to $^{186}\text{Re(V)}\text{-DMSA}$ injection and bio-distribution studies were carried out. However, no significant reduction in renal uptake was seen with probenecid injection. The major disadvantages seen with $^{186}\text{Re(V)}\text{-DMSA}$ were the significant uptake of it in bone (~ 16 % at 24 h p.i.) and the retention of the activity by the kidneys. These might come in the way of its use as a therapeutic agent.

Bio-distribution studies with $^{186}\text{Re-HEDP}$ showed faster blood clearance with ~71% excretion of the activity at 3 h p.i. The bone uptake was ~30% at 3h p.i. which remained almost constant even at 48 h p.i. No significant uptake in any organ other than bone was seen at 48 h p.i. It is reported that due to in-vivo oxidation of rhenium, $^{186}\text{Re-HEDP}$ is washed out faster from normal bone than it does from abnormal bone, thereby increasing the ratio of uptake of abnormal to normal bone a favourable feature for a therapeutic bone agent (3).

In addition to the chemistry of complexation, the success of the development of therapeutic radiopharmaceuticals will also depend upon the availability of the isotopes in adequate quantities with sufficient specific activity. In the present studies, natural Re is used for the preparation of ^{186}Re . The calculations leading to the production of ^{186}Re and ^{188}Re at different fluxes are given in Table IV. While using natural Re for irradiation,

**Table III: Results of the bio-distribution studies of ^{186}Re (V)-DMSA,
 $^{99\text{m}}\text{Tc}$ (V)-DMSA and ^{186}Re (V) HEDP.**

Product	Time	Blood	Muscle	Bone	Liver	Gut	Kidney	Excretion
^{186}Re (V)-DMSA	1h	6.5 (0.9)	15.8 (2.5)	29.7 (10)	1.8 (0.2)	2 (0.4)	3.0 (0.7)	39 (6.5)
	3h	1.8 (0.3)	4.3 (0.8)	19.6 (5.6)	1.4 (0.20)	2.3 (1.2)	3.8 (0.5)	68 (5.6)
	24h	0.22 (0.1)	1.4 (0.4)	15.9 (5.0)	1.2 (0.1)	4.0 (2.4)	6.4 (1.5)	76 (10)
^{186}Re (V)-DMSA (With probenecid)	1h	6.7 (1.8)	17 (5.3)	27 (11.5)	1.8 (0.3)	1.9 (0.4)	3.0 (0.4)	31 (11)
	3h	2.0 (0.1)	4.4 (0.7)	26 (2.7)	1.3 (0.1)	1.0 (0.1)	3.9 (0.5)	71 (10)
	24h	0.7 (0.5)	0.8 (0.5)	15 (2.9)	1.0 (0.2)	4.2 (1.1)	5.4 (0.2)	80 (0.6)
$^{99\text{m}}\text{Tc}$ (V)-DMSA	1h	5.5 (0.3)	13.2 (1.4)	26.2 (1.7)	1.5 (0.2)	1.5 (0.9)	3.6 (0.5)	36.4 (0.4)
	3h	2.4 (0.2)	6.6 (0.7)	26.6 (3.2)	1.3 (0.2)	3.3 (0.9)	3.0 (0.7)	61.7 (3.1)
	24h	0.4 (0.1)	2.0 (0.9)	15.9 (3.0)	0.7 (0.1)	3.6 (1.4)	2.8 (0.2)	82.3 (2.0)
^{186}Re -HEDP	3h	1.0 (0.1)	2.1 (0)	30.7 (0.4)	0.5 (0.1)	3.3 (0.5)	1.3 (0.1)	71 (4.6)
	24h	0.4 (0.1)	0.8 (0.7)	28.2 (6.0)	0.3 (0.1)	2.7 (1.3)	0.6 (0.1)	75 (2.0)
	48h	0.3 (0.2)	0.4 (0)	29.5 (4.0)	0.3 (0)	1.6 (0.5)	0.7 (0.1)	80 (1.0)

Values reported are percent administered dose/organ Mean(\pm SD) n = 3-7 Total blood, muscle and bones are taken as 5, 45 and 6 % of the body weight, respectively.

Table IV: Production of Re isotopes by irradiation of natural Re target

	EOB			One Day Cooling			Four Days Cooling		
Flux (neutron /cm ² /s)	^{186}Re (mCi)	^{188}Re (mCi)	Specific activity (mCi/mg)	^{186}Re (mCi)	^{188}Re (mCi)	Specific activity (mCi/mg)	^{186}Re (mCi)	^{188}Re (mCi)	Specific activity (mCi/mg)
3×10^{13}	781	1215	200	649	456	111	373	24	40
6×10^{13}	1562	2430	400	1298	914	221	745	48	79
1×10^{14}	2603	4050	665	2164	1522	368	1243	81	132

^{188}Re is an inevitable radionuclidic impurity, which can be reduced to $\sim 6\%$ by allowing a cooling period of four days. However, such long cooling is not advisable as there will be reduction in activity and specific activity of ^{186}Re . 100 μg of Re activity was used in the present studies for the preparation of the complexes which will give a total activity of $\sim 4, 8$ and 13 mCi at neutron fluxes of 3×10^{13} , 6×10^{13} and 1×10^{14} neutrons/ cm^2/sec , respectively. This specific activity is grossly inadequate for therapy assuming the dose to be 0.5 to 1 mCi/kg of body weight. These calculations clearly indicate that irradiation of natural Re and cooling to reduce the ^{188}Re contamination will not be a practical solution for the production of ^{186}Re based radiopharmaceuticals. Irradiation of enriched Re at 6×10^{13} neutrons/ cm^2/s (an irradiation position ordinarily accessible in the Dhruva reactor) for seven days followed by 24 h cooling will give a product with adequate specific activity (~ 350 mCi/mg). However, the high cost of the enriched target ($\sim \$10,000$ per g) is a major handicap. Hence, a better alternative is to use a mixture of ^{186}Re and ^{188}Re for therapy which is obtained by irradiating natural Re for seven days followed by one day cooling which is used for radiochemical processing, quality control etc. Under these conditions a specific activity of ~ 220 mCi/mg could be obtained resulting in a radioactive concentration of 22 mCi/mL for the final radiopharmaceutical. About 60% of the activity will be ^{186}Re and the rest will be ^{188}Re . We are exploring the possibility of following the second option as ^{188}Re is an equally effective isotope for therapy.

Conclusion

The present studies indicate that rhenium form complexes with DMSA as well as HEDP in high yield. Conditions required for preparation of stable ^{186}Re -HEDP complexes were more stringent than required for the preparation of $^{186}\text{Re(V)}$ -DMSA complexes. Bio-uptake in major organs were found to be similar for $^{186}\text{Re(V)}$ -DMSA

and $^{99m}\text{Tc(V)}\text{-DMSA}$. A major disadvantage for using Re-DMSA for therapy of medullary carcinoma appears to be the high uptake of it in the kidneys and bone. Further work on reducing kidney uptake with proper blocking agents is in progress. $^{186}\text{Re-HEDP}$ has shown ~30% bone uptake with faster washout. Further studies on exploring the possibility of using these radiopharmaceuticals for clinical trials will be taken up soon.

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**CHALLENGES ASSOCIATED WITH Re-186:
FROM 'CARRIER-FREE' $^{99m}\text{Tc}(\text{V})$ -DMS TO
'CARRIER' CONTAINING $^{186}\text{Re}(\text{V})$ -DMS***

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Abstract

Efforts on radiolabeling with rhenium (Re-186, Re-188), a tumor agent to resemble the pentavalent polynuclear technetium complex of dimercaptosuccinic acid [$^{99m}\text{Tc}(\text{V})$ -DMS] have been reported, for radiotherapeutical use. Nevertheless, in spite of the periodic analogies between both radiometals, differences in the redox potential and the carrier concentration have made the radiolabeling of the rhenium counterpart difficult. Based on the drug-design bases set for the Tc-complex, the present work is centered on the study of the carrier effect on the radiolabeling of the pentavalent DMS complex of both radiometals at an alkaline pH. Gathered data indicated very interesting effect of the carrier present in either the Tc- 99m eluate or the reactor produced Re-186; although no effect of the carrier present in the generator eluate on the Tc-complex radiolabeling was detected, the great dependence of ligand and reducing agent on the Re amount present (two to four order higher than Tc) as carrier was noticeable in the Re-complex labeling. Under a good control of the above parameters, $^{186}\text{Re}(\text{V})$ -DMS was liable to be labeled with high yield (93-97%) at alkaline pH and room temperature. The mice biodistribution (Ehrlich Ascites Tumor bearing animals) of $^{186}\text{Re}(\text{V})$ -DMS superimposed that of $^{99m}\text{Tc}(\text{V})$ -DMS in most tissue except the excretory organs. The interesting effect of radiometal carrier on the biological behavior of $^{99m}\text{Tc}(\text{V})$ -DMS and $^{186}\text{Re}(\text{V})$ -DMS will be discussed.

1. INTRODUCTION

In recent years, efforts on radiolabeling with rhenium (Re-186, Re-188), a tumor agent to resemble the pentavalent polynuclear technetium complex of dimercaptosuccinic acid ($^{99m}\text{Tc}(\text{V})$ -DMS) have been reported (1, 2). The published work on radiolabeling with rhenium

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radionuclides has indicated that in spite of the periodic analogies with technetium, rhenium complexes are more difficult to reduce and more kinetically inert toward ligand substitution (3). Most of the work with rhenium-186, has reported a specific activity in the range of 5.92-8.88 GBq/mg Re(160-240 mCi/mg Re) with the total concentration of perrhenate of 3.5×10^{-4} - 5.5×10^{-4} M (1, 2). Since the total concentration of pertechnetate in the $^{99}\text{Mo}/^{99\text{m}}\text{Tc}$ generator eluent has been traced to values in the range of 10^{-7} - 10^{-6} M (37-74 GBq/mg, 1000-2000 mCi/mg) or lower, our interest focused on the differential effect of carrier on the radiolabeling from the 'carrier free' $^{99\text{m}}\text{Tc(V)}$ -DMS to the 'carrier' containing $^{186}\text{Re(V)}$ -DMS.

Moreover, in the $^{99\text{m}}\text{Tc(V)}$ -DMS formulated at the optimal ligand DMS concentration of 5.0×10^{-3} M (7.13 μmole), the amount of the reducing agent, stannous chloride ranged from 0.2 - 5.0 μmole at pH 8 to 9 for the Tc-complex to have affinity with tumoral cells /tissue (4). Due to the various experimentals and clinical reports supporting the distinctive character of $^{99\text{m}}\text{Tc(V)}$ -DMS from that of $^{99\text{m}}\text{Tc(III)}$ -DMS kidney imaging agent radiolabeled at an acid pH (5, 6), the present work will be centered on the radiolabeling of $^{186}\text{Re(V)}$ -DMS at an alkaline pH (pH 8.0-8.5) in the presence of variable carrier containing Re-186.

2. MATERIALS AND METHODS.

2.1 Materials

The ligand, meso-2,3-dimercaptosuccinic acid, abbreviated DMS was supplied by Sigma Chemical Co. St Louis, Mo (Registered name, Succimer). Tin(II) chloride dihydrate, $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ used for the reduction of rhenium was the pure reagent, for Automatic Aminoacid Analyzer, from Wako Pure Chemical Ind. Ltda. Others' chemicals were of reagent grade, also, from Wako Pure Chemical Ind Ltd, Japan.

The rhenium-186 made available as perrhenate (HReO_4) at pH 4.0 to 4.9 containing diverse amount of carrier by Japan Energy Research Institute, Department of Radioisotopes, Tokai-mura, Ibaraki. The specific activity ranged between 16-20 GBq/mg (430-540 mCi/mg) with the rhenium concentration in the range of 21.6-32.2 $\mu\text{g/mL}$. As regard the technetium-99m, solution containing diverse amount of inactive Tc-99 eluted from generators with diverse elapsed time since the last elution were used (Table 1). The total technetium content calculated to be in the order of 1.6×10^{-8} M - 1.0×10^{-7} M (7). On the other hand, the Re-186

made available by the Japan Atomic Energy Research Center(Tokai, Ibaraki, Japan) ranged 17.0-19.0 GBq/mg (460-513 mCi/mg) with the total perrhenate concentration in the range of 8.4×10^{-5} M - 1.15×10^{-4} M, which means that the Re-186 differs two to three order with that of technetium eluted from generators.

2.2 Radiolabeling $^{99m}\text{Tc(V)}$ -DMS and $^{186}\text{Re(V)}$ -DMS

The preparation of $^{99m}\text{Tc(V)}$ -DMS carried out as previously reported (4); namely, it follows the procedure described in the preparation of $^{186}\text{Re(V)}$ -DMS as in Fig 1. Every comparative studies carried out with $^{99m}\text{Tc(V)}$ -DMS and/or $^{186}\text{Re(V)}$ -DMS prepared with pertechnetate- 99m eluted 24 hr post last elution (Table 1, B); and a perrhenate-186 solution adjusted to contain 10 μmole .

2.3 Analytical Methods.

The analysis were carried out using Merck silica gel strips (Merck, Art 5553), developed in the standard developing solvent of n-buthanol (BuOH) : acetic acid (AcOH) : H_2O at 30 : 20 : 30 ratio (4). Also, acetone 100% selected for the detection of free perrhenate or pertechnetate. The TLC radioactivity analyzed by cutting the strip in segments of 0.5 cm whenever necessary and the radioactivity determined in the gamma counter. The area under the curve (AUC) of peak calculated and expressed as AUC % of peak.

2.4 Tumor bearing mice

Ehrlich Ascites Tumor Cells (EATC) maintained in-vivo by weekly intraperitoneal transplant, subcutaneously injected into the left leg flank of 5 weeks old (23-25g), ddy mouse. After the growth of tumor for 7 days, the in-vivo biodistribution of $^{186}\text{Re(V)}$ -DMS and $^{99m}\text{Tc(V)}$ -DMS were tested in animal weighing 28-32 g. Radioactive agents injected through the tail vein. After one, three or 24 hours, mice were decapitated, different organs excised and counted in a gamma counter. For the urine and faeces collection, mice kept in metabolic cages with water and food feeding ad-libitum.

Radiolabeling of Re(V)-DMS

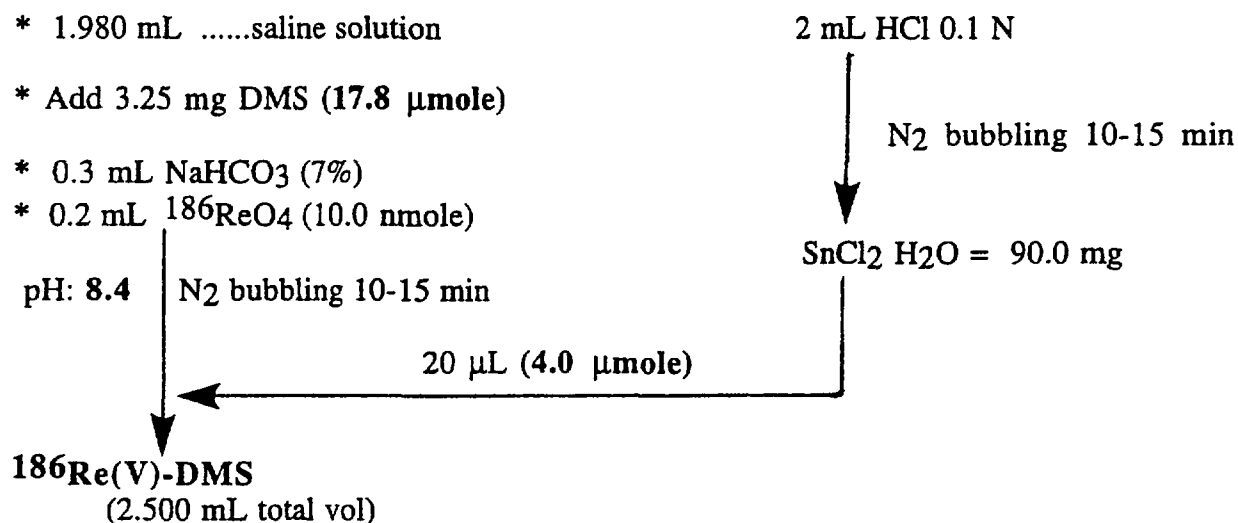


Fig. 1. Flow Chart of $^{186}\text{Re(V)}\text{-DMS}$ Radiolabeling in the Presence of 10 μmole of Rhenium-186.

3. RESULTS AND DISCUSSION.

The gathered data indicated very interesting effect of the carrier present in either the Tc-99m eluate or the reactor produced Re-186 on the radiolabeling of their pentavalent radiometal complexes of DMS and on their sequential biological effect tested in mice.

The radiolabeling of $^{99\text{m}}\text{Tc(V)}\text{-DMS}$ was carried out with eluates from generators with diverse elapsed time, as shown in Table 1. The labeling yield showed no alteration with the amount of carrier; also the TLC radioanalysis showed no detectable differences. The in-vivo biodistribution studies were carried out with EAT bearing mice. The radioactivity biodistribution studies indicated an enhanced radioactivity accumulation in the EAT tumor tissue with the increase of the inactive Tc-99 ('carrier') present in the eluate. The biodistribution in every excised organ indicated an increase of the clearance rate of the $^{99\text{m}}\text{Tc(V)}\text{-DMS}$ complex whenever prepared in the presence of higher Tc-99 'carrier'. Another tissue greatly sensitive to the presence of 'carrier' was the skeletal system.

On the other hand, the radiolabeling with Re-186 which contains much higher amount of carrier (lower specific activity than Tc-99m), demanded a critical control of various labeling parameters; namely, the

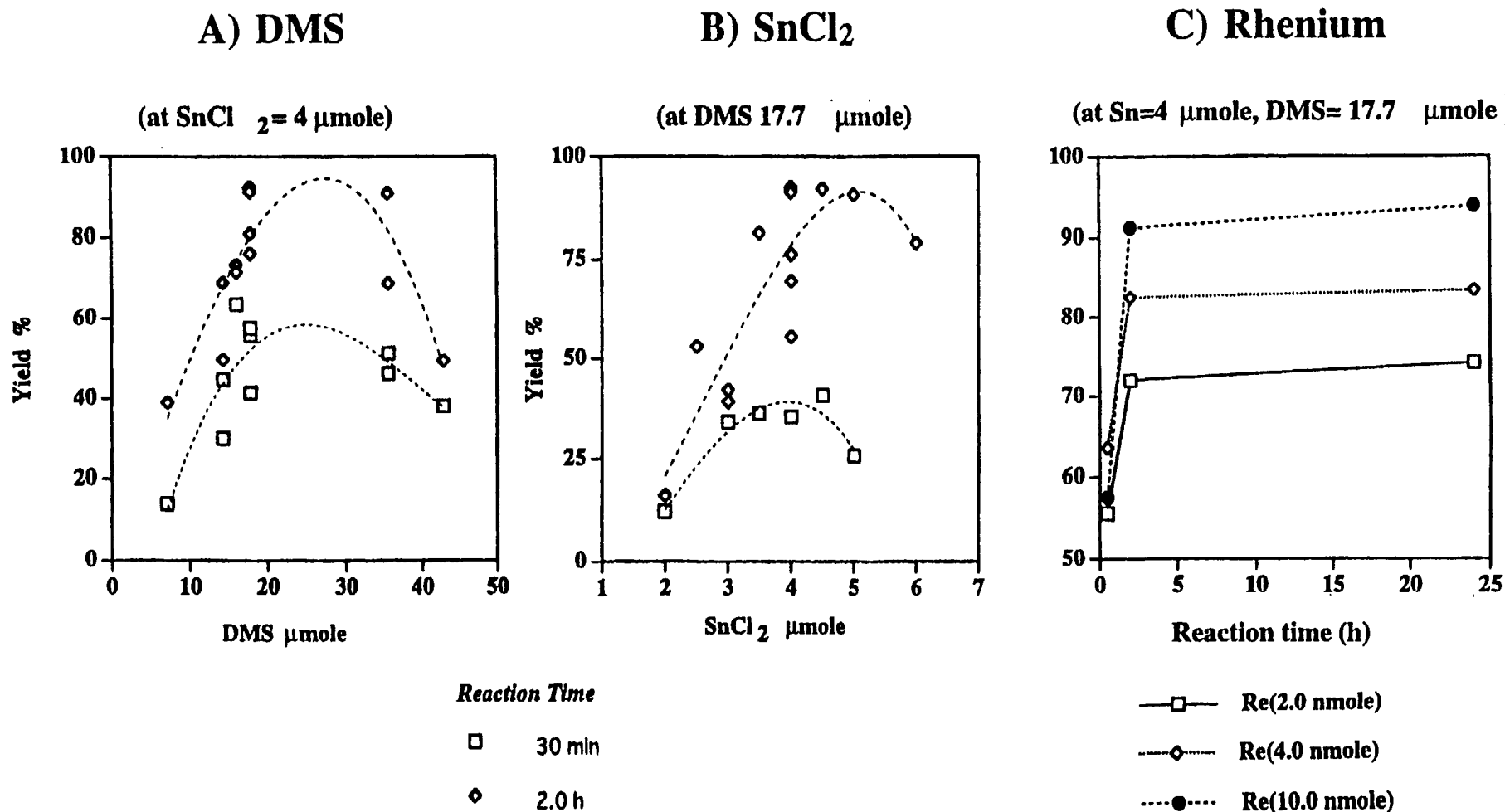


Fig. 2. Radiolabeling $^{189}\text{Re(V)}$ -DMS at Alkaline pH

A) Effect of DMS Ligand Concentration ($\text{Re} = 10 \mu\text{mole}$)
(At Stannous Chloride = $4 \mu\text{mole}$)

B) Effect of Stannous Chloride Concentration ($\text{Re} = 10 \mu\text{mole}$)
(At DMS $17.7 \mu\text{mole}$).

C) Effect of Rhenium Concentration
(At Stannous Chloride = $4 \mu\text{mole}$, DMS = $17.7 \mu\text{mole}$)

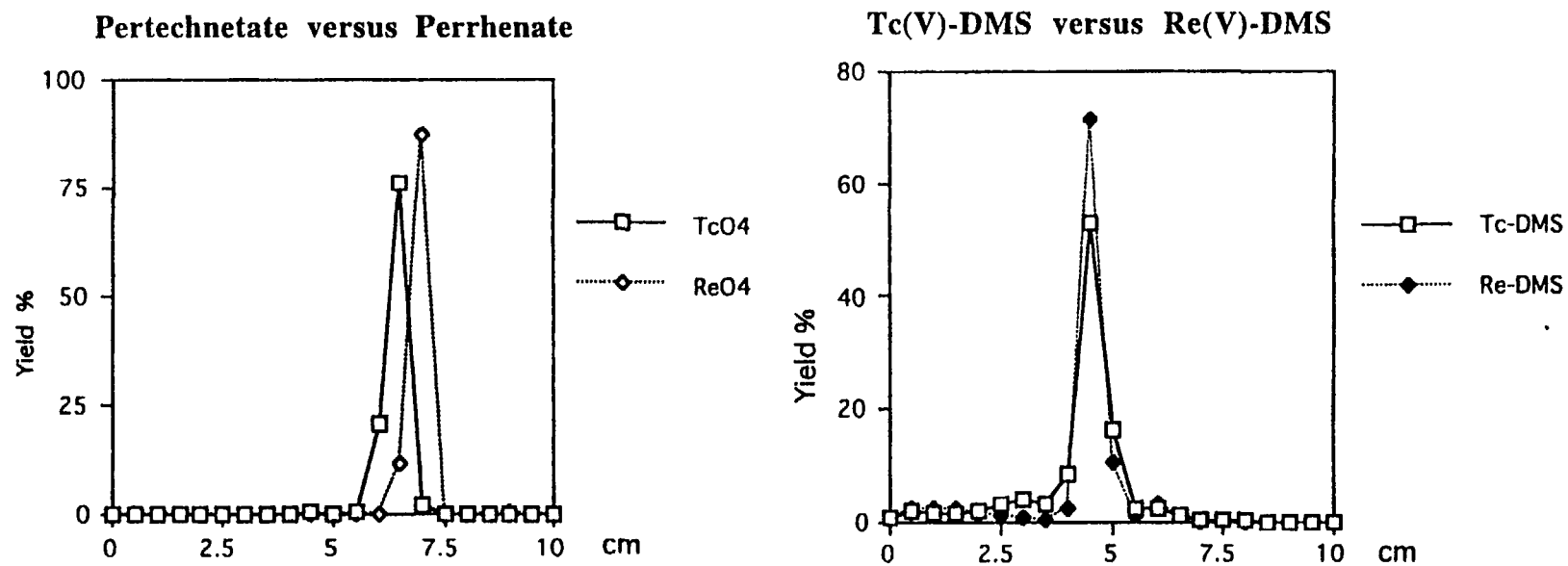


Fig. 3. Comparative Analytical Studies of $^{99m}\text{Tc(V)-DMS}$ versus $^{186}\text{Re(V)-DMS}$. Thin Layer Chromatography (TLC) studies carried on silica gel gtrips (Merck) in the following developing solvent : $\text{Bu(OH)} : \text{AcOH} : \text{H}_2\text{O} = 30:20:30$

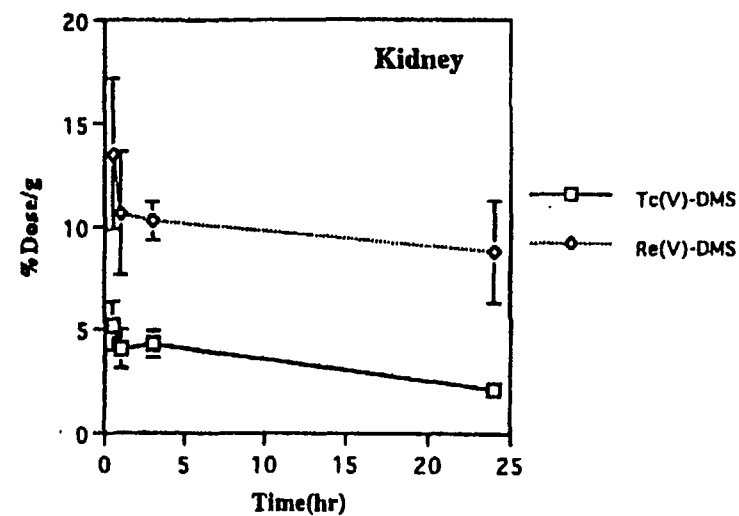
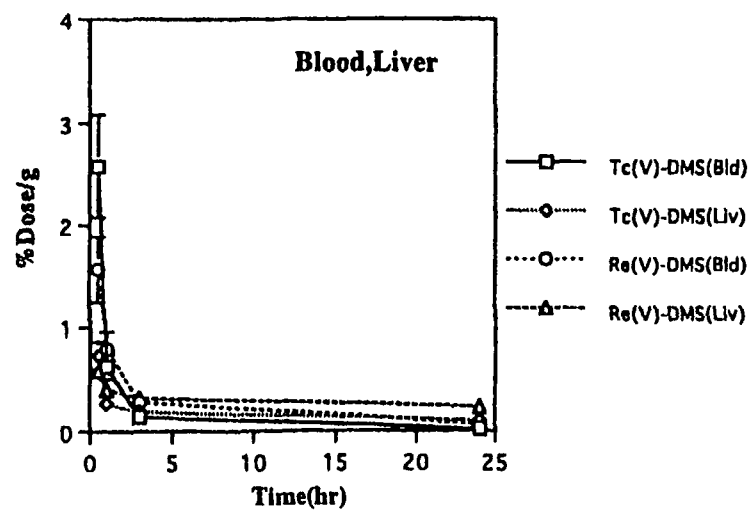
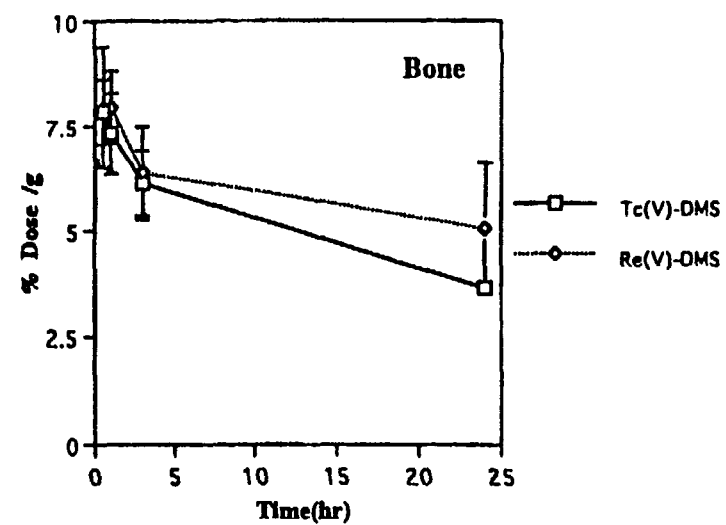
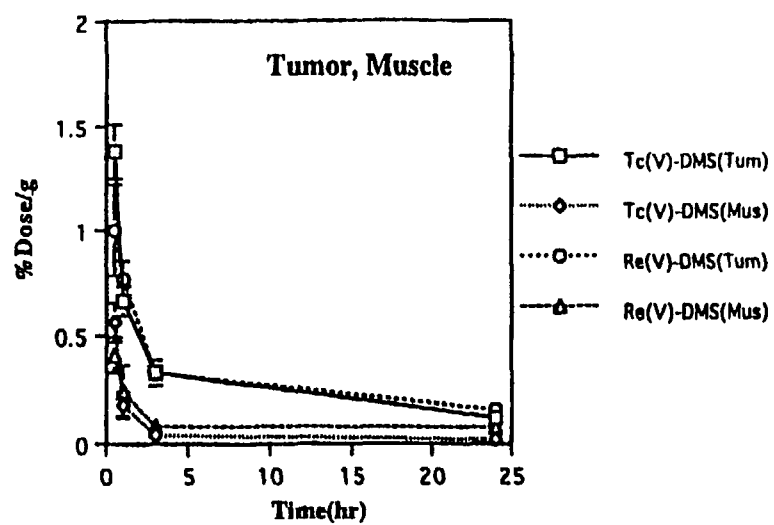


Fig. 4 Comparative Biodistribution Studies of $^{99m}\text{Tc(V)}\text{-DMS}$ versus $^{186}\text{Re(V)}\text{-DMS}$ in Ehrlich Ascites Tumor (EAT) Bearing Mice.

Table I.

**Effect of *Technetium Carrier* on Radiolabeling
Tc(V)-DMS**

Generator Time post- elution	Eluted ^{99m}Tc (mCi/ml)	@Ratio = $^{99}\text{Tc}/^{99m}\text{Tc}$	Total Technetium (M)	Tc(V)-DMS Labeling Yield %
A) 2.0 h	333.00*	0.29	0.165×10^{-7}	97.8 %
B) 24.5 h	4.60	2.65	0.324×10^{-7}	97.5 %
C) 192.0 h	0.96	55.00	1.04×10^{-7}	98.5 %

* Diluted 50 times

^{99m}Tc 1mCi = 1.91×10^{-7} mg

Table II.

**$^{99m}\text{Tc(V)}$ -DMS versus $^{186}\text{Re(V)}$ -DMS
Labeling Parameters**

	$^{186}\text{Re(V)}$ - DMS	$^{99m}\text{Tc(V)}$ - DMS	$\Delta =$ $^{186}\text{Re}/^{99m}\text{Tc}$
Radiometal Concentration nmole	2.0 - 20.0	0.04 - 0.08	50 - 250
Ligand DMS μmole	17.7 - 24.0	7.13	2.5 - 3.5
SnCl_2 μmole	4.0 - 6.0	0.2	20 - 30
$[\text{Sn}] / [\text{Tc}]$ $[\text{Sn}] / [\text{Re}]$	300 - 2000	2500 - 5000	—
$[\text{DMS}] / [\text{Sn}]$	4.0 - 4.5	~ 36	—

amount of the ligand DMS and the reducing agent, stannous chloride depended on the amount of the 'carrier' present in the provided Re-186 solution. There was a need to increase the ligand DMS concentration and the amount of the reducing agent (Fig 2) for the reaction to proceed. The time required for the reaction to reach 93-97% completion was basically a function of rhenium concentration (Fig 2C). Moreover, it required longer radiolabeling period than the Tc-99m (30-120 min for the Re-186 versus 10-15 min of the Tc-99m) (Fig 2A, 2B) for the reaction to proceed at room temperature. Also, the greater increment of the stannous chloride amount, might be probably due to the presence of higher amount of rhenium 'carrier', and/or its involvement in the complex itself. For the optimal labeling condition (Fig 1), the amount of the ligand DMS needed was 2.5-3.5 times, while that of the reducing agent increased 20-30 times (Table 2) (with regard the labeling with the radiometal, Tc-99m), under various DMS/Sn and Sn/Re mole ratio. The TLC analysis developed on BuOH:AcOH:HsO= 30:20:30 of the 186-Re(V)-DMS labeled under the optimal formulation clearly superimposed the analysis pattern of the $^{99m}\text{Tc(V)}$ -DMS (Fig 3).

The organ biodistribution study in EAT tumor bearing mice, indicated also the superimposition of $^{186}\text{Re(V)}$ -DMS to the $^{99m}\text{Tc(V)}$ -DMS biodistribution pattern (Fig. 4); namely, the radioactivity biodistribution detected in the tumor tissue was very similar; the excretory kidney tissue was the most sensitive organ to the presence of the carrier. Nevertheless, as a whole it can be said that the data supported the in-vivo behavior of $^{186}\text{Re(V)}$ -DMS labeled under the optimal condition described in Fig 1, to mimic that of $^{99m}\text{Tc-99m}$ radiolabeled agent.

In conclusion, the presence of the 'carrier' in either, the generator produced Tc-99m or the reactor produced Re-186 represented a great challenge for the radiolabeling reaction and the biodistribution of the pentavalent technetium or rhenium DMS tumoral agents. In the present work, the specific activity of Re-186 ranged between 17-19 GBq/mg, and the calculation indicated 50 to 250 higher concentration of the carrier than the generator eluate of technetium-99m. On the other hand, the effect of the Re carrier on the radiolabeling of $^{186}\text{Re(V)}$ -DMS seems to support the mechanistic step controlling the radiolabeled product as being other than first-order in the radiometal concentration (8), as depicted in the drug-design basis of the pentavalent polynuclear $^{99m}\text{Tc(V)}$ -DMS complex (4). Most of the attempt to radiolabel the

tumor agent, $^{186}\text{Re(V)}$ -DMS has been carried out at low pH (1, 2); in the present radiolabeling at an alkaline pH, the redox potential of the stannous chloride might be weaker than in the reported work (pH 2-3) for the rhenium reduction; so greater increment on the amount of stannous chloride was necessary for the reaction to proceed at room temperature. Under the presented optimal labeling condition of $^{186}\text{Re(V)}$ -DMS (Fig. 1), the biodistribution in tumor tissue mimic that of $^{99\text{m}}\text{Tc(V)}$ -DMS. Thus, rewards from the present studies on the carrier effect were the translation of Tc(V)-DMS drug-design approach into the preparation of $^{186}\text{Re(V)}$ -DMS, a simpler formulation liable to be carried out at an alkaline pH, and at room temperature, with yield over 92%.

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THERAPEUTIC APPLICATION OF NEW HOLMIUM-166 CHITOSAN COMPLEX IN MALIGNANT AND BENIGN DISEASES

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Abstract

The new holmium-166 chitosan complex ($^{166}\text{Ho-CHICO}$, DW- ^{166}HC) was prepared by reacting the aqueous acidic solution of chitosan with $^{166}\text{Ho}(\text{NO}_3)_3$ at room temperature with quantitative labelling yield. The progress of the reaction and labelling yield were determined by instant thin layer chromatography using silicic acid impregnated glass fiber (ITLC-SA) and developing solvent of $\text{MeOH:H}_2\text{O:HAC}$ (49:49:2). The high labelling yield of more than 99% was obtained by reacting chitosan solution (35 mg/4 ml) with $^{166}\text{Ho}(\text{NO}_3)_3$ in which 7 mg of $^{165}\text{Ho}+^{166}\text{Ho}$ were contained as a maximum content. The labelling yield was highly dependent on the pH of the chitosan solution. The optimal labelling could be obtained at pH 2.5~3.5. The characteristics of $^{166}\text{Ho-CHICO}$ were similar to those of chitosan, which is biocompatible, biodegradable, non-toxic, soluble and viscous in acidic condition but gelatinous at pH 6.0 and precipitating in alkaline conditions. $^{166}\text{Ho-CHICO}$ can be easily prepared by reconstituting freeze-dried chitosan (kit A) with $^{166}\text{Ho}(\text{NO}_3)_3$ solution (kit B) just prior to use. After intrahepatic administration of $^{166}\text{Ho-CHICO}$ to male rats, the radioactivity concentrations in blood were low and the cumulative urinary and fecal excretion over a period of 0 to 72 hours were 0.53% and 0.54%, respectively. The radioactivity concentration in tissues and the whole-body autoradiography images showed that most of the administered radioactivity was localized at the administered site, and only slight radioactivity was detected from the liver, spleen, lungs, and bones. An autoradiograph after intratumoral administration of $^{166}\text{Ho-CHICO}$ showed that radioactivity was localized at the administered site of the lesion without distribution to other organs and tissues. A biodistribution study in normal rabbits with $^{166}\text{Ho-CHICO}$ showed that most of the radioactivities were retained in the knee joint with negligible extra leakage at 72 hours after intra-articular administration to male rabbits. Biodistribution of $^{166}\text{Ho-CHICO}$ 48 hours after intraperitoneal administration to male mice showed most of the radioactivities were evenly distributed at the inner wall of the peritoneal cavity. The ease with which the $^{166}\text{Ho-CHICO}$ can be prepared as a kit form and its high *in-vitro* and *in-vivo* stability make it an attractive agent for radionuclide therapy in malignant and benign diseases.

1. INTRODUCTION

Radionuclides considered suitable for therapy may be divided into three categories, i.e. beta-particle emitters, alpha particle emitters, and Auger and Coster-Kronig electron emitters. The physical characteristics of the most commonly-used therapeutic radionuclides, along with others whose use is various stages of development, have been reported [1]. Most of the radionuclides in

routine use are beta emitters. Beta particles are of low LET and their RBE is unity. The path length is quite variable, ranging from 1.0 mm for the beta particles of ^{169}Er , to 12.0 mm for those of ^{90}Y . Nevertheless, the range of beta particles is considerably greater than the diameter of a typical cell (5~20 μm), and thus, the whole cell and surrounding cells are irradiated.

Many different radiopharmaceuticals have been applied clinically in the treatment of various malignant and benign conditions over the past 50 years. Only a small number of radiopharmaceuticals have been developed on a commercial basis and have become established as routine therapeutic agents. Falling into this category are ^{131}I -iodide for thyroid disorders ^{32}P -phosphonate for blood disorders, ^{89}Sr -Chloride for pain control in metastatic bone diseases, ^{131}I -MIBG for neuroendocrine tumors, ^{90}Y or ^{32}P -colloids for intracavitary therapy, such as the treatment of intra-peritoneal metastases and of malignant effusions, intracystic therapy, intrathecal therapy, radionuclide synovectomy, regional therapy of liver tumors, and radioimmunotherapy.

Among the various therapeutic applications, we focused on the intracavitary therapy and regional therapy of liver tumors. Direct intracavitary administration is a means of delivering radiopharmaceutical in high concentration to tumors which are spread out over the serosal linings of cavities and to tumor cells present in malignant effusions. In order to minimise leakage of the radionuclide from a cavity, it is usually given in the form of a radiocolloid or particulate. Even so, a proportion of the radionuclides will reach the blood stream by lymphatic drainage and by leakage of ionic radionuclides as the colloid is degraded. Intracavitary therapy is applied to the peritoneal, pleural and pericardial cavities as well as to cystic brain tumors and to the spinal canal. A non-cancer application of the method is the intra-articular injection of radiocolloids to treat inflammatory joint disorders.

In order to solve leakage problems in radiation synovectomy, the labelling of macroaggregates or microspheres of degradable particles (1~10 μm) as carriers of radionuclides has been attempted. Ferric hydroxide macroaggregates (^{165}Dy -FHMA) [2], hydroxide macroaggregates (^{165}Dy -HMA) [3], ^{166}Ho -PLA microspheres [4], and particulate hydroxyapatites (^{153}Sm -PHYP) [5] have been reported. In the case of hepatic tumors, ^{90}Y -resin microspheres [6], ^{90}Y -glass microspheres (22 μm) [7], [8], ^{166}Ho -Poly (L-lactic acid, 10~45 μm) [9], and ^{90}Y -Lipiodol [10] have been reported.

Even though the extra-leakage problem of radioactivity from an administered site has been mostly solved by controlling the size of particulates, there are many limitations to be overcome in its practical use such as large scale production, appropriate size and density control. When possible, soluble radiopharmaceuticals rather than colloids or particulates are more desirable for the even distribution within lesions after administration. If so, such radiopharmaceuticals should be used in both intracavitary and regional therapy. To our knowledge, aqueous soluble radiopharmaceuticals for these therapies have not been reported until now.

In this context, continuous effort has been devoted to develop new biodegradable, biocompatible, lanthanide radionuclides such as ^{153}Sm , ^{165}Dy , ^{166}Ho , and ^{169}Er . Among the natural polymers, chitosan, which is deacetylated chitin (Poly- β (1-4)-N-acetyl-D-glucosamine) [11], and naturally abundant, especially in the cuticle of marine crustacean, was chosen. In this report, we established the optimal preparative conditions of holmium-166 chitosan complex (^{166}Ho -CHICO) and determined its *in-vitro* and *in-vivo* stability.

2. MATERIALS AND METHODS

Chemicals

A reagent grade (>99.99%) of $\text{Ho}(\text{NO}_3)_3 \cdot 5\text{H}_2\text{O}$, was supplied by the Aldrich Chem. Co. Inc. Chitosan (400,000-500,000 MW, degree of deacetylation, 90%) was purchased from the Samcheon R1 Pharm. Co. Instant thin layer chromatography plates (glass fiber impregnated with silicic acid, ITLC-SA) were supplied by

Gelman Science, Inc. All other chemicals were of analytical grade and obtained from commercial sources.

Preparation of Lanthanide Radionuclides (^{153}Sm , ^{165}Dy , ^{166}Ho , ^{169}Er)

For *in-vitro* and *in-vivo* analysis, 40-200 mg nitrate salt of lanthanide was irradiated in the HANARO Reactor at the Korea Atomic Energy Research Institute, Taejon, Korea (reactor power=30MW) for an appropriate time depending on the above experimental purposes. Radionuclidic purity was determined by a Multichannel Analyzer and the radioactivity of the irradiated samples was determined by a radioisotope calibrator (Model CRC-12, Capintec Inc.). Irradiated sample was dissolved in a dilute HCl solution (pH 3.0) and then filtered using a $0.2\mu\text{m}$ membrane filter for sterilization.

Preparation of Holmium-166 Chitosan Complex ($^{166}\text{Ho-CHICO}$)

$^{166}\text{Ho}(\text{NO}_3)_3$ was chosen as a typical radionuclide for the preparation of the chitosan complex. $^{166}\text{Ho-CHICO}$ was prepared by adding 0.1ml of 10% $^{166}\text{Ho}(\text{NO}_3)_3$ solution in dilute HCl (pH 3.0) to a sterile 1% chitosan solution in 1% acetic acid. The resulting solution was stirred thoroughly by a vortex mixer or in a ultrasonic bath for 10 minutes. The reaction mixture was left standing for 30 min at room temperature and then the labelling yield and radiochemical purity of $^{166}\text{Ho-CHICO}$ was determined by Instant Thin Layer Chromatography [ITLC-SA, developing solvent, $\text{MeOH:H}_2\text{O:HAc}(49:49:2)$].

The Effect of pH on Formation of $^{166}\text{Ho-CHICO}$

The chitosan solution, in which 40 mg of chitosan was dissolved in 4 ml of 1% aqueous acetic acid solution, was prepared to give various solution (pH 1-5 range). A 0.1 ml of 10% $^{166}\text{Ho}(\text{NO}_3)_3 \cdot 5\text{H}_2\text{O}$ solution was added into the resulting solutions at room temperature by stirring.

The Effect of an Amount of ^{166}Ho on Formation of $^{166}\text{Ho-CHICO}$

At pH 3.0 and 35 mg/4 ml chitosan solution fixed, the chitosan solution was reacted with the $^{166}\text{Ho}(\text{NO}_3)_3 \cdot 5\text{H}_2\text{O}$ soln. increasing the amount of holmium (3.74 mg, 7.48 mg, 11.22 mg, 14.96 mg, 22.44 mg, 29.92 mg).

In-vitro Analysis

The Effect of Radioactivity on the Stability of $^{166}\text{Ho-CHICO}$

To the chitosan solution (40 mg/4 ml) various $^{166}\text{Ho}(\text{NO}_3)_3$ solutions containing 5 mCi, 10 mCi, 30 mCi and 50 mCi was added and then the radiochemical purity was determined 1 hour after the addition of $^{166}\text{Ho}(\text{NO}_3)_3$.

The Effect of Ascorbic Acid on the Stability of $^{166}\text{Ho-CHICO}$

To the chitosan solution containing various amounts of ascorbic acid (10 mg-40 mg) 50 mCi of $^{166}\text{Ho}(\text{NO}_3)_3$ solution was added and then the radiochemical purity was determined at appropriate time intervals after labelling.

In-vivo Studies

For the *in vivo* retention study, two normal New Zealand White rabbits weighing 3.5-4.5 kg were used. After the preparation of $^{166}\text{Ho-CHICO}$ solution in aseptic conditions, about 5 mCi of $^{166}\text{Ho-CHICO}$ was administered into the knee joint of rabbits. Immediately after administration, the rabbits were imaged using a gamma camera (Siemens Digitrac 37) fitted with a pinhole collimator. A 20% window was centered around the 81 keV γ -ray of ^{166}Ho . The background and decay-corrected counts per pixel at 2, 6, 24, and 48 hr, times of injection were determined and calculated as percents injected activity retained in the knee over time.

For biodistribution studies, $^{166}\text{Ho-CHICO}$ (1-2 mCi) was injected into one knee joint of a rabbit ($n=3$). Daily blood samples and the total urine excreted were obtained and counted for ^{166}Ho activity. It was assumed that the total blood volume for the rabbit was 57.7 ml/kg. Biodistribution data for ^{166}Ho were acquired by killing the rabbits at 48 and 120 hrs. after CHICO administration. The major organs were removed, weighed, and counted for activity in a NaI(Tl) well-counter. The percent injected dose in major organs per g was calculated. Extra-articular leakage was calculated as the sum of all activities in major organs, total urine excreted and the activity remaining in the circulating blood at the time of sacrifice.

Male Sprague Dowley rats (6 weeks old) and male ICR nude mice (5 weeks old) were purchased from Charles River Laboratories Japan, Inc. The animals were kept in stainless cages in a room with a maintained temperature of $22 \pm 1^\circ\text{C}$, a relative humidity of $60 \pm 10\%$ and an alternating 12 hr light/dark cycle. The animals were allowed free access to fresh tap water and laboratory animal chow, CE-2 (Clea Japan, Tokyo). After acclimation for approximately a week, healthy rats and mice were used for the experiments. Tumor transplanted nude mice were prepared with transplantation of B16 melanoma into the liver lobe using a previously described method. The transplanted mice were kept for approximately 10 days and then used for the experiments.

In intrahepatic or intratumoral administration, ^{166}Ho -CHICO solution was directly injected into the liver or transplanted tumor by surgical techniques. In intravenous administration, ^{166}Ho -CHICO solution was administered via the caudal vein.

The animals were sacrificed by diethyl ether at the predetermined time. They were frozen in liquid nitrogen and then mounted for sectioning. From each animal, thin sections were prepared with the Cryomacrocut (Leica). The obtained sections were then freeze-dried at -20°C . The sections were exposed to the imaging plates (IP; Fuji Photo Film Co., Ltd.). After exposure, each autoradiography image was obtained by BAS 2000.

3. RESULTS

Preparation of ^{166}Ho -CHICO

Thin layer chromatography. Irradiation of 40 mg of $^{165}\text{Ho}(\text{NO}_3)_3 \cdot 5\text{H}_2\text{O}$ in a neutron flux of $1 \times 10^{13} \text{ n/cm}^2 \cdot \text{sec}$ for 10 hr gave 100 mCi of $^{166}\text{Ho}(\text{NO}_3)_3 \cdot 5\text{H}_2\text{O}$ with radionuclidic purity ($>99.9\%$). The progress of reaction and labelling yield were determined by ITLC-SA. The R_f of ^{166}Ho -CHICO and free $^{166}\text{Ho}(\text{NO}_3)_3$ were 0.3 and 0.9, respectively in the solvent system of $\text{MeOH}:\text{H}_2\text{O}:\text{HAc}(49:49:2)$.

Labelling yield. The labelling yield was highly dependent on the pH of reaction mixture, chitosan concentration, the amount of $\text{Ho}(\text{NO}_3)_3$ and on the reaction time. A high labelling yield greater than 99% was obtained by reacting the chitosan solution ($>35 \text{ mg/4 ml}$) with $^{166}\text{Ho}(\text{NO}_3)_3$ solution ($<7 \text{ mg of } ^{166}\text{Ho} + ^{165}\text{Ho}$) in acidic conditions (pH 2.5-3.5) at room temperature for 20 min (Table I and II).

TABLE I. THE EFFECT OF PH ON LABELLING YIELD OF ^{166}Ho -CHICO*

pH of Reaction Mixture	Labelling Yield**
1.47	20
2.00	30
2.78	95
3.53	95
4.03	30
5.00	20
6.00***	.

*Ten mg of $^{166}\text{Ho}(\text{NO}_3)_3 \cdot 5\text{H}_2\text{O}$ in 0.1 ml of distilled H_2O was reacted with 30 mg of chitosan dissolved in 4 ml of 1 % aq. HAc at r.t for 30 min.

**Determined by Instant Thin Layer Chromatography. (ITLC - SA, $\text{MeOH}(49) : \text{H}_2\text{O}(49)$ Acetic acid(2))

***Gel was formed up to the pH of 6.0.

TABLE II. THE EFFECT OF AN AMOUNT OF ^{166}Ho ON LABELLING YIELD OF ^{166}Ho -CHICO*

Volume of $^{166}\text{Ho}(\text{NO}_3)_3 \cdot 5\text{H}_2\text{O}$ (ml)***	Amount of $^{166}\text{Ho} \cdot ^{165}\text{Ho}$ contained (mg)	Labelling Yield (%)**
0.1	3.74	99
0.2	7.48	99
0.3	11.22	70
0.4	14.96	66
0.6	22.44	45
0.8	29.92	17

*Chitosan (35 mg/4 ml) was reacted with various amount of $^{166}\text{Ho}(\text{NO}_3)_3 \cdot 5\text{H}_2\text{O}$.

** See Table 1

***Stock solution of 200 mg of $^{166}\text{Ho}(\text{NO}_3)_3 \cdot 5\text{H}_2\text{O}$ /2 ml water

TABLE III. PERCENTAGE OF ADMINISTERED DOSE OF ORGANS AND TISSUES AT 24HR AFTER INTRA-ARTICULAR ADMINISTRATION OF ^{166}Ho -CHITOSAN COMPLEX TO MALE RABBITS

Organs and Tissue	% of injected dose				
	1	2	3	mean	\pm S.D.
Blood	N.D.	N.D.	N.D.	N.D.	-
Plasma	-	-	-	-	-
Brain	N.D.	N.D.	N.D.	N.D.	-
Pituitary	N.D.	N.D.	N.D.	N.D.	-
Eye	0.00	N.D.	N.D.	N.D.	-
Hardarian gl.	N.D.	N.D.	N.D.	N.D.	-
Submandibular gl.	N.D.	N.D.	N.D.	N.D.	-
Thyroid	N.D.	N.D.	N.D.	N.D.	-
Thymus	N.D.	N.D.	N.D.	N.D.	-
Heart	N.D.	N.D.	N.D.	N.D.	-
Lung	0.00	N.D.	0.00	0.00	0.00
Liver	0.20	0.11	0.10	0.14	0.06
Kidney	0.01	0.01	0.01	0.01	0.00
Adrenal	N.D.	N.D.	N.D.	N.D.	-
Pancreas	N.D.	N.D.	N.D.	N.D.	-
Spleen	0.00	0.00	0.01	0.00	0.01
Carcass	N.D.	N.D.	N.D.	N.D.	-
Stomach	N.D.	N.D.	N.D.	N.D.	-
Administration site					
Synovia membrane	87.62	81.42	80.16	83.07	3.99
Synovia	0.82	1.76	1.75	1.44	0.54
*Others	6.82	17.02	17.87	13.90	6.15

*Others : Radioactive recovery from administration site except synovia membrane and synovia.

TABLE IV. PERCENTAGE OF ADMINISTERED DOSE IN ORGANS OR TISSUES
AFTER INTRAHEPATIC ADMINISTRATION OF DW-166HC TO MALE RATS

Organ/tissue	% ID			
	2 hr	24 hr	72 hr	144 hr
Blood	0.06±0.04	0.02±0.01	N.D.	N.D.
Brain	0.00±0.00	N.D.	N.D.	N.D.
Thymus	0.00±0.00	0.00±0.00	0.00±0.00	N.D.
Heart	0.01±0.01	0.01±0.01	0.02±0.03	0.02±0.01
Lung	2.63±2.40	3.29±3.64	1.45±2.25	0.57±0.77
Liver	0.40±0.18	0.63±0.49	0.27±0.21	0.15±0.06
Kidney	0.04±0.04	0.03±0.02	0.02±0.01	N.D.
Adrenal	0.00±0.00	0.00±0.00	0.00±0.00	N.D.
Pancreas	0.01±0.01	0.01±0.01	0.00±0.01	N.D.
Spleen	0.02±0.02	0.02±0.02	0.01±0.01	0.02±0.01
Skin	0.10±0.08	0.08±0.05	N.D.	N.D.
Testis	0.00±0.00	0.00±0.00	N.D.	N.D.
Carcass	0.38±0.21	1.09±0.71	2.51±0.83	2.72±0.36
Stomach	0.00±0.01	0.01±0.01	0.02±0.01	N.D.
Small intestine	0.08±0.09	0.03±0.01	0.04±0.02	N.D.
Large intestine	0.01±0.01	0.03±0.02	0.04±0.03	N.D.
Administration site	92.39±2.73	90.45±4.25	91.78±3.50	93.31±2.20

* All values were expressed as mean % ID±S.D. (n=3)

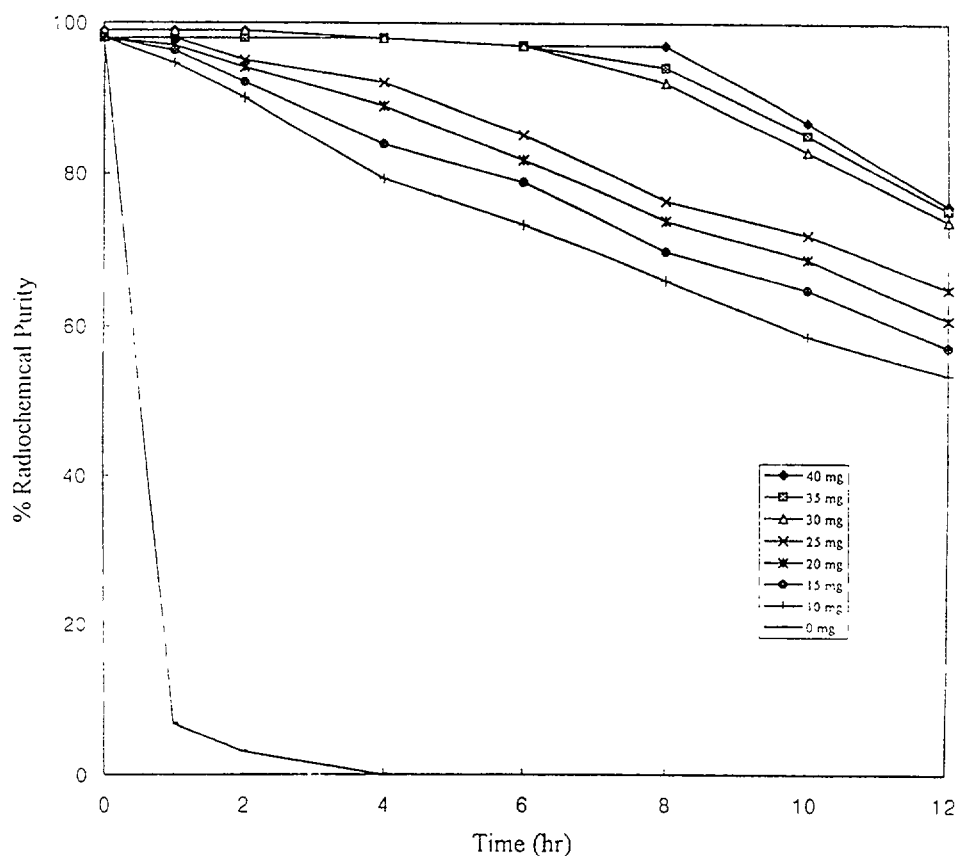


Figure 1. The Effect of ascorbic acid on *in-vitro* stability of ^{166}Ho -CHICO

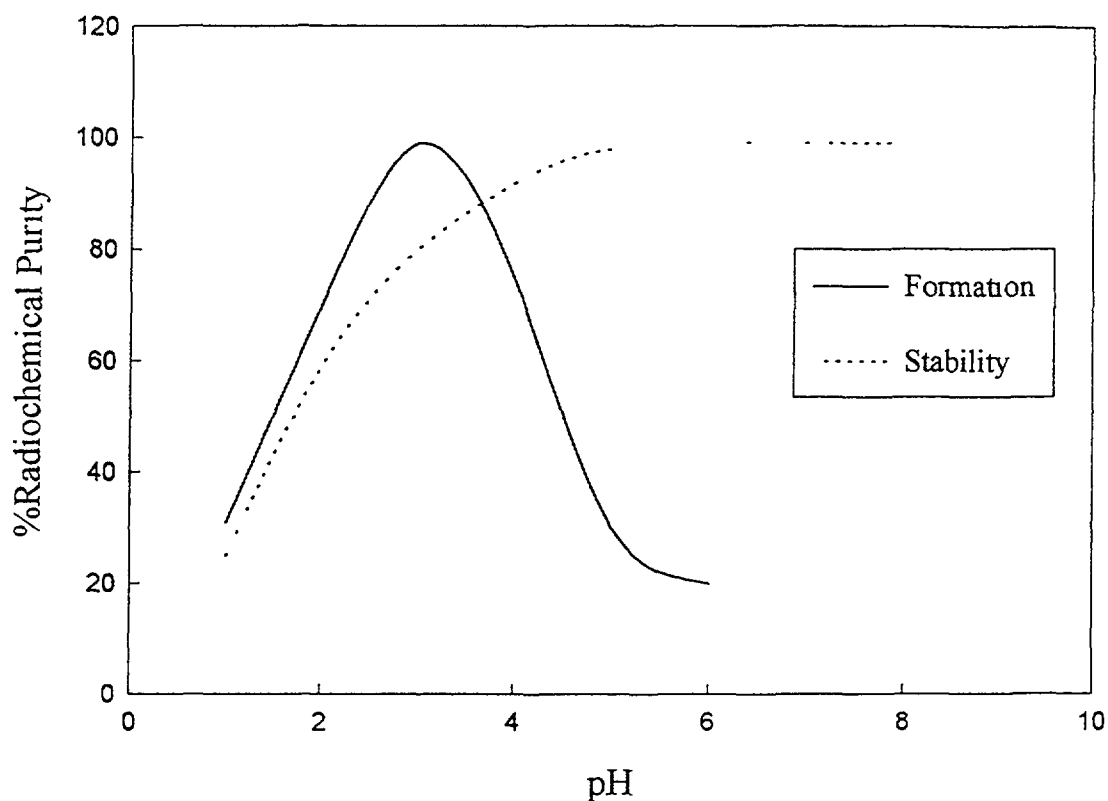


Figure 2. The Effect of pH on the formation and Stability of ^{166}Ho -CHICO

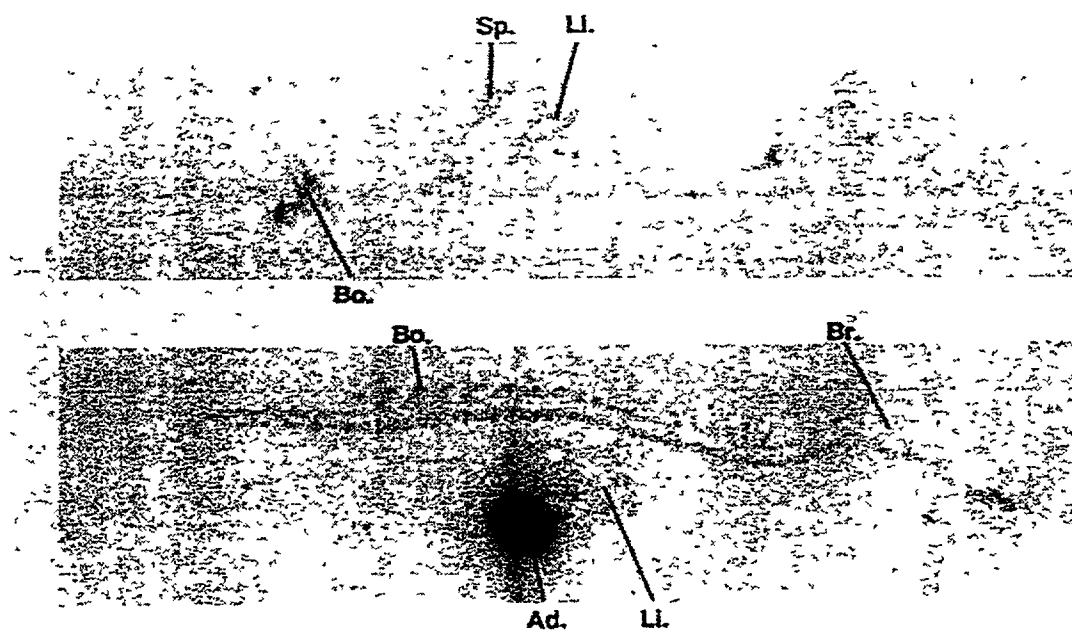


Figure 3. Whole-body autoradiographs at 72 hr after intrahepatic administration of ^{166}Ho -chitosan to a male rat.

Br.:Brain, Lu.:Lung, Li.:Liver, Sp.:Spleen, K.m.:Kidney medulla, K.c.:Kidney cortex, Bo.:Bone, U.b.:Urinary bladder, Ad.:Administration site, Upper:dorso-ventral section including kidney, Lower:dorso-ventral mesion section

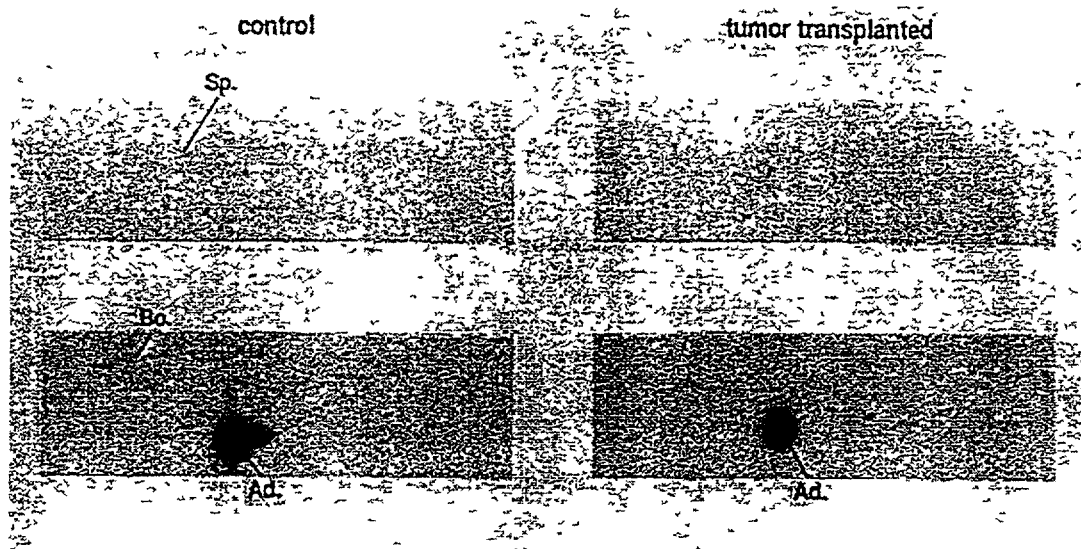


Figure 4 Whole-body autoradiographs at 72 hr after intrahepatic and intratumoral administration of ^{166}Ho -chitosan to a male control and tumor transplanted nude mouse, respectively.

Li :Liver, Sp :Spleen, Bo.:Bone, U.b.:Urinary bladder, Ad.:Administration site, Upper:dorso-ventral section including kidney, Lower:dorso-ventral mesion section

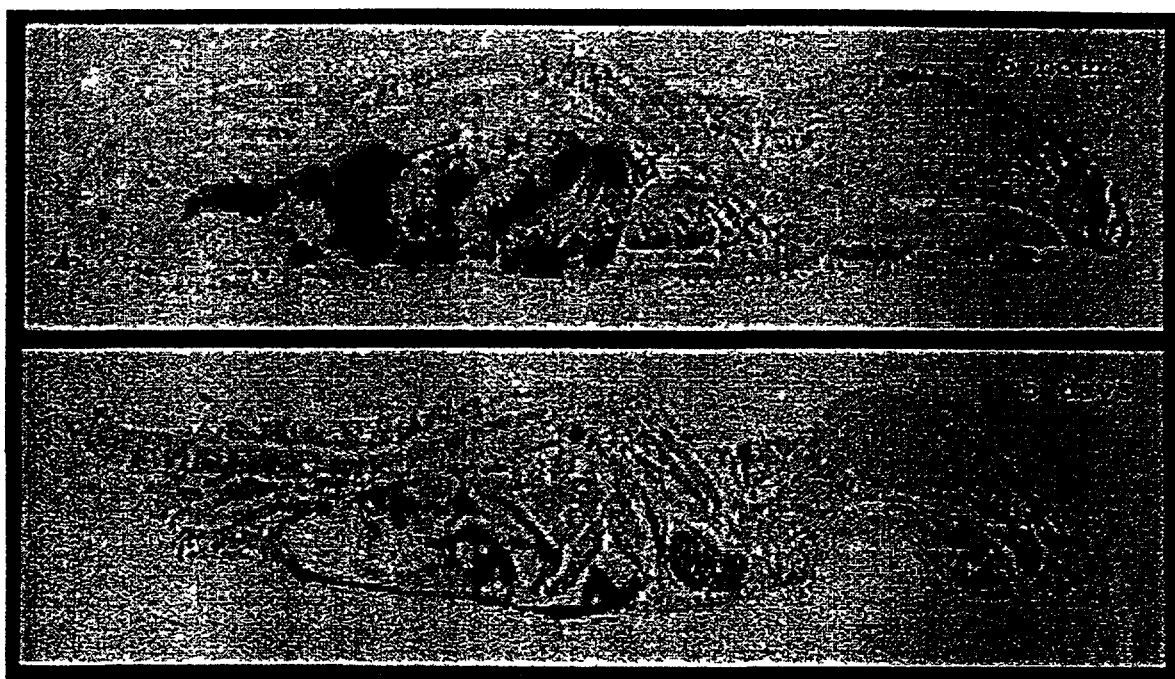


Figure 5. Whole-body autoradiographs at 6 hour and 5 days after intraperitoneal administration of ^{166}Ho -chitosan to rats.

In-vitro stability. Stability of ^{166}Ho -CHICO containing less than 2 mCi was examined in normal saline and in human plasma over four half-lives of each complex. In both the saline and plasma, no release of radioactivity from each complex was observed over the same period. The radiochemical purity(RCP) of ^{166}Ho -CHICO was highly dependent on the radioactivity of ^{166}Ho and the elapsed time after preparation. Its radiochemical purity decreased to 48% at one hour after preparation of ^{166}Ho -CHICO containing 10 mCi of ^{166}Ho . Similarly, in 50 mCi of ^{166}Ho , 5% of activity remained in ^{166}Ho -CHICO over the same time resulting in the high unstability of ^{166}Ho -CHICO under high activity conditions, probably due to the radiolysis and acid hydrolysis. On the other hand, in the presence of ascorbic acid (>35 mg), the stability of ^{166}Ho -CHICO, even though prepared from 50 mCi of ^{166}Ho , was dramatically increased, maintaining a high RCP (>98%) over 8 hours (Fig. 1). The ^{166}Ho -CHICO exhibited a high stability in neutral and alkaline conditions even in the absence of stabilizer (Fig. 2).

In-vivo stability and biodistribution study in rabbits with ^{166}Ho -CHICO showed most of the radioactivities were retained in knee joint with negligible extra leakage at 24 hr after intra-articular administration to normal male rabbits (Table III).

A biodistribution in tissues after intrahepatic administration of ^{166}Ho -CHICO was determined by radioactivity counting and autoradiography. The examination of organs and tissues showed that more than 90% of administered radioactivity was retained in the administered site for at least 144 hr after administration (Table IV). Autoradiography showed that most of the administered radioactivity was retained in the administered site of the liver lobe. Low levels of radioactivity were observed in the liver adjacent to the administered site, kidney, spleen and bones (Fig. 3).

An intratumoral administration study was performed using B16 melanoma transplanted nude mice as models. ^{166}Ho -CHICO was directly administered into tumors which had been transplanted into the liver lobe of mice and the radioactivity distribution was observed by autoradiography. In comparison, the tumor-transplanted mice showed a higher radioactivity retained at the administered site than in the control mice, and less radioactivity distributed to the body (Fig. 4).

Biodistribution of ^{166}Ho -CHICO at 48 hr after intraperitoneal administration to male mice showed most of the radioactivities were evenly distributed at the inner wall of the peritoneal cavity determined by autoradiography (Fig. 5).

4. DISCUSSION

^{166}Ho -CHICO was easily prepared by reacting the aqueous acidic solution of chitosan with $^{166}\text{Ho}(\text{NO}_3)_3$ or $^{166}\text{HoCl}_3$ at room temperature with a quantitative yield. The labelling yield and radiochemical purity were easily determined by instant thin layer chromatography.

The labelling yield was highly dependent on the pH of the reaction mixture, chitosan concentration, and the amount of $\text{Ho}(\text{NO}_3)_3$ used. A labelling yield of more than 99% was obtained by reacting chitosan solution (35 mg/4 ml) with $^{166}\text{Ho}(\text{NO}_3)_3$ in which 7 mg ($^{165}\text{Ho} + ^{166}\text{Ho}$) were contained as a maximum content. Three molecules (amine) of glucosamine in chitosan and one molecule of holmium are believed to participate in the complex formation. ^{166}Ho is not a carrier free radionuclide because it is produced by the $^{165}\text{Ho}(n, \gamma)^{166}\text{Ho}$ reaction. Therefore, it is important to label the chitosan with as large amount of holmium as possible to get a high radioactivity for therapy. ^{166}Ho -CHICO was found to have incorporated about 18% of the Ho. Compared to ^{166}Ho -poly(L-lactic acid) microspheres (about 10%) [4], it contained a higher amount of holmium. From a practical point of view, it is important that ^{166}Ho -CHICO can be easily prepared by reconstituting freeze-dried chitosan (kit A) with ^{166}Ho solution (kit B) just prior to use, for instance, $^{99\text{m}}\text{Tc}$ instant labelling kits.

The characteristics of ^{166}Ho -CHICO were found to be similar to those of chitosan, which is biocompatible, biodegradable, non-toxic, soluble and viscous in acidic condition but gel-forming at pH 6.0 and precipitating in alkaline condition.

The *in-vitro* stability of ^{166}Ho -CHICO was highly dependent on the radioactivity of the ^{166}Ho used. In the case of $<2\text{ mCi}$, it was very stable at room temperature or at 37°C for one week maintaining high radiochemical purity ($>99\%$). On the contrary, it was very unstable even at room temperature with increased the radioactivity ($>5\text{ mCi}$). Radiochemical purity was decreased to 50% at 10 mCi , and 15% at 20 mCi one hour after preparation. It is apparent that radiolysis leads to degradation of the polymeric matrix, especially in acidic conditions, resulting in decreased viscosity of chitosan. This may be the result of oxidative or hydrolytic cleavage of the ether linkages [12]. However, it was stable at alkaline conditions (Fig. 3) in which hydrolysis is prevented. These results indicated that acid hydrolysis is promoted by radiolysis and is the main reason for the degradation of ^{166}Ho -CHICO. Fortunately, it was very stable in the presence of anti-oxidants such as ascorbic acid, maintaining its nearly original radiochemical purity ($>99\%$) even at 100 mCi for 8 hours. This stability in neutral or alkaline conditions hints that even though ^{166}Ho -CHICO in acidic conditions (pH 3.5-4.5) is administered into body, the pH is changed immediately to neutral conditions by body fluids and forms a gel, resulting in *in-vivo* stability and organ specificity.

A biodistribution study and gamma camera image in normal rabbits with ^{166}Ho -CHICO showed that most of the radioactivities were retained in the knee joint with negligible extra-articular leakage 72 hours after intra-articular administration. Even though ^{166}Ho -CHICO is a solution type which is completely different from other known colloid or particulate radiotherapeutic synovectomy agents such as ^{165}Dy -FHMA[2], ^{165}Dy -HMA[3], ^{166}Ho -PLA microspheres[4], and ^{153}Sm -PHYP[5], it exhibited lower extra-articular leakage than these agents.

After intrahepatic administration of ^{166}Ho -CHICO to male rats, the radioactivity concentrations in blood were low and the cumulative urinary and fecal excretion over a period of 0 to 72 hours were 0.53% and 0.54%, respectively. The radioactivity concentration in tissues and the whole-body autoradiography images showed that most of the administered radioactivity was localized at the administered site, and only slight radioactivity was detected from the liver, spleen, lungs, and bones. An autoradiograph after intratumoral administration of ^{166}Ho -CHICO showed that radioactivity was localized at the administered site of the lesion without distribution to the other organs and tissues.

The biodistribution of ^{131}I -lipiodol infused via the hepatic artery of patients with hepatic cancer has been reported[13]. The radioactive concentration in blood after administration had been kept as low as $10 \times 10^{-4}\%$ dose/ml for 8 days after administration. In our results, the radioactive concentration of ^{166}Ho -CHICO decreased rapidly and became $10 \times 10^{-4}\text{ ID/ml}$ level within 2 days. The concentrations were continuously decreased, whereas in the case of ^{131}I -lipiodol, the concentrations were gradually increased. The overall radioactivity in blood over a long period suggested that ^{166}Ho -CHICO is likely to be in the same level as ^{131}I -lipiodol.

There have been many reports of intra-arterial administration of ^{90}Y -microspheres to primary or secondary liver cancer[6,7,8,14,15]. Some of these reports showed a tumor:liver ratio of 45:1 or less[14]. Comparison of the biodistribution with other internal radiation therapeutic agents strongly suggests that ^{166}Ho -CHICO has an extremely high administration site : tissue ratio. The whole body autoradiographs after intratumoral administration of ^{166}Ho -CHICO into the melanoma (B16) showed that most of the administered radioactivity was localized at the administration site. As mentioned above, since ^{166}Ho -CHICO is a high viscous solution which displays gelation characteristics at a neutral pH in the body, it can be retained in the administration site in either tissue, if transfer into blood is avoided.

The whole body autoradiography after intraperitoneal administration of ^{166}Ho -CHICO into male mice showed most of the radioactivities were evenly distributed at the inner wall of the peritoneal cavity. Installations of ^{32}P chromic phosphate (^{32}P -CP) have been used to treat intraperitoneal and pleural space malignancies, including ovarian cancer, for many years [16,17]. However, ^{32}P -CP has been shown that in animals about 85% of the colloidal ^{32}P was cleared from the blood in a single passage through the liver by phagocytosis in the reticuloendothelial system. A similar phenomena in which a large fraction of

the administered ^{32}P dose was absorbed by the liver was also observed [18]. In comparison with ^{32}P -CP, ^{166}Ho -CHICO showed a high radioactive concentration within the peritoneal cavity with relatively even distribution, which is rarely expected with radiocolloid or particulates.

5. CONCLUSION

The characteristics of ^{166}Ho -CHICO were similar to those of chitosan, which is well known to be biocompatible, biodegradable, non-toxic, soluble and viscous in acidic conditions but gel-forming in neutral conditions. The ease with which the ^{166}Ho -CHICO can be prepared as a kit form and its high *in-vitro* and *in-vivo* stability make it an attractive new agent for radionuclide therapy in malignant and benign diseases.

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CHILEAN EXPERIENCE IN PRODUCTION OF THERAPEUTIC RADIOPHARMACEUTICALS LABELLED WITH ^{153}Sm AND ^{166}Ho

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Abstract

$^{153}\text{Samarium}$ (^{153}Sm) and $^{166}\text{Holmium}$ (^{166}Ho) were produced at the Nuclear Center of La Reina Research Reactor, Chilean Nuclear Energy Commission. ^{153}Sm -EDTMP (Ethylenediaminetetramethylene Phosphonate) used for clinical trial of therapy for painful skeletal metastases and labeled particles such as ^{166}Ho -FHMA (ferric hydroxide macroagregattes) and ^{153}Sm -HAP (hydroxiapatite particles) used for radiation synevectomy, were labeled.

Radionuclide purity of both radionuclides was analyzed by gamma spectrometry using a multichannel gamma spectrometer.

Radiochemical labeled reaction parameters of ^{153}Sm -EDTMP such as: Sm/EDTMP molar ratio, ^{153}Sm specific activity, Labeled pH and temperature, were determined in order to get high radiolabeling yields. Radiochemical Quality Controls of ^{153}Sm -EDTMP using different chromatographic systems were carried out in order to determine labeling yields. Biodistribution studies were achieved in mice by dissection of animals and by autorradiography of histological slices in rats, after 2h post injection.

^{153}Sm -HAP and ^{166}Ho -FHMA labeled particles were prepared using the methods described in [2-3 -4 and 5]. Radiochemical purity, in case of

radiolabeled particles was carried out by centrifugation, measuring activity in the supernatant and in particles pellet. Physical parameters, such as particle size and range of the radiopharmaceuticals based on particles labeling were evaluated in order to determine the ideal conditions to obtain particles size range between 10 - 40 μ . In vitro labeling stability for over seven days and wash out activity by incubation in human synovial fluid after 6 and 24h post labeling, was also studied.

¹⁵³Sm-EDTMP was easily labeled with a Radiochemical purity over 99.5% and stable for over 7 days. Biodistribution studies in mice give more than 50% of ID uptake in bone and less than 0,1% in liver this was correlated by autorradiographic image.

¹⁵³Sm-HAP and ¹⁶⁶Ho-FHMA were also labeling obtaining radiochemical purity over 95%. Comparative in vitro studies of wash out activity for both radiopharmaceuticals, by incubation in synovial fluid, was less than 1% after 6 and 24h. Sterilization by autoclave does not change the particle size.

Over than 200 patients studies has been done using ¹⁵³Sm-EDTMP in our country. Patient studies using ¹⁵³Sm-HAP and ¹⁶⁶Ho-FHMA are in progress (phase 1).

INTRODUCTION

The availability of a 5MW Nuclear Research Reactor in our country allowed us to develop the production of radionuclides by direct neutron activation, such as: ¹³¹I, ¹⁵³Sm, ¹⁶⁶Ho, ¹⁶⁵Dy which can be used for labeling of radiotherapeutic agents (Table I).

Table I **β EMITTERS RADIONUCLIDES USED FOR THERAPY**

Radionuclide	Half Life	β Energy Max. (Mev)	γ Energy (KeV%)	Nuclide Target	Cross Section
⁶⁷ Cu	2.44 d	0.57	184 (48%)	⁶⁸ Zn	0.0012
¹⁰⁵ Rh	1.44 d	0.57	319 (5%)	¹⁰⁴ Ru	0.5
¹³¹ I	8.00 d	0.61	364 (81%)	¹³¹ Te	0.3
¹⁵³ Sm	1.93 d	0.80	103 (29%)	¹⁵² Sm	220
¹⁶⁵ Dy	2.30 d	1.34	95 (4%)	¹⁶⁴ Dy	1697
¹⁶⁶ Ho	1.10 d	1.60	81 (6.33%)	¹⁶⁵ Ho	58
¹⁸⁶ Re	3.78 d	1.07	137 (9%)	¹⁸⁵ Re	106
¹⁸⁸ Re	17 h	2.11	155 (15%)	¹⁸⁷ Re	73.2
¹⁷⁷ Lu	6.70 d	0.50	113 (6.4%)	¹⁷⁷ Lu	2100
³² P	14.3 d	1.71	-----	³¹ P	0.18

Therapeutic Radiopharmaceuticals give the possibility of localized irradiation permitting a systemic, non invasive and relative lack of toxicity treatment in comparison with external radiotherapy and chemotherapy. These characteristics make them an attractive product for therapy of malignant disease, as well as in some benign disorders. ¹⁵³Sm and ¹⁶⁶Ho have relatively short half-life(46.3h and 26.4h respectively) and both radionuclides emit beta particles permitting therapy effect and also its gamma rays emission of suitable energy for scintigraphy could be utilized to estimate the radiation absorbed dose.

MATERIALS AND METHODS**RADIONUCLIDE PRODUCTION**

¹⁵³Samarium (¹⁵³Sm) and ¹⁶⁶Holmium (¹⁶⁶Ho) were produced by 20h irradiation of enriched ¹⁵²Sm₂O₃ (95.2% ORNL) and ¹⁶⁵Ho₂O₃ of high purity at $2 - 3 \times 10^{13}$

n/cm²/seg, using quartz ampoules, at the Nuclear Center of La Reina Research Reactor, Chilean Nuclear Energy Commission, and later dissolution in HCl at pH 4.0 for ¹⁵³Sm and HNO₃ 4% for ¹⁶⁶Ho. Both radionuclides were evaporated to dryness in a quartz ampoule before irradiation. The specific activity of Sm obtained was between 64 - 156 mCi/mg (48h EOB). Radionuclide purity was analyzed with a multichannel gamma ray spectrometer using a Ge(Li) well detector.

¹⁵³Sm-EDTMP LABELING

¹⁵³Sm-EDTMP was labeled at different molar ratio of ¹⁵³Sm : EDTMP/(1:1; 1:5, 1:15 and 1:30) using different specific activities (5, 25, 50 and 100 mCi/mg) in order to determine the ideal labeling conditions. All preparation were done following a general labeling methods:

1. Evaporate the ¹⁵³SmCl₃ under a N₂ stream to dryness
2. Add EDTMP (4% solution; pH 7.8). The amount of solution was determined as described in (*)
3. Add 2.0 ml of buffer phosphate pH 7.6 mixing thoroughly and heat 30 min at 75°C or autoclave 20 min at 121°C

$$(*) \text{ mg EDTMP} = \frac{A \times R}{S} \times F$$

where: A = ¹⁵³Sm activity

R = Molar relation

S = Specific activity

$$F = \frac{M(\text{EDTMP})}{M(\text{Sm})} = \frac{463.13}{150.4} = 2.9$$

In vitro labeling stability for over 7 days was studied by Ionic Exchange Chromatography. Biodistribution studies were carried out in mice (25 - 30g) and autoradiography of histological slices was studied in Wistar rats (230 - 250g).

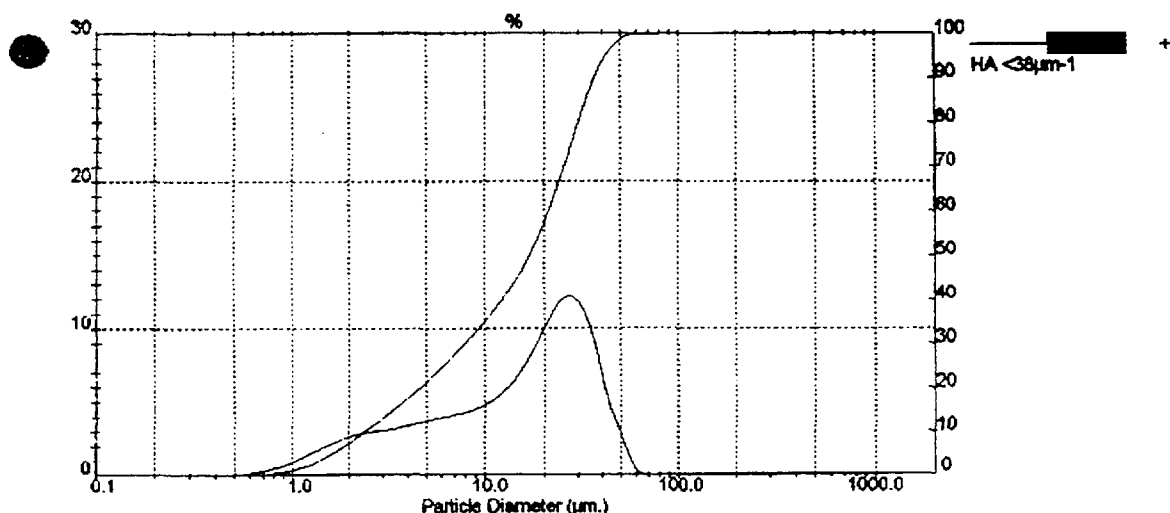
HAP SYNTHESIS AND ^{153}Sm -HAP LABELING

Hydroxyapatite was synthesized following the methods described in [1] and the particles size range was controlled in aqueous solution using a mini spray drying (BUCHI B-191) in order to obtain particles between 20 - 40 μ . The particles size distribution was evaluated in a Master Sizer before and after to sift them through a sieve. Particles size distribution used in these studies is shown in Fig. 1.

Labeling of ^{153}Sm -HAP was prepared [2 and 5] in two steps:

1. Preparation of ^{153}Sm -citrate by adding citric acid to the $^{153}\text{SmCl}_3$ solution in order to give a final concentration of 15 mg/ml of citric acid and leaving 30 min for reaction time at room temperature.
2. Add 250 μl of ^{153}Sm -Citrate prepared in 1. to a vial containing 750 μl aqueous suspension of 40 mg HAP particles. The vial was sealed, incubated at room temperature for 30 min and gently agitated using a rotator.
3. Separate free $^{153}\text{SmCl}_3$ by centrifugation of labeled particles in a 15 ml conical tube, using 4 ml of saline to rinse, during 8 min at 1000rpm.
4. Finally the particles were resuspended in 5ml of saline and autoclaved 20 min at 121°C.

Residual = 0.281%
 d(0.5) = 19.82 μm
 D[4,3] = 18.18 μm
 Sauter Mean (D[3,2]) = 6.58 μm
 Specific Surface Area = 0.9122 sq. m./gm
 d(0.1) = 2.44 μm
 Mode = 26.86 μm
 Density = 1.00 gm. / cc



Malvern Instruments Ltd.
 Malvern, U.K.

MasterSizer I Ver. 1.1a
 Serial No. 6230

P. 3
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Fig. 1

SIZE AND RANGE DETERMINATION MASTERSIZER

^{166}Ho -FHMA LABELING

The preparation of ^{166}Ho -FHMA was done (3 and 4) in two steps:

1. Prepare a stock target solution mixing 1.1ml ^{166}Ho solution and 3.5ml of FeSO_4 (4mg/ml) solution. This stock solution could be used to prepare 5 doses.
2. In a 12 - 15ml centrifuge tube with 2.2ml of saline add 1.1ml of NaOH 0.1N, 0.9ml of the stock target solution and 1.1ml of PVP (16 mg/ml) solution. Mix thoroughly for one min and centrifuge 5min x 1400rpm. Separate the

supernatant and add 6ml of a PVP (16 mg/ml) solution at pH 9.5, mix thoroughly and centrifuge 3min x 1000rpm. Discard supernatant and wash the particles with 6 ml of saline solution. Centrifuge, discard the supernatant and resuspend the particles in the desired volume of saline . Sterilization can be achieved by autoclaving 20 min at 121°C.

QUALITY CONTROLS METHODS

RADIOCHEMICAL PURITY DETERMINATION

- I. ^{153}Sm -EDTMP: Several methods of chromatography were evaluated to determine the radiochemical purity:

1. Whatman 3MM / NH_3 : EtOH : H_2O = 0.1 : 2 : 4

$$R_f \text{ } ^{153}\text{SmCl}_3 = 0.00$$

$$R_f \text{ } ^{153}\text{Sm-EDTMP} = 1.00$$

2. TLC-Celullose / NH_3 : EtOH : H_2O = 0.1 : 2 : 4

$$R_f \text{ } ^{153}\text{SmCl}_3 = 0.00$$

$$R_f \text{ } ^{153}\text{Sm-EDTMP} = 1.00$$

3. Whatman 3MM/ Py : EtOH : H_2O = 1 : 2 : 4

$$R_f \text{ } ^{153}\text{SmCl}_3 = 0.00$$

$$R_f \text{ } ^{153}\text{Sm-EDTMP} = 1.00$$

4. TLC Celullose / Py : EtOH : H_2O = 1: 2 : 4

$$R_f \text{ } ^{153}\text{SmCl}_3 = 0.00$$

$$R_f \text{ } ^{153}\text{Sm-EDTMP} = 1.00$$

5. Ionic Exchange Column using SP Sephadex C-25 (3 x 1cm) eluted with 20 ml Saline

6. Solid phase extraction Set-Pak

Radiochemical purity must be over 99%.

- II. ^{153}Sm -HAP: The labeling yield was determined measuring the activity remaining in the supernatant and in the precipitate after centrifugation. Overall yield was always around 95%.
- III. ^{166}Ho -FHMA: The labeling yield was determined measuring the activity of the three discarded supernatant, obtained during the preparation, and the precipitate. Overall yield was always around 95%.

PARTICLES SIZE DETERMINATION

- I. ^{153}Sm -HAP: After sterilization, using autoclave, the particle size was analyzed by filtration through nylon filters of 20 and 40 μ .
- II. ^{166}Ho -FHMA: The particle size was analyzed by filtration methods using a battery of different pore size nylon filters (0.45 , 1.2 , 5.0 , 11.0 and 40 μ) before and after sterilization by autoclave.

BIODISTRIBUTION STUDIES

^{153}Sm -EDTMP biodistribution studies in mice of 25 - 30 g weight were carried out by dissection of animals 2 h post injection. Autorradiographic studies of histologicals slices using a Cryomicrothome were also achieved using Wistar rats of 230 - 250g.

IN VITRO STABILITY OF RADIOLABELED PARTICLES

In-vitro stability of ^{153}Sm -FHMA and also for ^{166}Ho -HAP was measured every day during a week by centrifugation. After separation of supernatant the particles were resuspended again in saline solution. Comparative In-vitro studies of both radiopharmaceuticals, based on radiolabeled particles, were done by incubation at 37°C , of ^{166}Ho -FHMA or ^{153}Sm -HAP with 3ml of human synovial fluid diluted 1:1 with saline solution, measuring the wash out of activity after 6 h and 24h.

RESULTS AND CONCLUSIONS

Radiolabeling of ^{153}Sm -EDTMP can be easily labeled at basic pH 7.8, obtaining radiochemical purity over 99%. Free $\text{Sm}(+3)$, at pH over 7.0 could produce colloidal hydroxide ($\text{Sm}(\text{OH})_3$) given hepatic irradiation. Liver irradiation uptake should be avoided when using therapeutic doses. Several methods of chromatography were studied and correlated with SP Sephadex C $^{\circ}$ 25 and with biodistribution in mice, working at different Sm/EDTMP molar ratio and specific activities Fig. 1 in order to choose a reproducible quality control system to be used in a routinely production. Working at high specific activities using paper 3MM paper and/or TLC cellulose are not a reproducible systems Fig. 2 but could be used when working at low specific activity. The recommended system must be Ionic Exchange column SP Sephadex C25 Chromatography Fig. 3, either at high or at low specific activities. Biodistribution studies in mice after two hours post injection demonstrated rapid bone uptake, urinary clearance was over 50% and liver uptake was less than 0.1%, Table II. Biodistribution

Table II
Sm-153 BIOLOGICAL DISTRIBUTION IN RATS
2 H POST INJECTION

ORGANS	*%Act./Organ	*%Act./g
Blood	0.09 ± 0.005	
Spleen	0.28 ± 0.15	
Stomach	1.15 ± 0.56	
Gut	1.54 ± 0.09	
Liver	0.05 ± 0.005	
Kidneys	0.37 ± 0.05	
Urine + Blader	54.39 ± 6.4	
Femur	2.27 ± 0.11	23.83 ± 3.45
Carcas	39.78 ± 5.44	

* Average of five animals

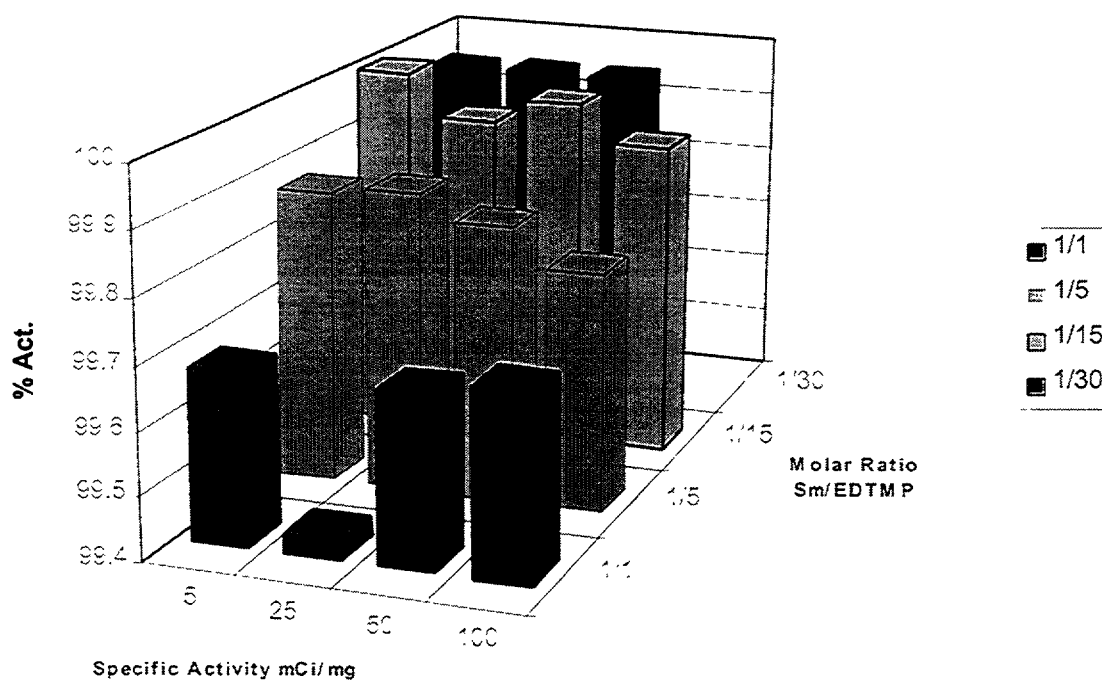


Fig. 2
RADIOCHEMICAL PURITY
Ionic Exchange Chromatography
(Sephadex C-25)

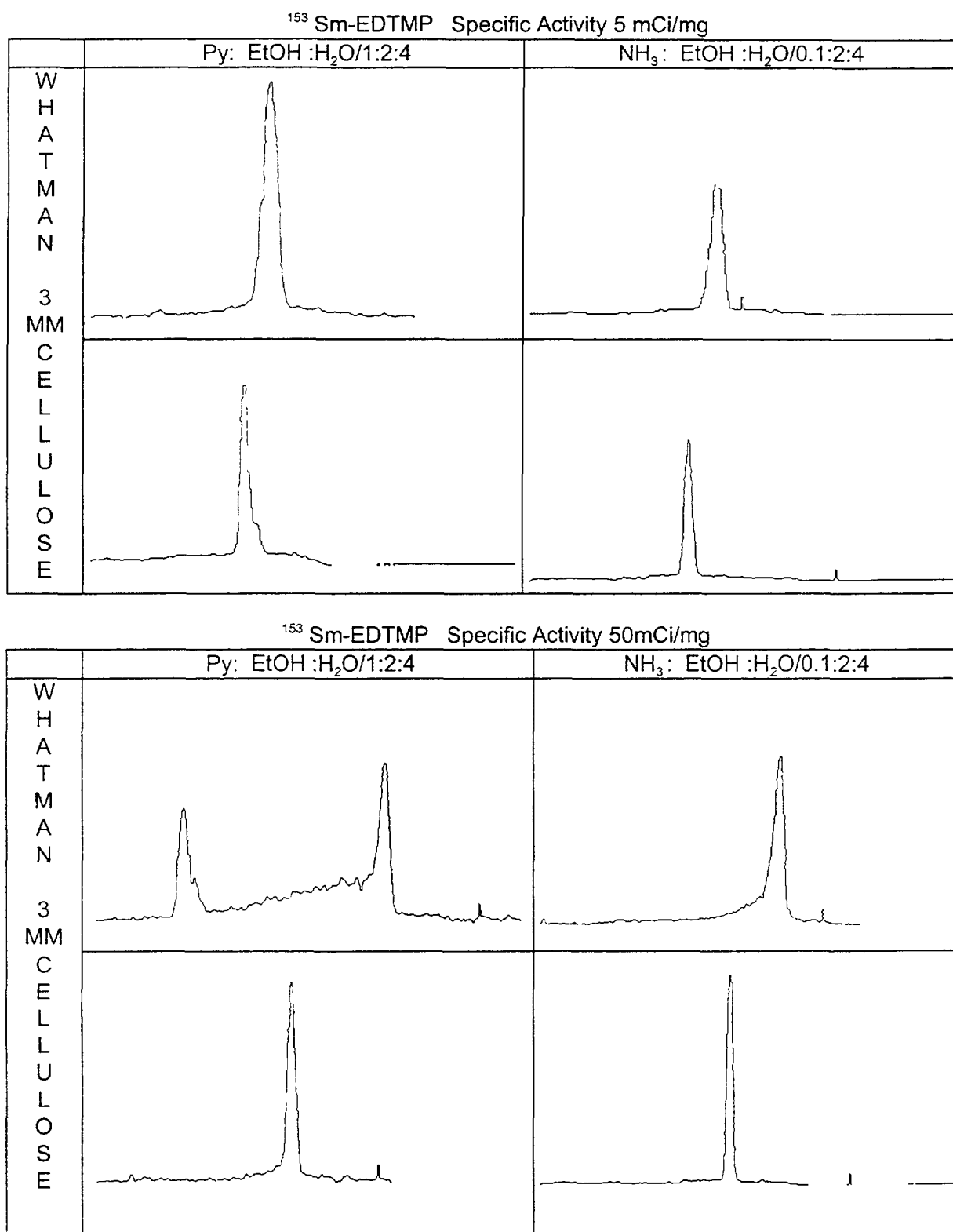


Fig. 3
RADIOCHEMICAL PURITY

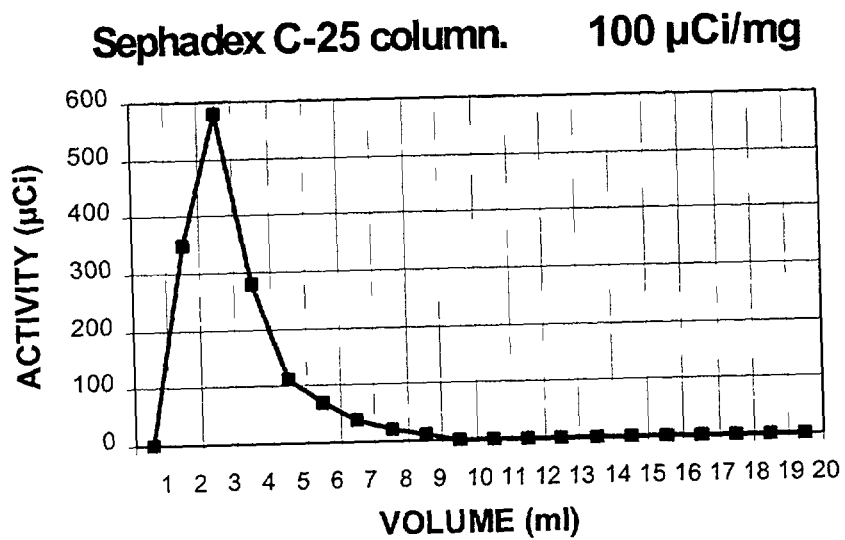


Fig. 4
RADIOCHEMICAL PURITY Sm153-EDTMP



Fig. 5
 ^{153}Sm -EDTMP Autoradiography in Rats (250 g)
2h Post Injection

studies results in mice were correlated Autorradiographic image in rats histological slices bone image Fig. 4. Radiochemical purity should be over 99% in order to be used for bone metastasis painful relief treatment in-patients. Over 200 patient studies have been done using ^{153}Sm -EDTMP in our country [7].

Labeled particles such as ^{153}Sm -HAP and ^{166}Ho -FHMA were also easily prepared following the methods described in references [2; 3; 4 and 5]. Particles size obtained were inside desired range: 20-40 μ for HAP and 10-40 μ for FHMA. Radiochemical Purity determined by centrifugation methods, for both radiopharmaceuticals was always over 95% and stables for a week. The wash out of the activity from the particles incubated in human synovial, fluid diluted 1:1 with saline solution, was less than 1% after 6h and 24h. Sterilization of the radiopharmaceuticals by autoclave did not produce growing of the particules.

Radiosynovectomy treatment in patients using radiolabeled particles are in progress.

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**LOW COST TECHNOLOGY FOR THE RAPID AND SAFE IN-HOUSE
(HOSPITAL-BASED) PREPARATION OF DUAL —
RADIOTHERAPEUTIC (R_x) AND RADIODIAGNOSTIC (D_x) —
DOSAGE FORMS OF HIGH SPECIFIC ACTIVITY ¹³¹I-mIBG
FOR CLINICAL APPLICATION**



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Abstract

Radioiodinated mIBG is finding increasing utility in nuclear medicine. However, its widespread use in developing countries is precluded by logistic constraints owing to the relative instability of the labelled molecule with respect to time and temp., and high costs. This prompted us to develop a low cost in-house batch process technology that could be used for the small-scale preparation of ¹³¹I-mIBG even in a less equipped hospital-based radiopharmacy. The production of large amounts of ¹³¹I-mIBG for clinical use requires sophisticated infrastructure (a scarce resource) to contain / safeguard against internal and external radiation exposures. We have indigenously designed a semi-automated, self-shielded, remote-controlled and safe microplant + process assemblies using easily accessible and cheap inputs, and developed the complete technology for the rapid and safe production of dual dosage forms of ¹³¹I-mIBG, a radiotherapeutic (R_x) single dosage form of high activity along with 1-3 low activity radiodiagnostics (D_x) as multidosage forms. The radioiodide exchange reaction was effected in the solid / melt phase at 190 °C in 1.0 h. The radiolabelling yield was ~80-86.0 %, and the radiochemical purity > 99.5 % and specific activity of R_x / D_x = 900-1300 / 60-800 MBq / mg. The product control was achieved by using fast methodologies, viz. electrophoresis (< 1.0 h), and paper chromatography, as well as pharmacokinetic profiles involving specific tissue indices (5 min-2.0 h). The rapid process operations helped ready the product for same day patient use, thus saving on losses caused by decay and possible instability. By storing the D_x dosage forms at < -35 to -45 °C we have been able to extend the useable shelf life of this dosage form to > 3 weeks. The dosage forms (especially R_x) were only made against firm patient appointment(s). Thus far we have prepared 14 R_x (49.0 GBq) and 70 + 14 batches (43.43 GBq) of (~150 nos.) D_x forms, and used them in 14 and > 1100 patients respectively.

1. INTRODUCTION

Radioiodinated metaiodobenzylguanidine (mIBG) is finding increasing utility both as a radiodiagnostic (D_x) and a radiotherapeutic (R_x) in the practice of nuclear medicine. ¹²³I-mIBG is extensively used for the diagnosis of neural crest tumours with high sensitivity and specificity, assessment of cardiotoxicity in patients undergoing certain chemotherapeutic regimens, evaluation of the sympathetic integrity in the myocardium in a variety of diseased states, determination of the status of autonomic re-innervation following cardiac transplantation, etc. ¹³¹I-mIBG has found wide acceptance as a radiotherapeutic for the management of neuroblastoma, pheochromocytoma,

paraganglioma, carcinoid, etc. [1-5]. In the early years this agent was also employed as a radiodiagnostic for neuroendocrine tumours. The versatility of the molecule is derived both from its catecholamine- mimetic component and as modified by the presence of the iodo substituent in the molecule. Since the iodine atom is capable of being tagged with different radioisotopes of diverse physical properties the labelled compounds so obtained have the potential of being put to a variety of D_x and R_x applications. These favourable attributes and uses have made it a much desired radiopharmaceutical. Unfortunately, its widespread application is restricted to a few developed countries owing to the high costs and relative instability of the radiolabelled species both with respect to time and temp., (often requiring storage in dry ice / low temp. [6-10], rapid transportation and necessitating its quick use) These limitations of logistics have put the agent outside the reach of many in developing countries. Although the incidence of neural crest tumours is low it is exacerbated by the burgeoning population in many developing countries. This is especially so when a department (R.M.C.) is juxtaposed with an oncological hospital. To mitigate the problem to some extent and in the absence of an indigenous source of supply for the agent we initiated a modest programme for the preparation of the ¹³¹I-mIBG radiopharmaceutical in our in-house hospital-based radiopharmacy. It was to be labelled with the reactor-produced radionuclide ¹³¹I, the only one available to us.

The processing of ¹³¹I-radiopharmaceuticals in large amounts is a hazardous operation best carried out by laboratories having good infrastructure- dedicated 'hot' cells with good exhaust, and radiation attenuation devices, besides personnel and expertise. Such facilities are rather scarce in a hospital radiopharmacy. Paucity of these made us to search for alternative simple low cost technologies that could be improvised and adopted in a hospital pharmacy scenario with limited resources. We were interested in a method that could be performed quickly and in high yields and purity. The method had to be amenable to scaling-up so as to afford at least a single R_x dosage form and / or one or more multidosage D_x dosage forms of high specific activity. Of the available methodologies the one by Mock and Wiener [11] attracted our attention as a viable option. However, the indicated technique dealt with only low activity levels and required the manual intervention of the 'hot' reagents to ensure good yields. Also, there was a distinct possibility of small amounts of ¹³¹I escaping from the reaction vial. Since our requirements were for a number of multidosage forms of D_x and / or possibly one R_x dosage form per batch we were required to use high amounts of ¹³¹I activity in the reaction (> 5000 MBq). In the absence of safeguards the handling of these high levels of activity could have possibly resulted in environmental contamination in addition to external and (plausible) internal radiation exposure(s) to the operator(s). To drastically reduce and contain these we modified the process and redesigned the process assembly. An indigenous prototype microplant was developed using readily available and cheap (disposable) inputs. A dual instead of single dosage option - a single R_x and multidosage D_x forms of ¹³¹I-mIBG per every batch operation, was envisaged to reduce the occupational exposures. These were tailored to patients' requirements. This paper describes the low cost in-house technology employed by us to arrive at the dosage forms. It deals with the design of the process plant and its control with special emphasis on radiation health, safety and protection to operators and environment. unit process operations including the kinetics of the radioiodide exchange process, formulation and dispensing of the single / dual dosage forms, methods of assay - both radiochemical and biologic, process parameters of radiochemical yield, purity and specific activity, and its storage and use.

Facilities Standard equipment and amenities available in a hospital-based radiopharmacy were freely used along with a non-dedicated fume cupboard (for radioiodine) with exhaust, membrane (200 nm pore) filtration assembly [12], etc., while a few devices were got fabricated. Many of the inputs were autoclaved before the start of the operations. Good pharmaceutical manufacturing practices (cGMP) cum radiation safety norms were practised in all these process operations. In addition to the above animal house facilities were also freely availed of for performing biologic assays.

Patient(s) Single R_x dosage form was prepared only against specific patient appointment. The R_x form was used without delay. The D_x multidosage forms were also made against patient appointments, but were used over 2-4 weeks.

(Radio)chemicals All chemicals were procured from commercial sources. Water was of redistilled grade. Conditioned and rewashed Amberlite IRA 400 anion exchange resin (~ 10 g, 15 x 10 mm dia) loaded on a glass / disposable column was employed in the purification cycle. No-carrier-added $Na^{131}I$ (925 ± 185 and 5550 ± 925 MBq for the preparation of D_x and R_x) in dil. alkaline sulfate was obtained from B.R.I.T., Mumbai. It was consigned in sealed glass vials (10 mL capacity).

Prototype (radiation protected) process microplant assembly The labelling process was carried out in a specially designed semi-automated microplant assembly using cheap and easily available (and often disposable) inputs. The assembly was housed at the far end of a fume cupboard and had a 25 mm thick lead shield in front. It consisted of a reactor fitted with a condenser and receiver along with a train of traps (Fig. 1). The reactor was the very vial in which the requisitioned $Na^{131}I$ was consigned. The receiver and traps were also 10 mL capacity vials. The former contained only a strip roll of pH indicator paper, while the latter contained 2-3 mL of a saturated solution of $Na_2S_2O_3$ and activated charcoal respectively. The assembly was a closed system and was interconnected with the other vials. The condenser (appropriately configured-250 mm long, doubly bent with long bevel on both ends) used was specially fabricated from 304 stainless steel capillary (10 G). A 45 mm rod was welded to it at the bend so that it could be kept suspended prior to its insertion into the bung of the reactor vial. This was achieved by employing a needle injector. This fabricated contraption could firmly grip the vial at the neck and by activating a lever insert the needle condenser (suspended in a groove of a collet) into the bung of the reactor vial. The device could then be extricated from the system. All these manoeuvres were remotely carried out from the distal end of the device.

The metal (heating) block was a 30 mm thick lead pot cut to vial size. This also doubled as a self-shield and was placed on a magnetic stirrer. It was electrically heated by enveloping the block with an electric heating coil (suitably insulated) and connected to a thermostat. The temp. (digitally displayed) could be accurately measured / controlled / monitored from a distance. Efficient heat transfer from the block to the reactor vial was facilitated via a close fitting copper annular tubing inserted into a hollow of the lead block to snugly accommodate the vial. The top portion of the vial was further covered with a suitably cut lead cap. The operator was further shielded from the reactor assembly by working behind a lead cum lead-glass bench. A suitably cut lead screen was placed in between the 'hot' shielded reactor and not-so-'hot' receiver end. The other traps could be placed in suitably cut lead pots with slits, if desired.

Extra long-handled tools were employed for performing the 'hot' operations. Close-fitting, interlocking modular type lead shields were custom designed and fabricated for the resin column and receiver, as well as for the membrane filtration assembly. These

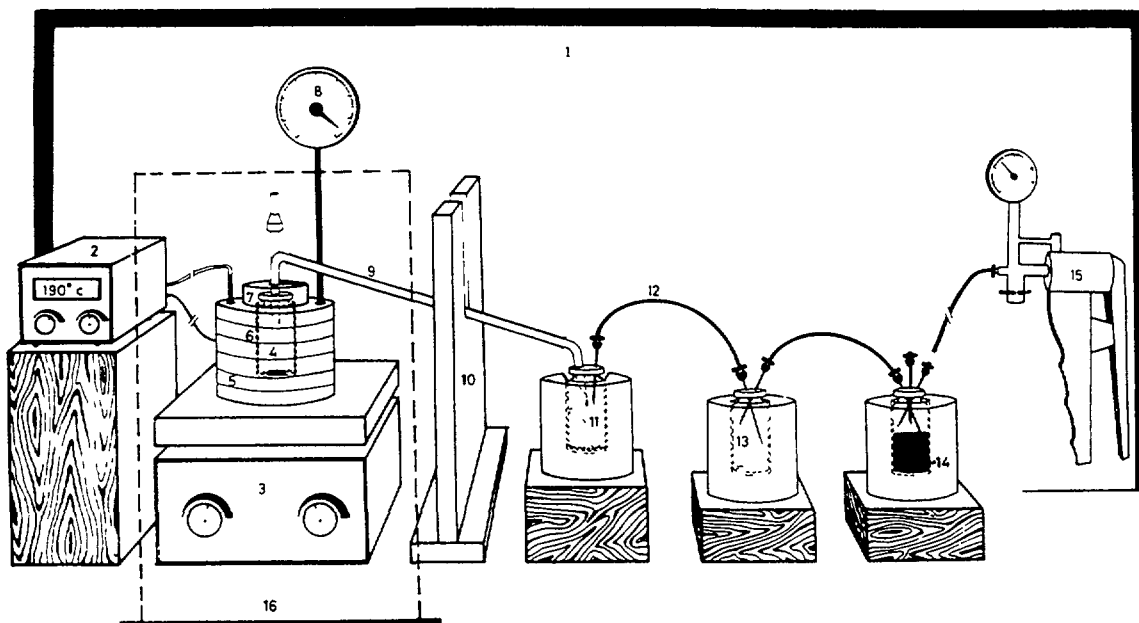


FIG.1 The semi-automated prototype process micro-plant used in the production of ^{131}I -mIBG at R M C (A few of the cheap disposable assemblies are shown.)

Key 1 - Mirror, 2 - Remote thermostat control (digital display), 3 - Stirrer, 4 - Reactor, 5 - Lead block with heating coil, 6 - Copper insert tube, 7 - Lead cap, 8 - Dial thermometer (standby), 9 - Insulated needle condenser, 10 - Lead screen, 11 - Empty trap with pH sensor, 12 - Silicone tubing with adapter (autoclaved), 13 - Trap containing sat. $\text{Na}_2\text{S}_2\text{O}_3$, 14 - Trap containing active charcoal, 15 - Low vacuum pump, 16 - Table top barrier shield with leaded glass

lead assemblies had slit openings that faced a mirror so that the liquid flow patterns could be monitored from the images. Similarly, L bench and movable shields as well as close-fitting vial shields were also employed in these operations. Liquid / vapour transfers was achieved with the aid of low vacuum (hand pump)

Chemical and pharmaceutical unit operations and processes

(i) *Transfer of reactants and promoters into the reactor assembly:* The shielded Na^{131}I reactor vial containing the desired activity was decripped and ~ 4 MBq removed for assay. Thereafter a mixture of cold mIBG + $(\text{NH}_4)_2\text{SO}_4$ + a mini stir bar + reaction promoter, were transferred into the reactor using extra-long handled / remote devices The reactor vial was sealed with a bung + aluminum closure. Thereafter, one end of the needle condenser was remotely injected into the bung of the reactor, and a close-fitting lead cap remotely placed on the top of the reactor. The other end of the condenser (positioned on the other side of a Pb screen) was manually inserted into the empty receiver containing a strip of pH paper and the assembly put in place

(ii) *Chemical reaction :* The reaction was initiated by slowly heating the mixture of reactants by remote control (ambient to 100°C , and maintained at $120 - 125^\circ\text{C}$ for 0.25 h during which time ammonia was liberated. This could be sensed by the pH indicator paper. The temp. was then raised to 130°C , at which temp. the aqueous content began to distill off into the receiver The removal of the remaining aqueous content was facilitated by the gentle application of low vacuum. Thereafter, the temp was progressively increased in steps of 10°C from 140 to 190°C . over a few min. and was then maintained at this temp. for 1.0 h, after which it was cooled to ambient temp.

(iii) *Leaching* The reactor vial was cooled, and decrimped. The contents of the vial were dissolved in 100 mL water, stirred, and an aliquot (~4 MBq) removed for assay (Process control sample)

(iv) *Purification*: The above solution was passed down a preconditioned column of Amberlite IRA 400 anionic resin, and the eluate collected. The reactor vial was rinsed with 5 x 10 mL portions of water (eluant) and the solution poured onto the resin bed and the eluates pooled

(v) *Terminal membrane filtration*: The shielded eluate was removed to a bioclean environment and membrane (200 nm pore) filtered into an empty apyrogenic and sterile vial either by remotely applying low vacuum (especially to obtain the R_x dosage form) or positive pressure in the case of D_x dosage forms. Small aliquots of the above dosage forms (product control) were also removed by aseptic techniques for radiochemical, biologic and other pharmaceutical assays

The 'empty' flask which had contained the pooled purified eluate was further washed with water and the solution membrane filtered using the same assembly to salvage small amounts of the adhering activity which could be dispensed as a D_x dosage form

(vi) *Dispensing of the dosage forms*: The primary filtrate containing high activity levels of ^{131}I was dispensed as the R_x dosage form. The washings obtained after dispensing the above, or sometimes small aliquots of the above (suitably diluted with water for injection) were dispensed as the D_x forms. Many a time only the D_x dosage form was prepared, diluted as required and dispensed into 2/3 vials as multidosage forms using aseptic techniques, and duly labelled.

(vii) *Assays*: The reagent Na^{131}I was subjected to both radionuclidic, radiochemical as well as radiobiologic assays while the process, product control samples including the final dosage forms were checked for radiochemical, radiobiologic and pharmaceutical purity. The radiochemical assays were performed by paper (Whatman Grade 1) electrophoresis (using phosphate buffer, 0.05 M, pH = 6.5 at constant voltage of 250 V, for 0.5 h), and ascending paper chromatography in $\text{BuOH} : \text{AcOH} : \text{H}_2\text{O} : 4 : 1 : 1$ and $n\text{PrOH} : 10 : 0\%$ $\text{NH}_4\text{OH} : 3 : 1$ as development systems

The radiobiologic assays were carried out by determining the pharmacokinetic tissue levels of the tracer in Wistar strain rats (300-525 g) injected with 0.5 mL (~1.8 MBq) of the respective product [13]. Standard pharmaceutical protocols were followed for confirming the sterility and apyrogenicity, and these were carried out on a post-facto basis especially with reference to the R_x dosage form

(viii) *Storage and use*: The dispensed dosage forms (mostly D_x) were generally stored in Pb pots at -35 to -45 °C until use. Whenever required for patient study the required shielded vial was removed, the contents thawed at room temp, assayed for activity, the required aliquot(s) withdrawn for i.v. injection, and the vial returned to the deep freezer. The second or third multidose vial was removed after the contents of the earlier vial was exhausted. These samples were periodically subjected to radiochemical assays in order to validate the levels of purity. The R_x dosage form was added to large volume saline-dextrose injectable and infused into the identified patient at the earliest- either on the same or the next day

3 RESULTS AND DISCUSSION

The pertinent technologies connected with the preparation of ^{131}I -mIBG dosage form could be classified under three categories - radiation health, safety and protection, chemical and pharmaceutical. The following aspects of the process, product and operator

interface as well as user requirements and constraints were studied while optimizing these technologies

3.1. Radiation health, safety and protection

Because of the intrinsic nature of the radionuclide and the levels of activity handled special safety systems had to be designed and in-built into the plant (Fig 1) and for various unit operations and processes to preclude external and internal radiation exposures to operators. Classical methods of radiation attenuation and safety procedures were invoked. A few of the assemblies were readily available, e.g. lead pots, etc., many others were custom designed and fabricated in a workshop, viz. needle condenser, needle injector, lead modules, etc.

The major hazards attending the manual handling of $\sim 1110 / 5550$ MBq (D_x / R_x) amounts of ^{131}I were the high radiation fields and plausible internal exposures of the radiotoxic ^{131}I in the event of a possible leak of the tracer (at high temp). One had to doubly safeguard against such an eventuality since ^{131}I is avidly concentrated in the thyroid gland. Therefore, a closed system with intervening traps was included in the process assembly (Fig 1). The operations were conducted in an efficient fume cupboard with good exhaust. To minimize any possible external radiation exposures the dispensing of ^{131}I was avoided primarily by making the Na^{131}I vial itself as the reactor. The reagents and reactants were added to this rather than vice versa. The needle condenser was inserted into the bung of the 'hot' reactor with the aid of a remote-controlled device. Further, the reactor was shielded with a lead pot which also doubled as a heated metal block, it was closed with a lead cap with a groove for the condenser outlet. The entire process assembly was located > 0.75 m away in the fumehood, which was further lead shielded in front. The reactor was further screened from the receiver + traps by a lead screen. Handling was effected via extra-long tongs of various sizes and descriptions, including mirror reflections and/or automation, gravity flow, low vacuum transfers from a distance, etc. Lead shielding was tailor-made and extensively used for each unit process / operation. Some of the modular type of assemblies (including those for traps) had wide slits which faced the mirror and helped to monitor the ongoing operation from a safe distance. Lead receptacles - modular design - were also made for storing the generated 'hot' wastes for decay, e.g. anion exchange column.

These measures were quite adequate in attenuating the radiation burden to the operators handling the above-mentioned activities. Both TLD as well as thyroid counts showed negligible exposures. Lack of better facilities in the hospital radiopharmacy including a dedicated fume cupboard precluded the use of higher activity levels to prepare a larger number of R_x forms per batch operation.

3.2. Chemical reaction

Quantitative yields were generally obtained when low (~ 75 MBq) activity levels of ^{131}I were employed [10]. However, these results could not be replicated at high activity levels. Since we were interested in obtaining high radioactive dosage forms we adopted the following process regimens: the reactants used were $1.0 / 3.0\text{--}4.0$ mg mIBG, $750\text{--}1110 / 4500\text{--}5550$ MBq Na^{131}I in the presence of $15.0 / 25.0$ mg of $(\text{NH}_4)_2\text{SO}_4$ in a typical batch process for the preparation of the D_x and R_x forms respectively. At times, $50 / 75$ mg of crushed porcelain / alumina / silica were also added to aid the reaction. The Na^{131}I used was requisitioned in the min. vol ($0.1\text{--}0.3$ ml), but was usually consigned in $0.8\text{--}1.5$ ml.

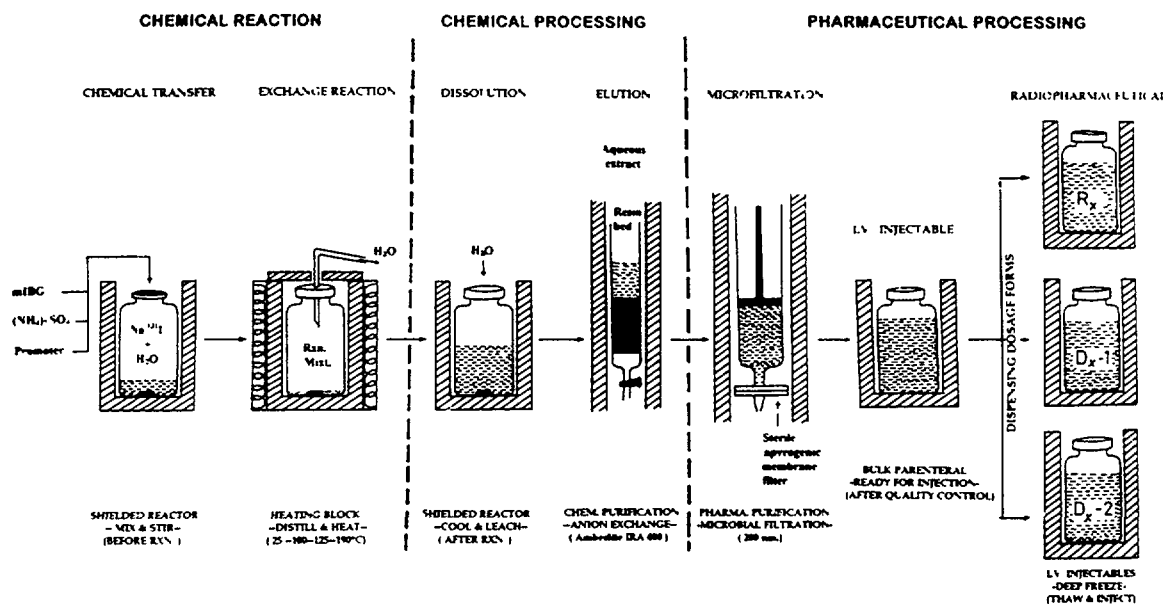


FIG. 2 Flow diagram depicting the various chemical and pharmaceutical unit processes and operations

A schematic of the flow diagram pertaining to the various chemical and pharmaceutical unit processes and operations is shown in Fig.2. The exchange reaction was found to take place at $> 120^{\circ}\text{C}$ in the solid phase in the absence of water. This necessitated the use of minimum vol. of solvent to start with, and the removal of the solvent already present in the reactor. In the absence of a steady stream of gas flowing through the reactor assembly it was found that the removal of the solvent was difficult because of the condensation of water occurring inside the narrow capillaries. Therefore, wider bore capillaries, viz. 10G for the condenser, available 16 G hypodermic needles, were employed. We adopted the following strategies to monitor the progress of the reaction. The evolution of NH_3 could be witnessed by the change in colour of the pH paper placed inside the receiver even from a distance. This preceded the distillation of the solvent at $\sim 120^{\circ}\text{C}$. To expedite the process of solvent removal the temp. was raised to 130°C , and maintained for a few min., following which low vacuum was applied very gently. Thereafter the temp. was progressively raised in steps of 10°C (5 min.). In our hands the yields obtained were not sufficiently high at $140\text{--}150^{\circ}\text{C}$. Therefore, we progressively increased the temp. to 190°C , i.e. the reaction took place in the solid / melt phase.

To ensure high yield in the shortest possible time the reaction run was carried out for a duration of 1.0 h at 190°C . This helped to complete the process operations and to inject the dosage form (especially the R_x) on the same day to save on losses occurring because of physical decay, apart from stability considerations. It was also found that increasing the reaction time did not result in higher yield. Unlike the method Mock and Weiner we were disinterested in intervening in the reaction sequence to save on radiation exposures.

The radiolabelling yield obtained following leaching was ascertained by radiochemical assays, using the process control sample. It was of the order of 80.0-86.0 %. The unreacted activity was in the form of iodide.

3.3. Specific activity

mIBG being a catecholamine analogue is known to exhibit a pharmacodynamic response and is therefore normally injected / infused very slowly and preferably in a diluted form [1,7,10,14] This made it mandatory to label it at high specific activity, especially the R_x form Therefore, it was essential to use minimal amounts of the cold mIBG and high activity levels of a carrier-free or no- carrier-added $Na^{131}I$ in the reaction We used $<1.0 / <3.0$ – 4.0 mg mIBG per 750 – $1100 / 4500$ – 5550 MBq of the tracer to prepare the D_x / R_x dosage forms respectively To effect high yield it was also necessary that the reactants were efficiently mixed in the microscale reaction This was a slightly difficult proposition for a solid-phase reaction taking place in a flat-bottomed reactor vial To ensure that the reacting species were indeed properly mixed, and did not remain on the walls of the reactor inert solid phase promoters such as crushed porcelain / alumina / silica were added alongwith the reagents and reactants These inputs facilitated the reaction The specific activities of the ^{131}I -mIBG dosage forms obtained were - R_x 888 - 1295 MBq / D_x 55 - 148 MBq / mg at the time of calibration

3.4. Radiochemical purification

Purification was effected by passing the solution over a small bed of anion exchange resin which efficiently retained the free iodide impurity The radiochemical purity of the product was thus enhanced to $>99.5\%$ (chromatography)

3.5. Pharmaceutical processing (purification)

GMP and aseptic techniques were instituted whenever and wherever required in the processes and assemblies In addition, the heat processing of the product at $190^\circ C$ for 1.0 h itself constituted a sort of an apyrogenizing process Furthermore, the solution was subjected to a terminal membrane filtration through a microfilter (200 nm) in the precincts of a bioclean work bench to remove any microbial load, if any present, and the filtrate collected in a sterile vial using aseptic techniques of work-up These processes resulted in the dosage forms

3.6. Dispensing the dual dosage forms

The first conc filtrate (~ 15 mL) obtained subsequent to the processing of high activity levels of ^{131}I was designated as the R_x single dosage form This was shielded in appropriate lead pots In addition, 1 – 3 D_x dosage forms of varying radioactive concs were redispensed by removing small aliquots from the above into sterile vials, and suitably diluting them with water for injection These were the D_x single / multidosage forms, and were also kept in shielded containers In actual practice the process assemblies always contained small amounts of adhering activity (upto 400 – 550 MBq) Hence, these were rewashed with water-for-injection and refiltered (200 nm) to afford additional D_x multidosage forms These too were reassayed for radiochemical and pharmaceutical purity by chromatography and established conventional / rapid pharmaceutical protocols

The radioactive concs. of typical dosage forms dispensed were, R_x 2.7 – 4.9 GBq / 15 mL, and D_x 185 – 740 MBq / 5 – 10 mL

3.7. Storage and use

The dispensed dosage forms were stored in shielded Pb pots at -35 to -45 °C in a deep freezer. In most cases the single dosage form was utilized (i.v. injected into the designated patient) at the earliest (preferably soon after preparation) following a rapid quality control regimen consisting primarily of radiobiologic and chromatographic assays. The multidosage D_x forms were utilized over a period of 2-4 weeks depending on the activity levels, number of D_x forms dispensed, patients scheduled for study etc.. It was observed that the low temp. delayed the process of deiodination of the D_x dosage forms. It was $< 5.0-6.0$ % at the end of the corresponding period. However, the dosage forms were constantly checked for their radiochemical purity status during the period of their use.

The identified patients were administered with Lugol's iodine prior to R_x / D_x . About 18 / 37 MBq in 0.5-3.0 mL amounts were i.v. injected for D_x study in children / adults and the patients were scinti-imaged after 48-72 h. In the case of the R_x form $\sim 75-130$ mCi / 15.0 mL (added to pharmaceutical grade 5% dextrose -saline) was slowly infused into the concerned patient over a period of 1.5 h.

3.8. Assays

The radionuclidic purity of Na ^{131}I raw material was reconfirmed by γ ray spectrometry. The migratory patterns of the radiotracers under the conditions of assay are given in Table I. The systems were thus able to well differentiate between the two species. The presence of small amounts of the $^{131}\text{I}^-$ impurity could also be easily quantified. Thus, any one of these physico-chemical methods could be utilized to assay the purity levels. The determination of the electrophoretic mobilities could be speedily performed (0.75 h) while the chromatographic analytical assays were slow ($\sim 3.0 - 4.0$ h).

Apart from the physicochemical assays the pharmacokinetic biologic profiles of the tracers were also studied in Wistar strain rats at three different time periods - early (5 min) and at 0.5 and 2.0 h respectively (Table II). It can be seen that the tissue profiles are different from that of the iodide anion. The iodide anion showed a progressive and selective concentration in the thyroid, stomach and intestines etc. with respect to time whereas it fell in the other tissues. ^{131}I -mIBG, on the other hand, demonstrated a low persistent and steady accumulation in the muscle, especially the heart muscle, salivary glands and pancreas even as early as 5 min. It remained in these tissues for a considerable period before being washed-out. Also, there were negligible uptake in the thyroid and stomach to begin with (at 5 min, 0.5h). Thereafter, the molecule displayed a tendency for slow in-vivo deiodination which was evident at 2.0 h. The localisation in the heart muscle and the different profiles were taken as positive indices of biologic specificity of ^{131}I -mIBG.

We have extensively relied on these to redefine the concept of biologic purity of ^{131}I -mIBG. The safe administration of a comparatively large dose in the animal (~ 0.5 mL / rat) also gave us a measure of the relative safety as far as toxicity is concerned. It may be added that these assays being quick, reliable and specific helped to shorten the time element.

3.9. Specifications

The general product specifications of the two different dosage forms are outlined in Table III.

Table I Paper electrophoretic and chromatographic migratory patterns of the radiotracers on Whatman Grade 1 paper at 25 ° C

Radiotracer	Electrophoretic behaviour (Dist traversed in 0.5 h at 10 V / cm, 0.05 M phosphate buffer), cm	Chromatographic (ascend) mobility parameters			
		BuOH	AcOH	H ₂ O	nPrOH 10 % NH ₄ OH
		4	1	1	3
		R _f values			
¹³¹ I-mIBG	Origin	0.90 - 0.95			0.90 - 1.00
¹³¹ I-Iodide	5.0 - 6.0	0.15 - 0.25			0.55 - 0.65

Table II A comparison of the pharmacokinetic profiles* of i.v administered ¹³¹I-mIBG versus ¹³¹I⁻ in select tissues of Wistar strain rats at early time periods

Organ	% administered dose in whole organ at different time periods					
	5 min	0.5 h	2.0 h			
	¹³¹ I-mIBG	¹³¹ I-iodide	¹³¹ I-mIBG	¹³¹ I-iodide	¹³¹ I-mIBG	¹³¹ I-iodide
Blood ^a	5.42±1.05	17.24±4.10	1.45±0.15	8.20±0.88	1.29±0.15	5.60±1.10
Muscle ^a	29.41±9.35	29.24±3.45	29.12±5.59	17.15±1.22	29.03±8.65	11.57±2.98
Heart ^a	4.11±0.82	0.24±0.05	3.78±0.66	0.10±0.05	2.64±0.33	0.11±0.06
Salivary glands	1.41±0.21	0.54±0.11	1.98±0.06	0.29±0.07	2.10±0.65	0.24±0.06
Thyroid	0.09±0.02	0.72±0.15	0.07±0.01	2.48±1.40	0.42±0.13	7.07±1.33
Pancreas	0.69±0.17	0.33±0.14	0.72±0.07	0.15±0.06	0.39±0.10	0.14±0.05
Lungs	8.42±1.32	1.11±0.17	4.50±0.63	0.63±0.11	1.93±0.59	0.39±0.21
Liver	14.93±2.23	5.92±1.57	14.69±0.69	2.39±0.40	6.60±2.08	1.79±0.36
Spleen	0.48±0.11	0.28±0.06	0.54±0.11	0.12±0.04	0.59±0.14	0.12±0.08
Stomach	0.97±0.14	4.64±1.74	1.15±0.28	17.49±3.41	1.90±0.53	17.32±5.17
Small Int	9.56±3.05	7.28±2.88	10.47±1.84	10.87±2.33	16.63±3.27	8.53±3.26
Large Int	2.68±0.48	1.63±0.39	2.50±0.44	1.16±0.41	2.73±0.80	0.96±0.24

* Results expressed as Mean ± S.D. · n = 7-8 rats

^a Assumed that the total blood and muscle is 5.0 and 45.5 % total body weight respectively

^a The excised heart was washed with water saline and blotted dry prior to counting

Table III. The product characteristics of typical R_x and D_x i.v. parenteral dosage forms of ^{131}I -mIBG prepared at R.M.C (at calibration), and the norms presently followed in their preparation and in-house use

Feature	R_x	D_x
1 Chemical / pharmaceut form	Formulated in sterile aqueous solution	
2 pH	6.0 - 6.5	6.0 - 6.5
3 Specific activity	888 - 1295 MBq / mg	55.5 - 814 MBq / mg
4 Radioactive conc	~ 275 MBq / mL	13.7 - 107.2 MBq / mL
5 Radiochemical purity	> 99.5 %	> 99.5 %
6 Pack size	> 2775 MBq	55.5 - 296 MBq
7 Single vial contents	~ 15.0 mL	~ 3.0 - 8.0 mL
8 No. of vials dispensed / prep	1	1-3
9 Dosage form	Liquid dosage form or stored deep frozen, thaw and inject	Liquid dosage form or stored deep frozen, thaw and inject, return vial with remaining contents quickly to deep freezer
9 Usage	Mostly injected soon after preparation	One - three patients injected soon after preparation
10 Storage conditions	Advised immediate / early use, or as in D_x	< -35 to - 45° C or lower temp (Deep frozen)
11 Recommended dosage form(s)	Single	Multiple
12 Recommended dose	> 2775 MBq	18.5 / 37.0 MBq (child / adult)
13 Expiry	Advised immediate use	2- 3 weeks, one vial at a time
14 Preparation schedule	Only against firm patient appointments	
15 Availability (at R.M.C)	Single patient / 3-4 wks.	5-15 patients / 3-4 weeks

3.10. Patients

To date we have prepared 70 exclusive D_x + 14 dual batches of R_x + D_x dosage forms totaling about 43.43 GBq (D_x) + 49.0 GBq (R_x) = 92.4 GBq amounts over the past 7.5 years. About 150 nos. of D_x multidosage vials were made available to clinicians during this period. These have been i.v. injected in ~ 1100 patients [15]. Similarly, we have dispensed 14 R_x dosage forms which have been administered into these patients.

3.11. Low cost technology

The above described technology has evolved over the past 7.5 years. In the early years the emphasis was only on the preparation of the D_x dosage form. During this period each of the technologies were standardized so that it could be scaled-up to levels that could deliver a R_x dosage form with high yields. Also, the use of easily accessible, cheap,

disposable substitutes for each process was explored. Presently we are able to prepare sufficient quantities of the D_x form to cater to ~ 180 patients / year (prepared once / 3–4 week interval), but are not fully geared and equipped for the enlarged production of large amounts of the R_x dosage form because of resource constraints. Besides, a programme for the manufacture of the R_x dosage form has also to reckon with the management of the generated ‘hot’ wastes, and their safe - keeping and disposal. In the present scenario we can at most prepare about 15 R_x doses / annum.

Being a near-physiologic analogue of catecholamine and one bearing an iodine label makes radioiodinated mIBG one of the more important radiopharmaceuticals. It can be labelled with a variety of radioisotopes of I, viz. ^{123}I , ^{124}I , ^{125}I , ^{131}I , etc. having different physical attributes. These endow the labelled products with broad spectrum capabilities - both for diagnostic applications with diverse type of instrumentation, as well as for therapeutic applications with ^{131}I and perhaps even with ^{125}I . Over the years the radiodiagnostic capabilities of the molecule have considerably increased (vide infra).

4 CONCLUSION

Radiodinated mIBG was first introduced in 1981 by the Michigan team [16,17]. The availability of commercially manufactured ^{123}I / ^{131}I - mIBG enlarged its application, but its relative instability and associated logistics and high costs put it beyond the means of vast segments of the patient population in developing countries. To remedy this situation we embarked on the indigenous in-house preparation of the radiopharmaceutical. We adopted / adapted one among the several process technologies [10] to arrive at a simple low cost technology for the rapid and safe small-scale production of relatively large amounts of ^{131}I -mIBG per batch operation in a hospital-based radiopharmacy. It resulted in assured high yield, radiochemical purity, and specific activity. The activities were adequate for obtaining a single R_x alongwith 1–3 multidosage D_x forms. The major constraints were the containment of the radiation burdens - both external and internal, and resources. These were overcome by designing and developing in-house a fool-proof, self-shielded remote-controlled, semi-automated prototype microplant for the chemical reaction and other downstream chemical and pharmaceutical process operations. The complete radiopharmaceutical technology was systematically worked out for execution in a hospital-based environment with available in-house (plus a few additional) resources. The process resulted in high yields (80–86 %) and radiochemical purity of $> 99.5\%$, and the entire sequence of operations could be performed within a relatively short time. Specific and reliable biologic indices obtained by pharmacokinetic profiles (along with physicochemical parameters) aided the assay process. These helped to hasten the control of the product and, in turn, to utilize the R_x and even the D_x radiopharmaceutical dosage forms on the same or the next day.

Presently we have been able to provide a fairly comprehensive ^{131}I - mIBG radiopharmaceutical service (mainly diagnostic and to lesser extent therapeutic) to patients attending our Centre. Thus far we have dispensed $49.0 - 43.43 = 92.4$ GBq amounts of ^{131}I -mIBG of high specific activity- 14 single dosage R_x and 70 – 14 batches (consisting of ~ 150 nos.) D_x multidosage forms respectively. The latter have been safely used in >1100 patients over a 7 year period. The injectable was safe and efficacious. The process was also safe from the health physics point of view. This semi-automated technology can be replicated by dedicated radiopharmacists in any other hospital radiopharmacy with a few additional inputs. Furthermore, it could be adopted for the in-house preparation of ^{123}I -mIBG (a relatively short-lived SPECT radionuclide).

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SELECTIVE SPLENIC TARGETING OF In-114m BY HEAT-TREATED RED BLOOD CELLS FOR THE TREATMENT OF LYMPHOID CELL MALIGNANCY

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Abstract

Spleen targeted In-114m, using labelled autologous lymphocytes, has produced a significant antitumour effect in patients with chronic lymphocytic leukaemia and Non-Hodgkins lymphoma (*Sharma et al, Anti-Cancer Research 17, 1815-1822, 1997*). Heat treated red blood cells could be used as alternative vectors for splenic targeting of In-114m, making the technique easier, more universally applicable and furthermore, may reduce the myelosuppression seen with labelled lymphocytes.

Red blood cells from HO3T rats were labelled with In-114m-oxine, incubated at 49.5°C for 15 minutes and their distribution investigated in the spleen, liver and blood of recipient animals. The splenic uptake in the spleen at 24h was 64.08%, remained unchanged at 7days, cleared slowly after that, clearly demonstrating the specificity of HTRBC to target In-114m to the spleen.

The depletion of peripheral blood lymphocytes was measured in two groups of HO3T rats following the administration of 1.6 and 3.2 MBq of In-114m-HTRBC respectively. Compared to the controls, ~ 70% of lymphocytes were depleted in the treated animals within one week and remained unchanged for 6 weeks. Using a rat T-cell lymphocytic leukaemia model, with resemblance to the clinical disease, an anti-leukaemic effect of this method of treatment, was monitored. An average life span of the treated group (1.85 MBq of In-114m-HTRBC) was 17.1 days, compared to the 13.5 days for the untreated group. These results are similar to the ones reported by targeting In-114m with labelled lymphocytes.

In summary, the project has shown that In-114m-HTRBC can be used to deposit the radioactivity, selectively in the spleen, which in turn, depletes the peripheral blood lymphocytes and produces an anti-leukaemic effect in terms of enhanced life span.

The bone marrow toxicity from In-114m therapy is under investigation and a pharmacokinetic study in selected cancer patients is planned following which, a clinical trial will be considered.

1.0 Introduction and Background

Lymphocytes, involved in lymphoid cell malignancy, are radiation sensitive cells and their migration involves continuous re-circulation between the blood and the lymphatic tissue. About 70-80% of the blood lymphocytes migrate to the spleen and

lymph nodes [1, 2, 3]. By virtue of these two properties, depletion of re-circulating lymphoid cells could be achieved by siting an irradiation field in a major lymphatic organ, eg the spleen. This would offer a potential treatment for the lymphoid cell malignancies such as chronic lymphocytic leukaemia (CLL) and Non-Hodgkin's lymphoma (NHL).

Principle of splenic radioactivity to induce lymphocytopenia was first demonstrated by Roser and Ford [4] in the rat by intrasplenic injection of ^{32}P and ^{185}W . Birch et al [5] used In-114m labelled lymphocytes as vectors to deposit radioactivity in the spleen which was subsequently shown to produce a rapid depletion of peripheral lymphocytes. Use of the lymphocytes to selectively deposit radioactive In-111 in the spleen had been demonstrated earlier by Sparshott et al [6]. Furthermore, Birch et al [5] clearly demonstrated that spleen localised In-114m increased the life span of leukaemic rats in a dose dependent manner. The animal leukaemia model used in these experiments was a T-cell acute lymphocytic leukaemia (ALL) called Roser's leukaemia [7], with a close resemblance to the human ALL [8].

A subsequent pharmacokinetic study [9] in patients with lymphoid cell malignancy confirmed that the results obtained from the animal experiments were applicable to human subjects. A clinical trial using autologous In-114m labelled lymphocytes to treat patients in the terminal phase of CLL, produced a 70% response [10]. The patients suffered no immediate side effects; none experienced fever, nausea or vomiting; there was no evidence of hepatic destruction. The major toxicity was myelosuppression, predominantly thrombocytopenia; some patients required platelet support.

In the above-mentioned investigations [5, 9, 10], the In-114m labelled lymphocytes were used as vectors to target the activity to the spleen. The clinical outcome has provided ample evidence, that spleen targeted In-114m, results in profound

lymphocytopaenia, and is successful for the treatment of patients with lymphoid cell malignancies. Although autologous lymphocytes have proved to be good vehicles for transporting the In-114m to the spleen, the number of lymphoid cells required, necessitates the use of a cell separator on some patients. In addition, approximately 5% of the administered radioactivity localizes in the bone marrow, which is probably the reason for myelosuppression.

There is an obvious need to search for new vehicles to create the selective internal lymphoid irradiation field in the spleen. The criteria would be: i) easy to harvest, ii) more universally applicable, iii) low uptake in the bone marrow and iv) avoid the ethical dilemma associated with the re-injection of malignant cells. Of several possibilities, the most practical one appeared to be the use of heat-treated red blood cells (HTRBC). In 1980, the specificity of using Tc-99m-HTRBC for imaging the spleen was reported [11], and four years later, [12] the optimum conditions for splenic localisation of HTRBC in man were described using Cr-51 label. This three-phased investigation, was undertaken, to create the lymphoid irradiation field in the spleen, with In-114m-HTRBC, and to test its potential for the treatment of lymphoid cell malignancy.

- Phase 1: The distribution and retention of In-114m in the spleen following the injection of labelled HTRBC.
- Phase 2: The assessment of lymphocytopenia from the localised In-114m in the spleen.
- Phase 3: Monitoring the increase in life span of the leukaemic animals treated with In-114m-HTRBC.

2.0 Materials and Method

In phase 1 of the project, red blood cells from HO3T rats were radiolabelled with In-114m-oxine and their distribution investigated in the spleen of recipient animals. In-

^{114}m has a half-life of 49.5 days and emits β -particle of 777 keV average energy in addition to a prominent γ -ray of 190 keV. The cell labelling efficiency with In-oxine was >95% in less than 5 minutes. The labelled cells were incubated at 49.5°C for 15 minutes and injected into 3 groups of rats. The uptake of In- ^{114}m was measured in the spleen, liver, kidneys, lungs, testes, and blood at 24h, 7d and 28d post-injection. Autoradiograph of the spleen section was obtained from an animal from the 7d group.

In phase 2, depletion of peripheral blood lymphocytes was measured using three groups of adult HO3T rats (8 animals per group). In group 1, each animal received 1.6MBq of In- ^{114}m labelled HTRBC intravenously, in group II, each received 3.2MBq and the third group served as controls. Peripheral blood samples (10 μ l) were taken weekly from the tail vein. The red cells were haemolysed and the total white cell count monitored using a Coulter counter. Since the lymphocytes and polymorphs were not distinguished by the Coulter counter, blood smears were made for differential cell counts. This enabled the proportion of lymphocytes to be calculated in control and treated animals.

In phase 3, an anti-leukaemic effect of this method of treatment was monitored using the rat T-cell lymphocytic leukaemia model. Three groups of rats (10 per group) were inoculated with 10³ leukaemic cells. Group 1 served as controls, whereas groups II and III received 0.9MBq and 1.8MBq of In- ^{114}m labelled HTRBC respectively. Following the injections, the animals were observed frequently for the enlargement of neck glands and disease related severe lethargy. Animals at this stage of the disease do not survive for more than a few hours and therefore have to be killed to prevent undue suffering.

3.0 Results

3.1 Distribution of In-114m

The uptake of In-114m following the administration of labelled HTRBC for three time points (24h, 7d and 28d p.i.) is shown in Table 1. The results clearly show that at 24h ~64% of the injected activity localized in the spleen and remained virtually unchanged for 7 days followed by a slow elimination. The uptake in the liver is lower by an order of magnitude and remained static for up to 28 days. The blood concentration declined dramatically from day 1 to day 7, after which it remained unchanged. The other organs monitored in these experiments i.e. the kidneys, lungs and testes, had very low uptake at all three time points.

Autoradiographs of the spleen section is shown in Figure 1. The radioactivity is concentrated in the marginal zone (MZ) and red pulp (RP), whereas, the white pulp (WP) containing the central arteriole (CA) is relatively free from the radioactivity. The concentration of β^- -emitting In-114m in the MZ would obviously provide a strong field of irradiation to the re-circulating pool of lymphocytes.

3.2 Effect of In-114m-HTRBC on the peripheral lymphocytes

The differential cell counting of several blood smears revealed that, in the control group, the mean lymphocyte count was $66 \pm 5\%$, polymorphs $34 \pm 5\%$. This situation had reversed itself in both the treated groups, in these the average lymphocyte count was $38 \pm 10\%$, whereas the polymorph count was $62 \pm 10\%$. These results are consistent with the selective elimination of highly radiation sensitive lymphocytes. Figure 2 shows the peripheral lymphocyte count Vs time in the three groups of rats. A dramatic fall in the lymphocyte counts can be seen at one week, which was maintained for up to 6 weeks and then begins to show a slight increase.

Table 1 Uptake distribution of In-114m from labelled HTRBC

Organ	Day 1	Day 7	Day 28
Spleen	64.08 ± 1.01	62.07 ± 0.55	40.34 ± 11.02
Liver	5.86 ± 0.39	7.58 ± 0.44	6.24 ± 0.29
Blood / mL	2.14 ± 0.29	0.37 ± 0.05	0.33 ± 0.02
Kidneys	0.39 ± 0.09	0.80 ± 0.05	1.17 ± 0.07
Testes	0.04 ± 0.004	0.08 ± 0.004	0.27 ± 0.05
Lung	0.21 ± 0.02	$0.07 \pm$	0.13 ± 0.004

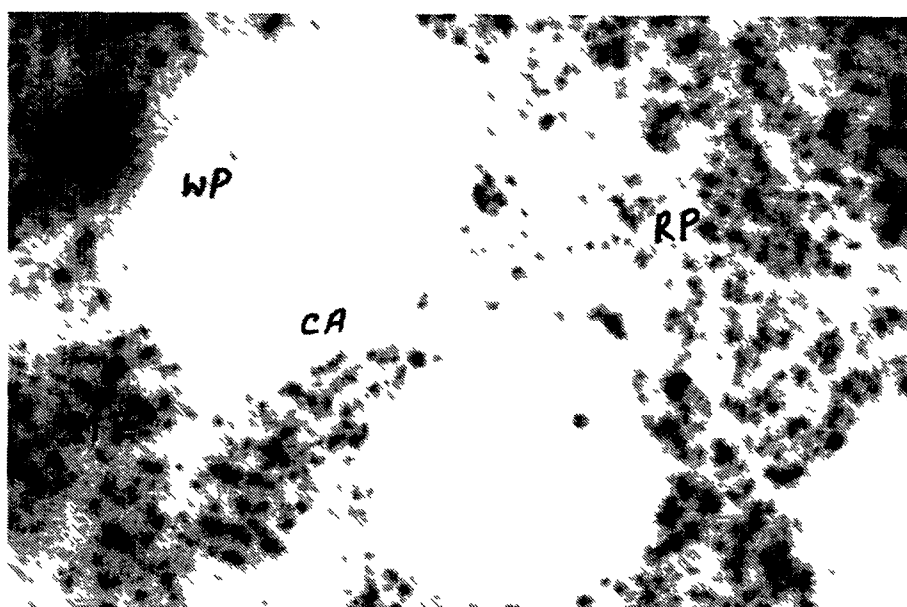


Figure 1 Spleen Autoradiograph

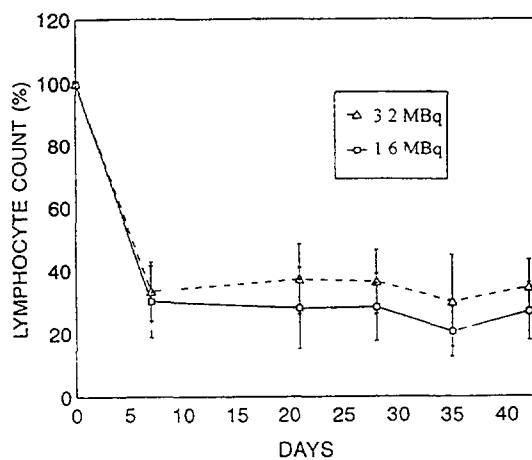


Figure 2 Lymphocyte count using In-114m HTRBC

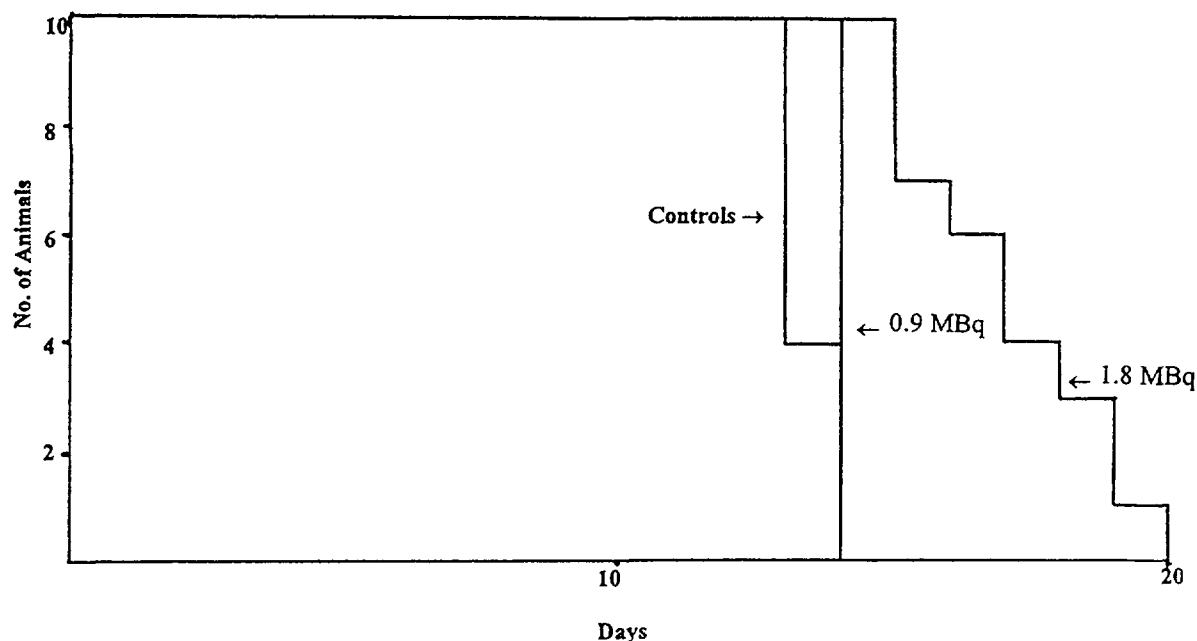


Figure 3 Survival time (days post-injection)

3.3 Anti-leukaemic effect of In-114m-HTRBC

To monitor the potential of In-114m-HTRBC as a radio-therapeutic treatment of lymphoid cell malignancy, relative increase in life span of leukaemic animals was investigated at two dose levels compared to the control group. Survival time histograms of the three groups of animals is shown in Figure 3. Six animals of the control group died on day 13, the remaining on day 14. All the animals treated with 0.9MBq of In-114m-HTRBC died on day 14 i.e. no significant increase in life span was seen for this group. In comparison, out of the ten animals treated with 1.8MBq, 3 animals died on day 15, 1 on day 16, 2 on day 17, 1 on day 18, 2 on day 19 and 1 on day 20. The average life span for this group was 17.1 days compared to 13.5 days for the control group.

4.0 Discussion

The results obtained from the distribution study, have clearly demonstrated that the radiolabelled HTRBC can be used to selectively deposit a large proportion of the

administered radioactivity in the spleen. Elimination from it is slow and therefore the spleen would be expected to continue to act as an internal field of irradiation for the re-circulating lymphoid cells. It should also be mentioned that the microscopic distribution of In-114m (Figure 1) in the spleen section, deposited by HTRBC is identical to the one seen with labelled lymphocytes (Figure 2 in Ref.5).

Profound depletion of peripheral lymphocytes resulted from the spleen deposited In-114m within one week, which was maintained for up to 6 weeks i.e. the duration of the experiment. Furthermore, the decrease in lymphocyte counts observed with 1.6MBq, is similar to the one obtained with 3.2MBq of In-114m indicating that the dose levels used in these experiments produced a saturation depletion. Much smaller doses may be sufficient to produce a similar response. These results are consistent with those reported by Birch et al [5] in which 95% of peripheral blood lymphocytes got depleted within 3 weeks of 0.74MBq(20 μ Ci) of In-114m administered as radiolabelled thoracic duct lymphocytes.

Depletion of peripheral lymphocytes is in itself a good indicator of therapeutic efficacy of this method against lymphoid cell malignancy. The significant increase in life span of leukaemic animals observed with 1.8MBq of In-114m-HTRBC has provided a further evidence of the anti-leukaemic effect of the technique. For comparison, administration of 2.2MBq(60 μ Ci) as labelled lymphocytes increased the life span of leukaemic animals from 7 to 11 days [5].

5.0 Summary

In a series of experiments, the administration of In-114m labelled HTRBC have been shown to deposit around 2/3 of activity in the spleen of the recipient rats thus creating an internal source of irradiation for recirculating lymphocytes. This in turn produced profound lymphocytopenia in normal animals and significantly increased the life span

of animals inoculated with rat T-cell lymphocytic leukaemia, clearly demonstrating an anti-leukaemic effect.

An investigation of bone marrow toxicity following In-114m-HTRBC therapy has now been undertaken. A pharmacokinetic study of In-114m-HTRBC in selected cancer patients is planned following which, a phased clinical trial of the technique would be considered.

ACKNOWLEDGEMENTS

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**Sm-153 EDTMP — A NEW PALLIATIVE THERAPY AGENT
AGAINST PAIN IN CASES OF BONE METASTASES**



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Abstract

During the last years exist an increasing interest in employing radiotherapeutic agents as palliative skeletal pain caused by disseminated bone metastases. Selection of an appropriate radiotherapeutic agent for one application is directly related to the biolocalization of carrier molecules.

Is estimated that the 50% of patients with mamma, prostate, and lung carcinomas can develop single or multiple bone metastases. The multiple bone metastases frequently cause intense pains that must be calmed with different analgesics.

The Physical characteristics of Samarium 153 permit be considered as excellent radiotherapeutic and diagnostic image agent.

Sm - 153 EDTMP is one of the most interesting products in the treatment of the bone metastases by the excellent biolocalization , including rapid blood clearance and bone affinity.

In some patients the relief is obtained during the first or second week of the application improving in progressive form. The benefit of the treatment can be maintained durign some months.

This radiotherapeutic agent has been applied success fully till now in near 300 patients from differents hospitals to Lima. Most of them have been experimented a progressive improvement. All patients were treated in single rooms. The dose was 1 mCi / kg in all cases according the protocol established. Images were obtained 3 hours after receiving Sm - 153 EDTMP.

INTRODUCTION

During the last years exist an increasing interest in employing radiotherapeutic agents as palliative skeletal pain caused by disseminated bone metastases. [1], [2]. Selection of an appropriate radiotherapeutic agent for one application is directly related to the biolocalization of carrier molecules.

In Perú is estimated that 50 to 60 % of patients with mamma, prostate, and lung carcinomas can develop single or multiple bone metastases. The multiple bone metastases frequently cause intense pains that must be calmed with different analgesics ; some times combining two or tree of them .

The physical characteristics of Samarium 153 permit be considered as excellent radiotherapeutic and diagnostic image agent.

Sm - 153 EDTMP is one of the most interesting products in the treatment of the bone metastases by the excellent biolocalization , including rapid blood clearance and bone affinity [3].

In some patients the relief is obtained during the first or second week of the application improving in progressive form. The benefit of the treatment can be maintained durign some months.

Peruvian experiences with Sm - 153 EDTMP became at the end of 1995 . This radiotehrapeutic agent has been applied success fully till now in near 300 patients from seven hospitals located in Lima and one out side of Lima . Most of them have been experimented a progressive improvement. All patients were treated in single rooms. The dose was 1 mCi/kg in all cases according the protocol established. Images were obtained 3 hours after receiving Sm - 153 EDTMP.

On the other hand in 1997, Peruvian Nuclear Center sent $\text{Sm } 153$ as Samarium Trichoride to Five Latinoamerican countries under agreement IPEN and AIEA . The EDTMP was labelling with this Samarium in each country according to protocol proposed by AIEA .

ADVANTAGES OF $\text{Sm } 153$ EDTMP

The penetration range in the bone tissue is approximately 3 mm , enough to penetrate in the zone where are localized the malignant cells.

The beta and gamma emission permit the obtaining of clear images in a detection system.

Short half life in comparison with the 50.5 days of the Strontium 89 before used in Perú.

The rapid product's elimination by the kidney and the long retention in the bones make of the $\text{Sm } 153$ EDTMP an ideal radiopharmaceutical in Therapeutic Nuclear Medicine.

The therapeutic dose is 30 times more economic than the dose of $\text{Sr } 89$ SrCl_2 according to Peruvian radiopharmaceutical market. After 24 hours of the injection , the patient's urine will contain very low $\text{Sm } 153$ concentrations so that the treatment may be ambulatory.

METHOD AND MATERIALS

1. Irradiation facilities

Samarium Trioxide enriched to 98.7% in ^{152}Sm from ISOTEC INC was irradiated in the RP-10 Reactor (Nuclear Center of Huarangal - Lima) at neutron fluxes of $1 - 3 \times 10^{14}$ n / $\text{cm}^2 \cdot \text{s}$ during 8 hours in same cases and 15 hours in others (no continued irradiation) . The average operating power level of the reactor during these irradiations was 7 Mw. After irradiation , the samples were left to cool for 24- 48 hours.

2. Target dissolution

Sm_2O_3 is dissolved in 1.5 ml $\text{HCl } 0.1\text{N}$. Ampoule quartz containing Samarium traces is washed two times with 1.0 ml bidistillate water. Both solutions are joined in a vial A.

3. Sm - 153 EDTMP Preparation

In the process, we usually dissolved an appropriate amount of EDTMP equivalent to 08 times molar respect to mass of Samarium irradiated [4]. This EDTMP is dissolved in NaOH 0.1N solution in a vial B.

Flask B is transfer to flask A. The pH of this solution was adjusted to 7.5 - 8.0 with NaOH 0.1N. The product thus obtained is put in a thermal bath during 25 minutes to 80°C, stirring several times the flask. The vial is opened and 2ml Buffer Phosphate (pH 7.0 - 7.5) is added into. The end product is sterilized by autoclave during 25 minutes to 120 °C. The bidistillate water must be free of pyrogenos as protocol of quality control.

QUALITY CONTROL

1. Radiochemical Purity

Take a small column containing 2ml of Sephadex G-25. Put upon sephadex bed 1 - 2 mCi of label product . Column radioactivity measurement was performed in a ionization chamber. Called A_1 to value thus determinated.

Washed the column with 15ml of NaCl 0.9% . The eluate contains the label product and column retains the ^{153}Sm free. The column activity measurement is called A_2

Radiochemical purity is calculated as:

$$\text{R.P} = 100 (1 - A_2/A_1) \% \quad [5]$$

The ionization chamber factor of Capintec is 241×1.12 . For Vinten chamber is 374.

2. Radionuclidic purity

The Radionuclidic purity is carried out by gamma spectrometry employing a mutichanel equipment, where the main pick of ^{153}Sm is found to 101.3 KeV.

Routinely were not detected ^{152m}Eu , ^{154}Eu , ^{155}Eu neither ^{156}Eu .

If the amount of lanthanides in target material is more to 0.01% could be detectd a significative activity of ^{155}Eu and ^{156}Eu .

3. Biodistribution

Biodistribution was performed in winstar rats weighing 280 - 350 g at 2 , 4, 16 , 24 and 48 hours after intravenous dose of 200 μ Ci of Sm - 153 EDTMP. Can observed a higher uptake in skeletal system , low dose in liver and rapid blood clearance . The results were expressed in % dose/organ. See Table No 01

4. Toxicity evaluation

A 5 mice group with an average weight 25 g are injected with a volume of 0.1 ml of Sm - 153 EDTMP . This dose is equivalent to 500 times more than human administration. Mice are maintain in observation by one week. If none animal die or none present severe reactions in behavior during evaluation period , the result is favorable.

TABLE No. 01

BIODISTRIBUTION OF 153 Sm EDTMP IN RATS (*)

ORGANS	TIME (h)				
	2	4	16	24	48
Blood	0.074	0.065	0.064	0.060	0.058
Liver	0.332	0.390	0.390	0.384	0.328
Spleen	0.012	0.013	0.012	0.011	0.010
Lung	0.030	0.028	0.026	0.024	0.020
Kidney	0.210	0.224	0.310	0.385	0.495
Stomach	0.048	0.045	0.042	0.039	0.034
Small Intestine	0.050	0.050	0.048	0.044	0.041
Large Intestine	0.162	0.160	0.152	0.148	0.144
Bladder	0.185	0.488	0.160	0.145	0.095
Heart	0.015	0.016	0.015	0.014	0.014
Muscle	0.340	0.245	0.244	0.244	0.238
Femur	1.856	1.989	2.112	2.224	2.229
Skeletal	46.22	47.18	49.62	50.16	51.48

(*) Biodistribution expressed as % Dose per organ . The data is the average for the last 12 production batches

PROTOCOL OF APPLICATION

A. SELECTION OF THE PATIENT

- Positive bone scan using Technetium 99m (MDP)
- Normal or near to the normal hemogram
- Fine renal function
- Life expectancy greater than 4 months
- Previous treatment with radiotherapy and chemotherapy at least one month before the application (if exist)

B. APPLICATION

- Dose:
 - 1 mCi / kg to localized metastases
 - 1.5 mCi / kg to multiple metastases
- Previous hydration of the patient by oral via (1 liter in 30 minutes)

TABLE No. 02

BIODISTRIBUTION OF Sm 153 EDTMP KIT IN RATS (*)

Batch	Time	Radiochemical Purity Sm 153 - EDTMP	Biodistribution		
			Liver	Blood	Skeletal
1	4 d	99.60 %	0.59 %	1.59 %	47.17%
1	65	99.85	0.58	1.61	45.27
1	120	99.83	0.64	1.69	42.21
2					
2	48	99.85	0.65	0.92	48.23
2	130	99.58	0.59	0.61	47.92
	150	99.41	0.58	0.95	45.96
3					
3	26	99.60	0.66	1.16	45.85
3	90	99.52	0.65	1.25	45.60
	125	99.41	0.71	1.12	43.69

(*) 02 Horas post injection. n = 2

- Intravenous injection in 5 minutes (is recommended make a dilution with physiological serum no more than 20 ml)
- Obtaining of images at 3 and 24 hours.

C. PATIENT POST OBSERVATION

- Control of the pain in a week post injection
- Blood sample:
Hemograms at 2 or 3 weeks and at 3 months
- Evaluation of the pain at 2 or 3 weeks and at 3 months
- Evaluation of the medication
- Proper evaluation of the patient

RESULTS AND DISCUSSION

Sm - 153 EDTMP has demonstrated be an excellent radiopharmaceutical to palliative metastatic bone cancer pain. Images obtained additionally allow a rapid diagnostic.

Availability of the label product in the Peruvian Nuclear Center added with an economic price permit to poor patients to access to their treatment.

Studies connecting EDTMP KIT have been development lately in order to obtain a new form of product [6]. Preliminary results are showed in the table No. 02

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THE USE OF $^{186}\text{Re}(\text{Sn})$ -HEDP FOR PAIN RELIEF IN THE PALLIATIVE TREATMENT OF BONE CANCERS



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Abstract

A common complication in patients with breast or prostate cancer is bone metastases causing pain. New radionuclide therapy methods have recently been proposed for palliation including $^{186}\text{Re}(\text{Sn})$ -HEDP. This paper reports on the local development of $^{186}\text{Re}(\text{Sn})$ -HEDP and the biodistribution studied in animals for eventual use in patients.

The ^{186}Re was labelled to HEDP using standard techniques. Initially the biodistribution in five Chacma baboons was studied. Doses ranging from 39.4 MBq/kg to 44.9 MBq/kg (mean 43.6 ± 2.8 MBq/kg) were administered which corresponded with an adult human dose of 2960 MBq (80 mCi). Whole body images of the animals were obtained with a dual detector scintillation camera on an hourly basis for six hours post injection and then daily for 3 days. The bone, soft tissue, kidneys and urinary bladder were considered source organs and data from these organs were used in a compartmental model to obtain the mean residence times of the radionuclide in the different source organs. Radiation dose estimates for $^{186}\text{Re}(\text{Sn})$ -HEDP were subsequently obtained with the MIRDose 3 program. Subsequent to the animal study, the palliative effect of the $^{186}\text{Re}(\text{Sn})$ -HEDP was evaluated in ten patients with primary prostate cancer using the pain scale suggested by the World Health Organisation.

Estimated absorbed radiation doses (expressed in mGy/MBq) to some of the organs of baboons were: bone surface 1.69; kidneys 0.09; liver 0.04; ovaries 0.04; red marrow 0.75; total body 0.12; urinary bladder wall 0.43. $^{186}\text{Re}(\text{Sn})\text{-HEDP}$ yielded an effective dose of 0.17 mSv/MBq in the animal study and 0.16 mSv/MBq in humans. Positive palliation was obtained in seven patients, but pain levels increased after 8 weeks.

The radiation dose delivered to the bone marrow in this study did not cause any detrimental effect to the baboons or the humans, indicating that the locally produced $^{186}\text{Re}(\text{Sn})\text{-HEDP}$ is suitable for clinical use.

1. Introduction

A consequence of bone metastases due to primary prostate cancer is severe pain, and it is therefore of great importance, and a challenge to increase the patients' quality of life. Analgesics, hormone therapy, chemotherapy, palliative surgery and radiotherapy are some of the options that can be used for palliation of these types of cancers. Significant palliation with minimal bone marrow toxicity was found with a range of radionuclides.

Cancer leading to bone metastases is also a large problem in South Africa (and Africa as a whole) due to limited funding for treatment and a low awareness for the problem in a large portion of the population. In this study, we used $^{186}\text{Re}(\text{Sn})\text{-HEDP}$ since the ^{186}Re isotope was available (long half-life, γ -rays with a low photon yield, β -rays $> 1\text{MeV}$), produced by the Atomic Energy Corporation. The utilisation of the $^{186}\text{Re}(\text{Sn})\text{-HEDP}$ in clinical trials illustrated by this study is primarily directed to local evaluation and possible production of low cost medicine, to enable the potential future development of new products in continued collaboration with research groups abroad and also eventually to help address this problem in Africa.

2. AIM

The aim of this study was to conduct the clinical trial for the in-house produced $^{186}\text{Re}(\text{Sn})\text{-HEDP}$. Radiation dose estimates and the biodistribution of the radiopharmaceutical were firstly obtained in an animal study. Thereafter the evaluation in relieving the pain as a result of bone metastases in patients with prostate cancer, was investigated.

3. METHODS

3.1 Chemistry: The preparation of the $^{186}\text{Re}(\text{Sn})\text{-HEDP}$ compound was reported previously [1,2] and consists of only minor modifications from that reported earlier by Maxon et al.[3]. In short, reactor produced ^{186}Re in the form of aqueous $\text{NH}_4^{186}\text{ReO}_4$ received from the South African Atomic Energy Corporation was purified to yield an

ethanolic solution of the tetrabutylammonium salt: TBA($^{186}\text{ReO}_4$); specific activity: ca 3700 MBq/ml (3.7 GBq/ml). The ^{186}Re was labelled to HEDP using commercially available chemicals. All procedures were performed under nitrogen atmosphere. The kits were prepared as follows: Hydroxyethylidenediphosphonic acid (HEDPH₂) (Fluka, 75 mg, 0.364 mmol), tin(II)chloride dehydrate (Fluka, 20 mg, 0.0886 mmol) and ascorbic acid (Merck, 10 mg, 0.0568 mmol) were weighed in a 10 ml vial which was subsequently capped and flushed with nitrogen. A de-oxygenated 1.00 ml sample of an aqueous solution of KReO₄ (Strem) containing 0.65 mg (3.5 μmol) rhenium (as metal), was added to the kit. The pH was adjusted to 3-4 by addition of 0.9 ml (36 mg, 0.90 mmol) of an aqueous NaOH solution. One ml of the TBA($^{186}\text{ReO}_4$) solution was added and heated at ca 100 °C for 15 min. After cooling, using preliminary TLC (Whatman) in normal saline and acetone/water (9:1) labelling of >95% was found. To this solution was added 3 ml of an aqueous normal saline/sodium citrate (M&B Pronalys, 90 mg, 0.306 mmol) solution, adjusting the pH to ca 7. The mixture was filtered through a millipore (22 micron) filter directly into a second vial under a nitrogen atmosphere. Again labelling of > 95% was found using TLC. This observed percentage labelling remained unchanged for at least a 24 h period.

3.2 In vivo biodistribution: The protocols for the animal study and eventual patient evaluation study were approved by the Ethics Committee and the Radiation Control Committee of the University of the Orange Free State.

Initially the biodistribution and radiation dose estimation of $^{186}\text{Re}(\text{Sn})\text{-HEDP}$ were evaluated in five Chacma baboons [1]. After the successful completion of this study, $^{186}\text{Re}(\text{Sn})\text{-HEDP}$ was administered to ten patients with primary prostate cancer to evaluate the palliative effect of the radiopharmaceutical. Radiation dose estimates were also determined.

Therapeutic activities of up to 2960 MBq have been used in humans by De Klerk et al. [4] as the maximally tolerated activity in patients with symptomatic bone metastases of prostate cancer. For a 70 kg patient, this value translates into 42.3 MBq/kg. In the animal study, doses ranging from 39.4 MBq/kg to 44.9 MBq/kg (mean 43.6 ± 2.8 MBq/kg) were administered to the baboons. In the subsequent clinical study, $^{186}\text{Re}\text{-HEDP}$ activities of 1295 MBq was initially administered to eight patients while two received activities of 1850 MBq.

The imaging study was performed using a dual-detector scintillation camera fitted with low energy high resolution parallel collimators. Simultaneous anterior and posterior images of the objects were obtained five minutes after administration of the radionuclide and repeated in the animal study at hourly intervals till 6 hours post-infusion. In

the patient study imaging was done at 1, 3 and 6 hours on day 1. Furthermore in both cases imaging was performed at 24, 48 and 72 hours. For the animal study, blood samples were drawn at 5, 15, 30, 45 and 60 minutes and hourly till 6 hours as well as at 24, 48 and 72 hours.

In order to evaluate the uptake of $^{186}\text{Re}(\text{Sn})\text{-HEDP}$ in bone, whole body imaging of the baboons with $^{99\text{m}}\text{Tc}$ methylene diphosphonate (MDP) was performed one week prior to the $^{186}\text{Re}(\text{Sn})\text{-HEDP}$ study.

3.3 Biokinetic modelling and dosimetry: As result of the whole body distribution of the $^{186}\text{Re}(\text{Sn})\text{-HEDP}$ it was decided to use the bone, soft tissue, kidneys and urinary bladder as source organs. The anterior and posterior images were analysed separately and the corresponding counts from the different source organs were obtained as follows.

(i) Whole body counts: Regions-of-interest (ROIs) were drawn which included the total body on each of the whole body images obtained during day 1. The whole body counts (WBC) per pixel were then obtained for each image and a mean WBC per pixel calculated for day 1. The mean number of pixels for the whole body was furthermore calculated and by multiplying this with the mean WBC per pixel, the total WBC for day 1 could be obtained.

(ii) Soft tissue counts: A ROI was drawn over a representative area of the upper thigh which clearly contained only soft tissue using one of the early images of day 1. Counts per pixel was obtained and multiplied with the number of pixels for the whole body to obtain the soft tissue counts.

(iii) Kidney counts: ROIs were drawn over both kidneys and the mean counts from both kidneys calculated.

(iv) Urinary bladder counts: A ROI was drawn over the urinary bladder on all the day 1 images and the total counts obtained. In order to correct for counts from soft tissue overlying the urinary bladder, the soft tissue counts per pixel was multiplied by the number of pixels for the urinary bladder and subtracted from the total urinary bladder counts. A similar correction was made for the contribution of counts from the bone, a ROI was drawn over a representative area containing bone and used to eventually obtain the corrected total urinary bladder counts. Urinary bladder counts for images at 24, 48 and 72 hours were obtained as follows. The whole body counts on the 24 hour image were decay corrected and subtracted from the mean whole body counts of day 1. This difference gives the amount of activity excreted in the urine during the first 24 hours. Similarly, the amount excreted during the subsequent imaging periods could also be obtained.

(v) Bone counts: Bone counts were obtained from the images acquired during day 1. This was done by subtracting the urinary bladder, soft tissue and kidney counts from the whole body counts in each image. For the later studies, minimal activity remained in the other three source organs and therefore ROIs were drawn around the bone which could clearly be recognised.

Table I: Radiation dose estimates for $^{186}\text{Re}(\text{Sn})\text{-HEDP}$

Target Organ	Absorbed Dose	Absorbed Dose
	(mGy MBq^{-1})	(mGy MBq^{-1})
	Animal study	Human study
Bone Surfaces	1.69	1.06
Kidneys	0.09	0.27
Liver	0.04	0.07
Red Marrow	0.75	0.52
Urinary Bladder Wall	0.43	0.55
Total Body	0.12	0.12
Effective dose (mSv MBq^{-1})	0.17	0.16

The above mentioned procedure was performed on the anterior and posterior images and the geometric mean counts were calculated for each source organ and expressed as a percentage of the whole body activity.

The biokinetics of the $^{186}\text{Re}(\text{Sn})\text{-HEDP}$ were determined using a multi-compartmental model consisting of five compartments representing the four source organs as well as a blood compartment. The simulation analysis and modelling was performed with the SAAM30 software (Washington State University) yielding the mean residence times of the radionuclide in the different source organs. These data were then used to calculate radiation dose estimates for $^{186}\text{Re}(\text{Sn})\text{-HEDP}$ using the MIRDOSE 3 software (Oak Ridge) which incorporated the dynamic bladder model. This model assumes that the bladder is voided every three hours during the first twelve hours after administration of the radiopharmaceutical.

3.4 Toxicity: Radioactive toxicity was evaluated during the animal study in each baboon. Blood samples obtained before radionuclide administration and at weekly intervals for 6 weeks were analysed for platelet count, neutrophils, lymphocytes, s-creatinine, s-urea, S-ALP, S-GGT, S-ALT and S-LD. No significant decreases in any haematologic parameter nor significant increase in kidney and liver function parameters were observed which indicated the safety for human use.

4. RESULTS

4.1 $^{186}\text{Re}(\text{Sn})\text{-HEDP}$ labelling: $^{186}\text{Re}(\text{Sn})\text{-HEDP}$ was prepared yielding labelling efficiencies of higher than 95% in all cases. The radiopharmaceutical was sterile and pyrogen free.

4.2 In vivo biodistribution: Whole body images of the baboons indicated a satisfactory uptake of $^{186}\text{Re}(\text{Sn})\text{-HEDP}$ in the bone in comparison to $^{99\text{m}}\text{Tc}$ MDP. Areas of metastatic uptake were clearly demonstrated on $^{186}\text{Re}(\text{Sn})\text{-HEDP}$ whole body images in patients.

4.3 Dosimetry: The absorbed radiation dose estimates for $^{186}\text{Re}(\text{Sn})\text{-HEDP}$ in the baboons and patients are given in Table 1. $^{186}\text{Re}(\text{Sn})\text{-HEDP}$ yielded an effective dose of 0.17 mSv/MBq for the animal study and 0.16 mSv/MBq for the patient study.

4.4 Palliative effect: The palliative effect of $^{186}\text{Re}(\text{Sn})\text{-HEDP}$ was evaluated in the patients using a three point scale according to the World Health Organisation. Seven patients reported positive palliation but in most cases the pain levels increased after 8 weeks.

5. DISCUSSION

This study showed that $^{186}\text{Re}(\text{Sn})\text{-HEDP}$ was successfully prepared and investigated in an animal model and subsequently used in patients. Absorbed radiation doses were calculated through the implementation of a multi-compartmental model to simulate the biokinetics and the MIRDOSE 3 programme. In the initial animal study, dose estimates for the bone surfaces 1.69 mGy/MBq, red marrow 0.75 mGy/MBq and urinary bladder wall 0.43 mGy/MBq supported the results reported by Stabin et al. [5] calculated from human data i.e. 1.9 mGy/MBq, 0.82 mGy/MBq and 0.57 mGy/MBq for the respective target organs. In the preliminary patient study, the corresponding dose estimates were 1.06 mGy/MBq, 0.52 mGy/MBq and 0.55 mGy/MBq. In other human studies Maxon et al. [6] calculated doses to target organs other than skeleton and bone marrow with the MIRD method. Mean doses very similar to those obtained in our patient study (values in brackets) i.e. kidneys 1.11 mGy/MBq (0.27 mGy/MBq), bladder wall 0.49 mGy/MBq (0.55 mGy/MBq) were reported. For the skeleton and bone marrow a “fixed” and a “variable” model were used yielding dose values of 0.79 mGy/MBq (fixed), 0.86 mGy/MBq (variable) and 0.62

mGy/MBq (fixed), 0.76 mGy/MBq (variable) for the skeleton and red marrow respectively, compared to 1.06 mGy/MBq to the bone surfaces and 0.52 mGy/MBq to the red marrow obtained in our patient study.

Radiation doses delivered with the locally produced $^{186}\text{Re}(\text{Sn})\text{-HEDP}$ corresponded well to those reported in the literature. The study also indicated that this radiopharmaceutical was suitable for clinical use and positive palliation of pain was demonstrated in an preliminary study of patients with primary prostate cancer.

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GRP, TRAINING AND REGULATORY AFFAIRS

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INVITED LECTURE

TEACHING AND RESEARCH IN RADIOPHARMACEUTICALS

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Abstract

Radiopharmaceuticals comprise a critical element of diagnostic and therapeutic clinical nuclear medicine. As well they contribute to more basic pre-clinical and clinical diagnostic studies such as the evaluation of new drugs and new drug formulations. Their development and utilization is based on the complex interaction of a number of disciplines including medicine, pharmacy, biochemistry, pharmacology, chemistry, physics and engineering. This technically-complex multidisciplinary base has impeded the development of a uniform curriculum of training for basic scientists and professionals who work with radiopharmaceuticals. The range of technical knowledge required is very broad; it ranges from chemical synthesis and radiolabelling, through a maze of biochemistry, pharmacology and now molecular biology, to GMP manufacture, dispensing and clinical consultation concerning use and interpretation of data.

Clearly, no single discipline can (nor should) be expected to undertake in-depth training of radiopharmaceutical scientists, but equally clearly, there is need for the development of curricula that will develop specific components of the essential knowledge base. The 'radiopharmaceutical' or 'product' orientation of both teaching and research can be used to provide a focus for academic and professional organizations to develop 'radiopharmacy' curricula that effectively train radiopharmaceutical practitioners for specific roles within the clinical, academic, government and industrial interests of radiopharmaceutical scientists.

Currently, there is a plethora of segmented training programs, many of which are inadequately positioned to be of great value to the field or its practitioners. Efforts to re-focus radiopharmacy programs and to build professional recognition for them are bringing about harmonization of performance objectives, and leading to didactic and experiential curricula. The impact and evolution of regulatory processes will demand new and better radiopharmaceuticals and at the same time, contribute to the professional recognition of radiopharmaceutical scientists through the implementation of clearly defined training strategies.

RADIOPHARMACEUTICALS AND RADIOPHARMACY

The terms 'radiopharmaceuticals' and 'radiopharmacy' may be considered to be synonymous in many respects, but in fact the former describes a class of medicinal agents, whereas radiopharmacy refers to a specialization of pharmacy practice. Indeed, from the radiopharmacy perspective, radiopharmaceuticals comprise a subsection of radiopharmacy practice. Increasingly in pharmacy terms, 'radiopharmacy' and 'nuclear pharmacy' have become interchangeable practice descriptors, the latter term paralleling the usage of 'nuclear medicine'.

Although one may expect that ‘radiopharmaceuticals’ are a subset of ‘pharmaceuticals’, and that with the addition of radiation considerations they would be equal in the eyes of professional and regulatory bodies, this is not necessarily the case. The qualifications of personnel dispensing radiopharmaceuticals, the requirements for product registration and even the site conditions for production may not only differ between the two, but the regulatory bodies (or divisions thereof) that are responsible for these matters may not be the same. For example, in Canada there is no requirement for an individual who dispenses radiopharmaceuticals to either be a pharmacist nor to work under the authority of one, whereas this is a requirement for one who is dispensing pharmaceuticals. Similarly, registration of radiopharmaceuticals, although handled by the Health Protection Branch (HPB) of Health Canada [1], is regulated by a different division than prescription drugs. Furthermore, regulatory (e.g. Alberta Pharmaceutical Association [2]) and professional associations (e.g. Canadian Pharmacists Association [3]) in Canada currently do not exert any influence on the practice of radiopharmacy or on the regulatory aspects governing radiopharmaceuticals. These situations are found, to greater or lesser degree, in many countries.

RESOURCES FOR RADIOPHARMACEUTICAL TEACHING AND RESEARCH

Training Programs

There are a number of approaches to provide training in the design, preparation and use of radiopharmaceuticals. Historically, as is the case in most disciplines, training was a matter of self-education that was based on, and limited by, the fundamental knowledge and insight (vision) of the practitioner, and the resources available. The earliest radiopharmaceutical practitioners were most likely to be physicians and physicists, with pharmacists, chemists and biochemical pharmacologists appearing as the field matured. This rich human resource required the development of professional training in order to gain recognition and to sustain itself.

Two medical groups, the physicians and the pharmacists, which both enjoyed legal recognition of their roles in professional health care delivery were obvious front-runners in the bid to develop recognized training and specialization programs. Medicine established their specialization quickly (e.g. Society of Nuclear Medicine [4]), whereas pharmacy faltered at the professional level, having built strong academic programs in the 1970’s but failing, in most regions, to build a professional practice base. Even today, the Nuclear Pharmacy specialization of the American Pharmaceutical Association [5] stands as one of the few exceptions to this failure to build a professional base for radiopharmacy practice. As a result, there are still several avenues for a non-medical practitioner in radiopharmaceutical science to enter the field (Table 1).

Table 1. Approaches to Training Radiopharmaceutical Practitioners

- On the job
- Academic (University)
- Professional qualification (Society)
- Residency (Society; University)

Academic Training Programs

It is natural that individuals currently involved in radiopharmacy and the radiopharmaceutical sciences, most of whom are University-educated, look to Universities as primary sites to meet their teaching and research needs. Where they look within the University, however, frequently depends on their own background and experience, rather than on a recognized program (consider that physicians would only look to Medicine for training in nuclear medicine). Within the Universities there are a number of established disciplines that are involved in teaching elements of what one might consider to be basic elements of the field (Table 2). In some departments this information is presented in isolation of the radiopharmaceutical sciences, whereas in others, it is presented as part of integrated programs in nuclear medicine or radiopharmacy.

Table 2. Academic Units that may provide Training in one or more Disciplines of the Radiopharmaceutical Sciences

- Pharmacy
 - Nuclear Medicine
 - Radiology
 - Chemistry
 - Medicinal Chemistry
 - Pharmacology
 - (Bio)physics
 - (Biomedical) engineering
 - Biology
-

To an outsider, the role of schools of Pharmacy in the teaching of radiopharmacy and the radiopharmaceutical sciences would appear self-evident, although it is clear to those already in the field that these natural links between pharmacy and radiopharmacy may be weak or non-existent in some geographic regions. None-the-less, a pharmacy perspective will be emphasized in this monograph. The material presented in the following sections is intended to be representative, not comprehensive, in either the nature or scope of the programs currently being offered around the world.

The science and medicine of radiopharmaceuticals involves many disciplines. Indeed, although this could potentially be said for other medical fields, it is a fact for everyday nuclear medicine practice. Therefore, the diversity of teaching sources is not only to be anticipated, but a necessary element in teaching and research in radiopharmaceuticals which are equally multidisciplinary in nature.

Teaching Radiopharmacy in Schools of Pharmacy.

Several schools of Pharmacy have, or have had, strong didactic programs in radiopharmacy. The Nuclear Pharmacy program at Purdue University in the USA is one of the original and most durable programs typical of radiopharmacy training by schools of Pharmacy. They have maintained the teaching of basic knowledge and concepts of nuclear pharmacy through an integrated undergraduate education. The radiopharmacy programs offered by many other Pharmacy schools in North America in the late 1960's and 1970's (e.g. Alberta, California) and

in the UK (Chelsea) appear to have diminished in impact within their Universities, although aspects of their programs may continue to flourish [6]. In other geographic regions (e.g. Japan, Australia, Europe), radiopharmacy (research) has become more prominent within pharmacy programs. University-based pharmacy programs which provide training in radiopharmacy and/or in radiopharmaceutical research utilize a range of academic platforms to this end (Table 3).

Table 3. Radiopharmacy-Related Academic Degrees and Certificates offered by Schools of Pharmacy

-
- undergraduate: B.Sc.(Pharm); Pharm. D.
 - post-graduate professional: M. Pharm.; diploma
 - post-graduate research: M. Sc.; Ph. D.
-

New emphasis in the Purdue undergraduate program reflects a growing global change in pharmacy education: the provision of clinical services and pharmaceutical care. In the Purdue pharmacy curriculum there is instruction in the broader subject of radiology in order to promote nuclear pharmacy roles and to emphasize the unique aspects of nuclear medicine in diagnosis and therapy relative to the competing technologies. Purdue, like other pharmacy schools in the USA, is moving rapidly towards the Pharm. D. program as the practice entry qualification and is phasing out the baccalaureate degree in Pharmacy. The Pharm. D. degree programs include a much larger component of experiential training than the baccalaureate programs, and Purdue, for example, has established an advanced clerkship in nuclear medicine for students in the final professional year in pharmacy. This nuclear medicine clerkship is designed to aid the undergraduate pharmacy student in relating to the patient and to the nuclear medicine technologist, in order to promote more clinical service rather than to promote only the clinical product viz. the radiopharmaceutical.[7-9]

Other Pharmacy programs in North America, where the Doctor of Pharmacy (Pharm. D.) degree will become, or already is the predominant practice-entry degree for Pharmacy, are adopting similar or somewhat modified approaches. At Alberta, for example, a parallel-track, non-practice baccalaureate degree in 'pharmaceutical sciences' is being developed. In this program, which will share some of the didactic curriculum of the Pharm. D. program [10], a series of specialties, including radiopharmacy, will be offered in order to provide in-depth scientific training for specialties like radiopharmacy in which practice is not regulated by a professional or licensing body. Unlike the American counterpart, nuclear pharmacy in Canada is not recognized as a professional specialty by any main-stream pharmaceutical organization. In fact, the Canadian Association of Pharmaceutical Scientists (CARS), a body comprising pharmacists, chemists, physicists and others is the only 'radiopharmacy' voice in Canada [11]. Although CARS deals effectively with regulatory agencies on behalf of its members, it does this directly and through nuclear medicine organizations, and not through the Canadian Pharmacists Association, the Canadian Association of Hospital Pharmacists or, in curriculum matters, the Association of Faculties of Pharmacy of Canada. The role of the newly formed Canadian of Pharmaceutical Sciences (CSPS)[12] could potentially include a voice for radiopharmacy, but this avenue remains undeveloped at the present time.

Although a number of Pharmacy schools are promoting their radiopharmacy courses via the Internet (e.g. 9,13,14], Purdue may be the first to have developed a distance learning program to

aid pharmacists that wish to learn the nuclear pharmacy basics after graduation from the practice-entry pharmacy degree program distance. Such certificate programs are likely to have broad appeal to informally qualified radiopharmaceutical practitioners already in the field, as well as to pharmacists, especially hospital pharmacists, who are legislatively compelled to assume responsibility for radiopharmaceuticals used within their institutions.

Non-Pharmacy Teaching of the Radiopharmaceutical Science.

As shown in Table 2, many aspects of radiopharmacy training are also taught in science and engineering departments. cursory examination of programs listed on the Internet suggest that in fact, more 'radiopharmaceuticals' training is offered through nuclear medicine and other programs than by pharmacy schools [e.g. 15-21]. Part of this subject material will be taught in introductory courses in physics (e.g. nuclear structure, nuclear activation reactions, interactions of ionizing radiation with matter) and chemistry (e.g. radiochemistry, radiolabelling chemistry, chemical kinetics, radiometry, chromatography). Other basic aspects are more likely to be found in second-tier courses such as biochemistry and pharmacology (metabolism and excretion, molecular targets, pharmacokinetics) and in engineering (kinetics, process control, materials, targetry). The medical subjects can be found in medical programs, especially in Radiology and Nuclear Medicine (biological effects of ionizing radiation, radiation dosimetry). Medical applications are taught not only in these 'radio-medical' departments, but also in medical specialty units such as cardiology, pulmonary medicine, internal medicine and oncology.

STRATIFICATION OF RADIOPHARMACEUTICAL TEACHING AND RESEARCH

Radiopharmaceutical sciences are taught through a number of academic departments, of which schools of Pharmacy probably offer the most comprehensive curriculum. Interestingly, the nature of teaching radiopharmaceutical science in non-pharmacy-based units is frequently biased by the research and/or clinical expertise of that unit. These specialization's are based not only on the medical specialty (e.g. cardiology) but also on features of the associated technology (Table 4).

Table 4. Medical Specialties and Technologies that Influence Teaching and Research in the Pharmaceutical Sciences

-
- - Diagnostic imaging
 - Radionuclide-based radiotherapy
 - Imaging technique (e.g. planar scintigraphy, SPE(C)T; PET)
 - Radionuclide source (e.g. generator, cyclotron)
 - Labelling techniques (e.g. shake & shoot, chemical synthesis)
 - Disease / target tissue (e.g. cancer, cardiology)
-

Greatly improved didactic and experiential radiopharmaceutical science training programs have been developed in recent years. This is due in large part to the individual efforts of a few radiopharmaceutical scientists who are active in either clinical departments or academic units. These efforts have been motivated by both the perceived need for high(er) practice standards and increasing regulatory constraint. Clearly there is a need for the development of standards for training, and these appear to be under regional development [22,23]. The result of these efforts has not only been to broaden the scope of 'radiopharmaceutical' curricula, but to provide an unprecedented degree of international harmonization of training programs [24].

International harmonization will not be addressed further in this monograph, except to say that the radiopharmaceutical community already owes a substantial degree of appreciation to a number of individuals, including Peter Cox, Knud Kristensen, David Laven, Steve Mather, Gerd Meyer and Stan Shaw, for their commitment and hard work to this end.

INFORMATION SOURCES FOR RADIOPHARMACEUTICAL TEACHING AND RESEARCH

It is both desirable and necessary to develop information data bases that will satisfy the needs of the potential student, the regulators, the current practitioners and the curious onlooker. Information is available from a number of sources, none of which is universally available to all who want details of the field. The most common information resources are presented in Table 5. Personal contacts are invaluable sources for the insider, but they may be virtually non-existent for the novice and the outsider. Personal bias is an integral part of the information that is obtained in this way, but this is frequently an advantage, especially in the selection of teaching sources, since official documents seldom convey 'rankings' or other information that may be accurate but critical in nature.

Journals are likely to be excellent sources of up to date information on research and product evaluation, but articles on professional practice and training are harder to find. Several excellent series [25] and monographs [26,27] that address both teaching material and research reviews are available.

Table 5. Commonly used Sources of Information about Radiopharmaceutical Teaching and Research.

- Personal contacts (small field)
 - Journals (pedagogical, professional, scientific)
 - Books and monographs
 - Institutions (hospital, university, regulatory agency)
 - Industry
 - Internet
-

The industry is generally an unlikely source of information on training programs, at least of programs that are not intended to promote their own commercial interests. The programs offered, of course, may be of vital importance to the function of the individual, for example, as in new radiopharmaceuticals for new indications [28-35]. Of the Institutional sources, the best materials are available from academic departments and research units active in the field [36-40].

The Internet as an Information Resource for Teaching and Research in Radiopharmaceuticals

The *Infobahn* (information highway) has become a global household phenomenon; access is universal in many countries and available to most scientists around the world. The cost is usually not prohibitive, the size of the data base is simply unimaginable and the speed of gathering information from world-wide sources is adequate for most users. There are, unfortunately,

several limitations to the technology. The main limitation is usually found in the ‘search engine’ used to find the key words that are used for the search. Both the quantity and quality of the ‘hits’ can vary from engine to engine, as shown in Table 6.

Syntax plays an important role when using multiple word search phrases. A search using the words radiopharmaceutical chemistry, without delimiters, will usually bring up several thousand hits, many or most of which will not be useful for the intended purpose. When delimiters are used properly, however, the hits are more likely (but not guaranteed) to be accurate, and small in number.

Other limitations of the Internet data base include the biases introduced by the author of the information package loaded on the source server. For example, extraneous phrases and keywords may be used (deliberately) in order to capture additional readers. This, in fact, leads directly to the major problem with today’s Internet data bases; there are virtually no standards to be met; thus, a search brings not only extraneous information, but also hits that are unacceptably outdated, devoid of information, speculative and even deliberately misleading.

When using an Internet search, the reader must be prepared to use critical judgment. Institutional, academic and industrial sources are usually dependable, whereas personal comments of an editorial nature are the most likely to have hidden agendas.

Table 6. Search Engine Variability for Predetermined Keyword Searches

Search engine	Search word		
	Radiopharmacy	Nuclear Pharmacy	Radio- pharmaceuticals
Infoseek*	264	229	1127
Lycos	82	43	285
Webcrawler	40	22	62
Yahoo	3	2	8

* 10,777 entries for “nuclear medicine”

RADIOPHARMACEUTICALS RESEARCH

Much of what has been stated in the preceding paragraphs was directed towards the teaching of radiopharmacy and radiopharmaceutical sciences. Clearly, however, it is essentially impossible to divorce teaching from research. This is true because of the rapid cycling of research into clinical practice, so that the teaching needs for today are based on current research. The major difference may lie in the venues where the two functions take place. Whereas teaching may be primarily the prerogative of academic institutions, research is spread quite uniformly among a number of players (Table 7).

Table 7. Venues for Radiopharmaceutical Research

-
- Radio (Nuclear) pharmacies
 - Nuclear Medicine departments
 - University laboratories
 - Government research laboratories
 - (Radio)Pharma industry
-

Research in radiopharmacies, nuclear medicine departments and the radiopharmaceutical industry laboratories is frequently product/process oriented. That is to say, these groups are interested in the development of a new radiopharmaceutical or image enhancement process, or to develop pragmatic improvements in existing products. In practice there is often good rapport among these organizations, to take advantage of the unique resources of each participating member. Resources of the industry include materials, data bases and money, whereas the medical institutions have medical knowledge, practical radiopharmaceutical knowledge and perhaps most importantly, access to patients. Shared intellectual skills may or may not be part of these arrangements.

Research in academic and government laboratories was traditionally oriented towards the generation of knowledge and less towards exploitable intellectual property. This has changed drastically, even in the most conservative institutions, in the past two decades. Although basic research is still being undertaken in all venues (Table 7), academic and government interaction with industry is a prominent feature today.

Within all research environments there is a range of projects that address the development of new diagnostic and therapeutic radiopharmaceuticals. Examples from groups in Canada include:

- development of new therapeutic radionuclide sources at TRIUMF (a national laboratory)
- radiopharmaceuticals for clinical neuro-PET and FDG for oncology; and SPECT; at TRIUMF, University of British Columbia (Pharmaceutical Sciences; Medicine) and Vancouver General Hospital
- radiopharmaceuticals for imaging in gene therapy at the University of Alberta (The Noujaim Institute, an academic research institute in the Faculty of Pharmacy and Pharmaceutical Sciences) and the Alberta Cancer Board [43].
- radiopharmaceuticals for melanoma imaging at the Edmonton radiopharmaceutical Centre (a not for profit radiopharmacy operating through the Alberta Cancer Board).
- nuclear medicine applications for 'neural net' technology at the University of Alberta (Faculty of Pharmacy and Pharmaceutical Sciences)
- assessment of hypoxia-sensitive diagnostic radiopharmaceuticals at the Ontario Cancer Centre and the University of Alberta
- assessment of diagnostic and therapeutic monoclonal antibodies at several institutions, including the Royal University Hospital and the Saskatchewan Cancer Board.
- design and synthesis of novel ^{99m}Tc radiopharmaceuticals, and solid-phase radioiodination precursors, at the University of Western Ontario (Department of Chemistry and Faculty of Medicine)
- neuroPET (^{18}F and ^{11}C) diagnostics at the Clark Institute, University of Toronto, and the Montreal Neurological Institute.
- although laboratories of governmental agencies at the provincial level are active in radiopharmaceutical research, particularly in the area of oncology, TRIUMF is the only

national laboratory where radiopharmaceutical research is undertaken; the laboratories of Atomic Energy Laboratories of Canada (AECL) are no longer active in this field, as a result of the privatization (Nordion [32]) of their isotope interests a decade ago. The Atomic Energy Control Board, the nuclear regulatory body in Canada, divested itself of (potential conflicts of) interest over 2 decades ago, and the Health Protection Branch (the radiopharmaceutical regulatory body) similarly has no research program.

- with few exceptions, the radiopharmaceutical industry in Canada has been that of multinational subsidiaries, and as such virtually no research has been undertaken and little has been sponsored by this important group. The most notable exceptions are Draxis [31] (formerly Frosst Radiopharmaceuticals, a division of Merck, Canada) and Nordion [32]. The former has concentrated on marketing licensed radiopharmaceuticals (including generators), with a long history of sponsoring collaborative research with University and clinical partners, while Nordion has concentrated primarily on the production and marketing of bulk radiotracers (^{99m}Tc and the radioiodines) for industrial end-users.

Clearly, a detailed review of Canadian, let alone international radiopharmaceutical research efforts lies well beyond the scope of this paper. The examples given above undoubtedly have their counterparts in many countries, and certainly there are projects and approaches elsewhere that are not identified in this brief review.

SUMMARY

The clinical practice of nuclear medicine is based on the complex interaction of a number of disciplines including medicine, pharmacy, biochemistry, pharmacology, chemistry, physics and engineering. Clinical nuclear medicine relies entirely on the radiopharmaceutical, which can be either therapeutic or diagnostic. By extension, the development and utilization of radiopharmaceuticals has an equally complex technical base, and it is therefore understandable that neither research nor teaching in the radiopharmaceutical sciences can lie in the province of a single discipline. It is equally apparent that the 'product' orientation of both teaching and research ensures that effective action comes about through the cooperative interaction of clinical, academic, government and industrial scientists. The development of regulatory process will continue to exert impact on the clinical realization of new and better radiopharmaceuticals and on the professional recognition of radiopharmaceutical scientists.

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Abstract

Production, control, distribution and clinical application of radiopharmaceuticals have not been subject to the same regulations and legislation developed for conventional pharmaceutical products. Generally the production of radiopharmaceuticals have started up at national nuclear energy research centres, where the products have been regarded more as radioactive tracers than pharmaceutical products. Gradually pharmaceutical philosophy and requirements have been introduced in the field and necessary changes in legislation have been introduced. The registration process to obtain official market authorisation for a radiopharmaceutical have been introduced in most countries. This process of registration is constantly changing with regards to the amount and the type of documentation required by the health authorities. Correspondingly the general requirements for the systems applied in pharmacy for the production, control, quality assurance and medical application have also become an integral part of the field of radiopharmacy. Is the present situation generally satisfactory? The ever increasing demands leads to increasing costs and thereby higher prices on the products for the end users. Do the present requirements generally ensure that the radiopharmaceuticals applied are safe and effective for the patient?

Radiopharmaceuticals have a short history compared to other drugs and medicinal products. The widespread use of radioactive nuclides for medical applications came as a direct result of the development of the atomic bomb during the Second World War. The construction of nuclear reactors in this context, opened up for the possibility of producing a whole range of new radionuclides by neutron activation of non-radioactive targets. Radionuclides that were not found in nature, could now be produced artificially in quantities that made it possible for use in scientific and medical applications.

Production, control, distribution and clinical application of radiopharmaceuticals have not been subject to the same regulations and legislation developed for conventional pharmaceutical products. The first important radiopharmaceutical product in routine clinical practice was Iodine-I-131, used for diagnosis and therapy of disorders of the thyroid gland. This was also the first true specific targeting radiopharmaceutical, and the spectacular success obtained in treatment of thyroid cancer brought the product and the practice of the new medical speciality nuclear medicine to the front page of newspapers and magazines. It was anticipated that new «Magic bullets» like the iodine-131 solution, which got the nickname: «The Atomic Cocktail», would be developed quickly and the diagnosis and cure for many other types of cancer would be obtained by using these radiopharmaceuticals. Time has

shown that it was not so easy as expected then and it is first in the last decade that a variety of new targeting radiopharmaceuticals have been introduced in routine clinical practice.

However the way radiopharmaceuticals were developed and introduced to the users were completely different from what was typical for conventional pharmaceutical products. The radioisotopes incorporated in the products were produced in reactors belonging to national nuclear research centres and institutes. Many regarded this production as a spin off of the general activities of the centres and as these reactors were built and operated with public funds, the radionuclides should be supplied to the public free of charge or at a very low cost. Therefore the radiopharmaceuticals developed during the first three, four decades of the new era were not priced according to the real costs of production of the radioisotope or the radiopharmaceutical. Furthermore it became prestigious for many countries to build and operate their own nuclear reactor. One of the ways to get political support and economic aid for such projects was the argument that the reactor also could be used for the production of radionuclides used in radiopharmaceuticals. This type of argument is one of the main reasons that we today find a widespread local production of radiopharmaceuticals all over the world, including many developing countries. However this hidden economic subsidy lead to the general expectation that radiopharmaceuticals should have a low price structure. This is clearly evident when the price of a multi dose vial of a generic technetium-kit is compared to the cost of a single dose vial with x-ray contrast media used in radiology. This tradition makes it very difficult to raise the price of the older radiopharmaceuticals to the correct level based on actual costs. This is an absolutely necessary requirement, now when many countries have privatised the production of radiopharmaceuticals

1. Good Radiopharmacy Practice

Generally the production of radiopharmaceuticals started up at national nuclear energy research centres and establishments. When looking at the major suppliers of radiopharmaceuticals in Europe, they all have their origin in such national research centres. However in these nuclear centres the products were more regarded as radioactive tracers than pharmaceutical products, and this had a had a major influence on the systems instituted for production, distribution and control of these medicinal products. Because the fields of nuclear science and pharmacy are far apart, the guidelines for Good Manufacturing Practice (GMP)

that were first introduced more than 30 years ago in conventional pharmaceutical practice, did not have any immediate impact on the general production of radiopharmaceuticals.

The first time the phrase «**Good Radiopharmaceutical Practice**» was coined, was around 1970, when a group of Scandinavian radiopharmacists came together to study how the GMP philosophy and guidelines could be applied within the field of radiopharmacy. The Good Radiopharmaceutical Practice Code was defined to comprise the application of the GMP guidelines for pharmaceuticals with the relevant guidelines for radiation protection during production, distribution and hospital handling of radiopharmaceuticals to ensure the safety and efficacy of the product administered to the patient. In the beginning the discussions put much emphasis on how different radiopharmaceuticals were from non-radioactive medicinal products, and why it was important to make special legislation and requirements for these drugs. But over the years the discussions lead to the realisation that radiopharmaceuticals could not be considered as something extraordinary, and that the same quality standards would have to apply for these products as for conventional pharmaceuticals. However the radiation protection aspects make the situation more complicated, as personnel involved with the production, transport and preparation and administration in the hospitals as well as the environment has to be protected against the products.

So what makes radiopharmaceuticals that different from non-radioactive drugs?

1. The preparation of the radiopharmaceuticals in its final form does most often take place in the hospital department immediately prior to the administration to the patient.
2. Radiopharmaceuticals have a very limited period of use. (Short shelf-life)
3. Radiopharmaceuticals are frequently shipped directly from the manufacturer to the end-user without any formal control in a pharmacy or hospital pharmacy
4. A quality control must often be performed in the hospital upon the preparation of the radiopharmaceutical in its final form.
5. Manufacture, distribution and use of radiopharmaceuticals require special expertise in handling of radioactive materials

Gradually general pharmaceutical philosophy and requirements have been introduced in the field of radiopharmacy and necessary changes in legislation have followed. As a basis for the elaboration of such changes, it was necessary to characterise the various types of products most commonly used:

- a) Ready-for-use radiopharmaceuticals, which contains radionuclides with a sufficiently long half-life to allow the product to be distributed in its finished form from the manufacturer
- b) Radiopharmaceuticals prepared from semi-manufactured products. The use of radionuclide generator systems and preparation kits allows the preparation of radiopharmaceuticals with a relative short half-life in the hospital department.
- c) Radiopharmaceuticals produced directly prior to patient administration
For radiopharmaceuticals containing radionuclides with ultra-short half-lives it may be necessary to both produce the radionuclide and prepare the radiopharmaceutical close to the patient for immediate administration. (PET radiopharmaceuticals are typical products of this group)
- d) Radiopharmaceuticals based on biological material from the patient. Cells or plasma protein fractions from a patient may be labelled with a radionuclide and re-injected in the same patient.

In the discussions on Good Radiopharmaceutical Practice the complexity of the handling procedures for these products in the hospital environment increases from the first to the fourth of these categories.

The practice of radiopharmacy in the hospitals can also be classified according to the various types of work that is performed:

- a) Dispensing of individual dosis from ready-to-use radiopharmaceuticals
- b) Preparation of radiopharmaceuticals from radionuclide generators and preparation kits
Preparation is carried out in accordance to manufacturer's instructions and the finished pharmaceuticals is only intended for use within the department.
- c) Centralised in-hospital dispensing and preparation
Dispensing and preparation of radiopharmaceuticals as described under a and b for use in other departments within or outside the hospital

d) Production of radiopharmaceuticals

Production and handling of radiopharmaceuticals not covered by a, b and c above. Included is the preparation and labelling of biological material from the patient.

How can all these requirements be taken care of without making impossible restrictions affecting the daily routines in the nuclear medicine departments? In most countries the radiation protection aspect is taken care of by the licensing of the premises by the national boards of radiation, which would define the radionuclides and quantities allowed in each particular case. The pharmaceutical aspects could be covered by inspections by the general medicinal inspectorate. But in many countries the legislation demands that a pharmacist should be registered as the qualified person for the radiopharmaceuticals and take the responsibility of the handling, production and preparation in the hospital. The authorities in Denmark have established an interesting system, that may serve as a model for other countries. Each nuclear medicine department is visited by a team of two inspectors/advisors. One of the members of the team is a representative from the National Institute of Radiation Hygiene while the other is a radiopharmacist from the national Isotope-pharmacy, which is a laboratory belonging to the national board of health. The inspectors review the premises and equipment available and the qualifications and training of the personnel. Based on their findings each department is given a licence describing the type of work they are authorised to perform taking into consideration all the above mentioned factors. The classification process is the most important task for the team, but they are also welcomed in the departments as advisors on practical topics, such as design of the various working areas, application of working techniques, local radiation protection issues etc.

2. Regulations and legislation

For radiopharmaceuticals the legal requirement for marketing authorisations or an official registration of the products came late. Some countries did include the new products under the general legislation for medicinal products from the start. But the introduction of special requirements on distribution and sale of the products often made a direct link between radiopharmaceuticals and the national nuclear research centres.

The first formal systems for obtaining market authorisation for radiopharmaceuticals were established in the USA, United Kingdom and Denmark. It was obvious that the requirements

used for non-radioactive drugs could not be applied directly to the radiopharmaceuticals. Several special characteristics of the radiopharmaceuticals had to be taken into consideration. The drugs were then mostly used for medical diagnosis. They were usually given to the patient only once or occasionally a few times and they contained only very small physical quantities of carrier substance and radionuclide. In general most requirements concerning documentation of the safety and efficacy of a non-radioactive drug would also apply to radiopharmaceuticals. But it must be recognised that most radiopharmaceuticals do not have a measurable pharmacodynamic effect. In addition to the general requirements it is also necessary to include staff and patient radiation protection aspects and patient dosimetry parameters in the documentation. Certain points therefore did differ considerably from the documentation of non-radioactive drugs:

- a) Radiopharmaceuticals have a changing composition with time due to the radioactive decay of the radionuclide. The radioactive concentration and the specific activity of the product would therefore change all the time from the production day to expiry date.
- b) For certain radiopharmaceuticals containing ultra-short lived radionuclides it would be impossible to perform quality control of the product before administration to the patient. For example radionuclide generators and cyclotrons may be used for production of an ultra-short lived radioactive gas which is inhaled directly after production by the patient. All quality control would have to be performed retrospectively and the quality assurance program would concentrate on the reproducibility of the chosen production process to provide a final radiopharmaceutical fulfilling all quality specifications.
- c) The labelling of radiopharmaceuticals do not follow the same pattern as for non-radioactive drug. The label on the inner container contains only basic information, as this label is not supposed to be studied in detail by the user. Most of the relevant information is contained on the label attached to the lead-shielding surrounding the inner container and in the packaging insert.
- d) The extent of toxicology studies would depend and the intended clinical use of the radiopharmaceutical. Part of the studies should be performed on the non-radioactive form of the radiopharmaceutical. Studies of chronic toxicity would only be required in special cases. If studies of mutagenic, oncogenic or carcinogenic potential are required, they should be performed using the non-radioactive form of the new chemical entity.

Radioactivity is in itself an important risk factor for the introduction of such biological changes.

e) Clinical documentation would normally be more limited than for non-radioactive drugs.

The administration of radioactivity to a patient would always be subject to a risk/benefit evaluation. No application of a radiopharmaceutical should take place if the benefit of a better diagnosis, better treatment etc., outweighs any risk induced by the radiation dose and other properties of the product. To give radioactive substances to healthy volunteers would have to be subject to ethical evaluation in each case. It would therefore not be ethical to perform large, comparative studies of radiopharmaceuticals with other diagnostic or therapeutic tools.

Now most countries have established national registration procedures to obtain official market authorisation for a radiopharmaceutical. But less than a decade ago many European countries did not have a drug legislation that did also cover radiopharmaceuticals. Several of these countries did finally get their legislation for these products as part of the directives on radiopharmaceuticals issued to the member-states of the European Union.

3. Status today

The procedures for obtaining market authorisation is a dynamic process, and the requirements set by the health authorities for documentation will change constantly over the years to come. For the manufacturers of radiopharmaceuticals it seems that there is an never ending process requesting more and more documentation, while the users in the hospital departments feel that the demands with regard to premises, equipment, education and supervision are getting stricter and stricter. It is obvious that the consequence of the situation will be higher prices for radiopharmaceuticals and increased problems in the nuclear medicine departments, which already are fighting with serious economic constraints. It is easy for authorities and regulators to introduce new demands and requirements, but these must be balanced against the benefits gained and the ultimate goal: To ensure a reliable supply of safe and effective radiopharmaceuticals at a price that is affordable for public and private health services. The standards we establish must be set with this goal in mind and must not be based on purely political or juridical motives.

4. The future

The question is therefore how to reach this common goal without using too much labour and economic resources. One evident solution must be global and regional collaboration in various fields of radiopharmacy. How can this be achieved?

a) Joint projects to elaborate manuals and guidelines for Good Radiopharmaceutical Practice.

Some do already exist! In Scandinavia the Nordic Council of Medicines formed a group of radiopharmacists that elaborated guidelines for the preparation and control of radiopharmaceuticals in the hospital environment. These guidelines have been the model for similar projects around the world. It has been realised that guidelines should be established for other areas of radiopharmacy as well, such as centralised radiopharmacies and the local, small-scale production of radiopharmaceuticals. Currently a manual is being established under the auspices of International Atomic Energy Agency covering these fields of radiopharmacy and intended for use in the region of Central and South America.

b) Collaboration and harmonisation on radiopharmaceutical monographs in the pharmacopoeias. These monographs are very important to establish the quality requirements for the products on the market. Today it is difficult to find experts to take part in the work on these monographs who have the access to laboratories that have the time and economic backing to perform the necessary laboratory work to establish and validate the various analytical methods that are to be included in the monographs. Working with radiopharmaceuticals the analytical equipment will be contaminated with radioactivity, and not many centres or companies are willing to work with this type of problem. It may therefore be of interest to look at this work on new monographs globally instead of regionally, in order to get a good scientific background for the new monographs. The harmonisation process has already been started on monographs for the Ph. Eur, the U.S.P and the Japanese Pharmacopoeia.

c) Could this work be extended to include a regional process for the granting of market authorisations? Yes, within the European Union this possibility does already exist. By applying through the «Centralised Procedure» a product could be granted a market authorisation in all the member states of the union. This type of collaboration is a very sensible idea. It must be regarded as a waste of time and money if all nations should designate personnel and money to evaluate the same data supplied as documentation in the

applications for marketing authorisation for a radiopharmaceuticals. A thorough study performed by a competent authority will be much more valuable than the study of an individual health authority with little experience and expertise to handle and evaluate applications for a radiopharmaceutical product.

- d) The question of joint projects for regional production of radiopharmaceuticals has been raised. It has become more and more evident that transport and logistics will become very important issues in the future of radiopharmacy. This year has showed clearly how difficult the transport by air will become, if firm commitments for long periods of time for shipment of radiopharmaceuticals are not granted by the various air companies. To avoid this problem the idea of setting up local production centres are getting more and more interesting. But this idea of local production does already exist because of national interests. Could this idea be of more importance by expanding it from a national interest to a regional interest? It is a very difficult topic, as nobody really wants to give up or transfer the production to another company in the region. Denmark, Norway and Sweden had such discussions 30 years ago. But the negotiations stranded as nobody at that time were ready to give up their production of ^{131}I -iodinated Hippuran! Political and professional issues will always make it difficult to make this type of arrangements. Could the establishment of commercial centralised radiopharmacies make an impact? These pharmacies are already well known in USA and are now being introduced in south-east Asia. But they are still an almost unknown entity in Europe. The practice of centralised radiopharmacy may change the way of marketing and distribution of radiopharmaceuticals in certain areas, but may also help to increase the standards of Good Radiopharmaceutical Practice and reduce the total costs in areas with the necessary population density and transport infrastructure.

THE IMPLEMENTATION OF A RADIOPHARMACY QUALITY ASSURANCE PROGRAMME IN THE LATINOAMERICAN REGION

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Abstract

During the last ten years the capacity of local production of radiopharmaceuticals in each country of the latinoamerican region has been increased because of a coordinated, joint action among them. This is a consequence of the implementation of the ARCAL XV Project "Production and Quality Control of radiopharmaceuticals". During this period attention has been focussed in the development of more defined agents for specific functional investigations such as the second generation ^{99m}Tc radiopharmaceuticals. Active research is also being continued to develop third generation radiopharmaceuticals based upon monoclonal and policlonal antibodies, peptides and other biomolecules. Group training in this field is also being carried out in this region. The enhancement of quality requirements and the globalization of the economy make it necessary to produce same quality radiopharmaceuticals and the harmonisation of the legislation and regulatory needs should be raised in a next future. The implementation of a quality assurance programme in each country of the latinoamerican region and the harmonisation of those programmes will be the principal goal of this project.

1 INTRODUCTION

Drugs, by definition, are chemical agents that work by pharmacological action within or on the body. Radiopharmaceuticals are radiochemical agents without an intended pharmacological effect. The vast majority of radiopharmaceuticals are diagnostic and exert no pharmacological effect on the body. Radiopharmaceuticals for therapeutic use exert their effect upon the body through their physical properties (radiation). Without suggesting specific language the definition of

radiopharmaceutical would have to reflect that they are used occasionally, never chronically, always administered by a physician or under the direct supervision of a physician and in a facility with the proper equipment to complete their use

Because radiopharmaceuticals are clearly different from traditional drugs, they need special regulations

2 GENERAL CONSIDERATIONS

The criteria will have to reflect that handling of radioactive material is regulated by the Nuclear authority, therefore, the issues of training workers to handle radioactive material and radiation safety for workers, the public and the patients are also carefully regulated. Throughout the regulations applicable to radiopharmaceuticals, the specific for licensing, manufacturing, compounding and quality control, as well as the inspectional processes, should be set out separately from traditional drugs

Expertises in radiopharmacy field of each latinoamerican country would be an appropriate body to write the regulatory standards for radiopharmaceuticals. This body would have to promote the harmonization of the legislation and administrative procedures relating to radiopharmaceuticals. The activities will focussed on legislation and guidelines for the drug regulatory authorities. Guidelines would issued also on the registration of preparations, drug applications, evaluation reports, bioavailability studies in man, etc

The following is a list of Institutions and the name of the Project Coordinator of each country involved in the Project

Argentina

Comisión Nacional de Energía Atómica

Silvia Castiglia

Uruguay

Centro de Investigaciones Nucleares Facultad de Ciencias Cátedra de Radioquímica, Facultad de Química

Silvia Verdera

Brasil

IPEN-Instituto de Pesquisas Energéticas y Nucleares

Nilda Sosa de Pereira

Colombia

Instituto de Ciencias Nucleares y Energías Alternativas. División de Aplicaciones Técnicas.

Mercedes Mendoza de García

Costa Rica

Hospital San Juan de Dios

Martin Jimenez Cordero

Cuba

Agencia de Energía Nuclear.

Jorge Cruz

Chile

Comisión Chilena de Energía Nuclear

Mónica Chandia

Ecuador

Hospital Eugenio Espejo

Julio Urresta

Guatemala

Dirección General de Energía Nuclear

Claudia Quintero

Mexico

Hospital Nacional de Nutrición

Consuelo Murphy

Peru

Instituto Peruano de Energía Nuclear

David Carrillo

Paraguay

Instituto del Cancer y el Quemado

Raquel Candia

Venezuela

Facultad de Farmacia. Universidad Central de Venezuela

Ligia Arrechedera

3. BACKGROUND OF THE PROJECT

In 1996 under the assistance of the Agency, expertises in radiopharmacy field of each latinoamerican country have joint and written some guidelines for registration of radiopharmaceuticals. This document has been sent to the drug regulatory authorities and it was the first attempt to promote the administrative procedures relating to radiopharmaceuticals.

Last year a handbook of Quality Control Protocols has also been written and it is now in its final revision. This handbook will be distributed among the different hospital radiopharmacies of each country as recomendations for setting radiopharmaceutical standards in order to obtain products with uniform properties.

Guidelines for the preparation and control of radiopharmaceuticals in radiopharmacies have been discussed and written by a working group last year, launching the concept of Good Radiopharmaceutical Practice (GRP). These guidelines will be published this year and sent to the drug regulatory authorities.

4. WORK PLAN

It would be necessary to centralize the information concerned to the radiopharmacy practise in private or public institutions of each country. In addition coordinated actions will be established with other public institutions of different disciplines that would have implemented a quality assurance programme. All these actions will conduct to colaborar with the health authority with the legislation and regulatory needs.

The elaboration of a technical document in collaboration with different institutions and under expertises assistance will be carried out . Open channels of communication must be maintained with all the institutions, public or privates, involved in the development, production, quality control and application of radiopharmaceuticals in order to obtain a final technical document of quality assurance. The technical document will be sent to the health authority in order to contribute to the implementation of the programme. At the same time the laboratories involved in the Quality Control of radiopharmaceuticals with a solid experience should be the reference body for the health authority in order to verify the special requeriments of the radiopharmaceuticals.

Special efforts will be done in the implementation of this programme at the hospital level.

The guidelines for preparation and control of radiopharmaceuticals in hospitals will set the concept of Good Radiopharmaceutical Practice (GRP) as a new approach that takes further account of the most essential aspects of the quality of the radiopharmaceuticals.

GRP relating to the preparation and handling of radiopharmaceuticals in hospitals combines the principles of Good Manufacturing Practice (GMP) for pharmaceuticals with aspects of radiation protection. Its purpose is to safeguard the quality of radiopharmaceuticals up to the point of administration to the patient.

The preparation of the radiopharmaceuticals includes a variety types of work. Requirements regarding facilities, equipment and training personnel are therefore dependent on the type of work to be done .It will be necessary to classify the hospital radiopharmacies according with different degrees of complexity, requiring different types of organization, skills and permises.

The rol of the radiopharmacist will be well establised and finally regulations should promote a continous improvement, encourage additional training and experience for workers and strive to achieve a level playing field for those wishing to enter medical/pharmacy practice or the drug manufacturing field.

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THE RADIOPHARMACIST AS A PROFESSIONAL SPECIALITY: PAST, PRESENT AND FUTURE

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Abstract

When the technetium generator developed in the 1960's, there was an urgent need for individuals in Nuclear Medicine Departments that were specialists in such agents. While training in the use and handling of labeled compounds had been offered at a number of institutions, the need to have Pharmacists specialized in such radiolabeled drugs determined that Radiopharmacy emerged as the first specialty in Pharmacy. The University of Southern California School of Pharmacy, which had taken the lead, in the late 1960's, of changing the profession of Pharmacy from a product-oriented to a patient-oriented paradigm, also took the lead in developing the notion that Pharmacy, just as Medicine or Dentistry, could have post-professional specialties.

The USC Radiopharmacy program, which included offerings at the MS in Radiopharmacy (a professional specialization degree), the short-term training programs (usually 30 days, not degree-oriented), and training for Radiopharmacy Technologists, operated from 1969 through 1986. 201 pharmacists and others graduated with the MS in Radiopharmacy, 15 individuals with a Technologist certificate, and well over 500 individuals who participated in one or more of the formal short-term courses. A number of other institutions, in the US and elsewhere, have and are offering such specialty education. However, in the same manner that USC closed its program in 1986, the number of institutions that offer formal degrees has also been decreasing, just at a time when the demand for radiopharmacists is increasing.

The changes in the how health care is being delivered in the 1990's and beyond requires new paradigms. There is now a need for pharmacists (and perhaps some new breed of professionals) to be specialists, not only in radioactive drug products, but in all drugs used in imaging studies. While that includes diagnostic radiopharmaceuticals, it also includes drugs used for therapy with internal emitters, as well as novel aspects of the study of drugs and of the pathophysiological determinants of drug distribution – pharmacokinetic imaging and functional imaging. The challenge is here to develop new directions.

Introduction:

When the technetium generator developed in the 1960's, and ^{99m}Tc started to become widely available to Departments of Nuclear Medicine, one of the problems that had to be

addressed was, who was competent for preparing, dispensing and advising on the use of these new radiotracers? Were they (radio)pharmaceuticals that also had to meet pharmaceutical regulations and operational principles? Contrary to what used to happen with products labeled with the 8-day ^{131}I , these products labeled with a short lived radionuclide ($t_{1/2}$ of 6 hrs) could not be prepared at a central location and then distributed all over the country. While courses devoted to the teaching of radioisotope methodology for chemical, biological and medical uses had existed for many years, a number of institutions started appreciating in the late 1960's the need of developing programs leading to a formal degree in radiopharmacy. This coincided with the recognition that Pharmacy had to move from a purely product-oriented profession to one which emphasized the provision of pharmacy services. The University of Southern California was active in pioneering developments in both areas. Pharmacy became clinically oriented, and USC instituted the first formal degree program in Radiopharmacy in 1969, with the first class graduating in 1970 (1). Over the years, the USC Radiopharmacy program graduated 201 pharmacists with the degree of Master of Science in Radiopharmacy and it trained several hundred other people (including many sponsored by the IAEA) in various aspects of Radiopharmacy, including the establishment of a formal program for training Radiopharmacy Technicians (2) and others. Other programs throughout the country and throughout the world developed concurrently and shortly thereafter, and now that more than a quarter of century has passed, it is timely to assess what had been the experience and what the future entails.

Nuclear Pharmacy

Radiopharmacy (or Nuclear Pharmacy, as it is called in many places) is now a well-established pharmaceutical specialty. Indeed, the very concept that the profession of Pharmacy could have specialties was a new idea in 1969, which took some time to be accepted, as was the evolution of Pharmacy into becoming a more clinically oriented profession. The American Board of Pharmaceutical specialties was established in the US in the 1970's, with Radiopharmacy as the first field attaining this recognition. By now, this has expanded to several other pharmaceutical specialties having board certifications (3). While each country has their own regulations concerning who is responsible for the preparation, dispensing and the safe utilization of radiopharmaceutical drug products, the central point is that such specialized training is now generally recognized as needed to prepare the individual to be fully competent in these areas. One area of particular interest is the rapidly growing field of PET radiopharmaceuticals. They all must, by necessity, be prepared "in house". Who regulates them? What is the relative role of the (radio)pharmacist in their preparation, dispensing and utilization? These are problems under active discussion all over the world (4).

A number of key principles were incorporated into the development of the MS in Radiopharmacy, a professional specialty of Pharmacy. The first was that the (radio)pharmacist was to be the drug specialist of the Nuclear Medicine team. His/her role was not to be limited to the preparation of the products, but to participate actively in their selection, monitoring the patient to determine drug effectiveness, and work with the physician, in a collaborative rather than in a subaltern manner, in providing clinical radiopharmacy services. The diagram in figure I illustrates how that relationship was supposed to work, and appended is a civil service class specification for the Radiopharmacist, as developed in the 1970's for the County of Los Angeles.

NUCLEAR MEDICINE PHYSICIAN
Diagnostic Considerations

RADIOPHARMACIST
Pharmacological Considerations

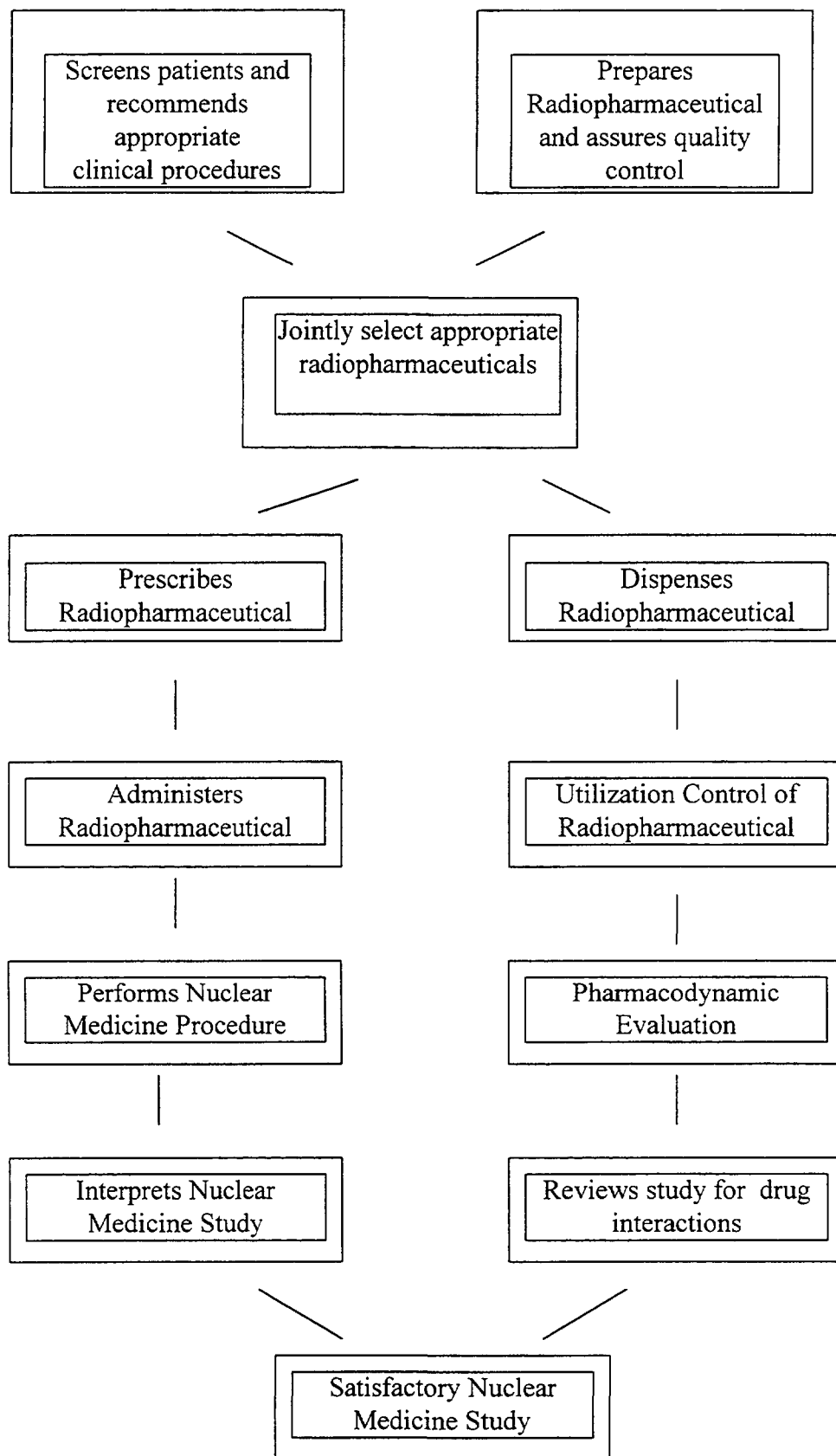


Figure I: The Relation Between Physicians and Pharmacists in Nuclear Medicine.

Practical realities and institutional traditions dictated a number of different variations of this idealized schema. While in some hospitals Radiopharmacy was part of Pharmacy services, the tensions created by the changes in the profession led a number of Pharmacy services to remain askance of anything that was new and different, whereas others, with greater vision, embraced wholeheartedly such new opportunities. In other hospitals a tradition in the Nuclear Medicine service determined that Radiopharmacy should become a section in such a service. The later evolution of Radiology, and the changing views of the relationship between Nuclear Medicine and the other Radiological Sciences complicated further this picture in the US. And in other countries, where the requirements did not exist that all pharmaceutical products had to be dispensed by licensed pharmacists, radiopharmacists could also come from other disciplines and scientific backgrounds, including chemistry, physics, etc.

Another major change started at the time these considerations were being sorted out: that of how the whole health care system should be managed. The rapidly increasing costs in health care lead to the development of cost-containment strategies which may be grouped, for this discussion, under the term of "managed care". In the swing of the pendulum from primary emphasis to quality patient care to primary emphasis on cost-effective patient care, many of the gains that clinical pharmacy and the pharmaceutical specialties had gained in the 1970's and early 1980's eroded. There were also developments unique to Radiopharmacy: the emergence of the commercial radiopharmacies, and the emergence of PET (positron emission tomography) as a rapidly growing branch of nuclear medicine.

The commercial radiopharmacies were based on the principle that the nuclear medicine services (and especially, the radiology services which did some limited nuclear imaging) would require products, with little or none of the clinical pharmacy services that were included in Figure I. It is a cost effective method for the distribution of products, and has taken significant hold in the US and a number of other countries. The commercial operations are different, however, from the shared services, where a number of institutions pool their resources. The role and the future impact of both sets of services is under constant evolution, and differs from country to country.

The rapid growth of PET centers, and hence, the need for the in-house preparation of radiopharmaceuticals with very short half-lives, under conditions where the primary emphasis has been on chemistry, has brought in a group of highly trained individuals into the field, many of whom do not have a professional Pharmacy background. The emphasis shifted to product preparation, purity and identity, rather than to clinical aspects of utilization and pharmacological monitoring. The more recent shift from ^{18}F -FDG (2-fluoro-deoxy-glucose) to ^{18}F - and ^{11}C -labeled receptors suggests another area where the need for persons properly trained in the radiopharmaceutical sciences will maintain or increase.

Future Directions

Both Pharmacy and Radiopharmacy are again at major crossroads. The delivery of health care and the nature and role of its providers are undergoing momentous changes, not only in the nature of the services they are asked to provide, but in the overall relations to other providers - physicians, technician, nurses -, to patients, and to the system. Clinical Pharmacy is evolving into Pharmaceutical Care (5), and Radiopharmacy is evolving now into

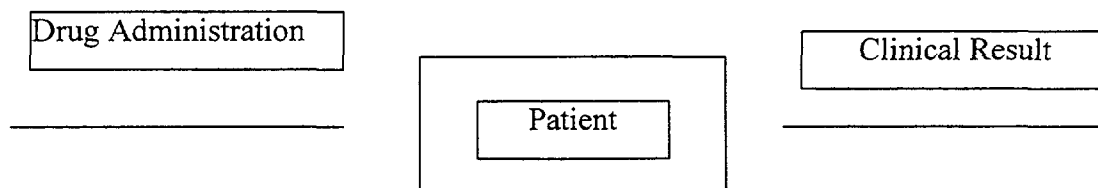


Figure II: Current scheme of drug development and treatment.

providing “poly-pharmacy” services. These will include not only expansion of the traditional roles of preparing, of dispensing and advising on the use of radiopharmaceutical drug products for diagnostic purposes, but much more frequently for their use in radiotherapy, as well as for monitoring the pharmacokinetics of drugs (6) as well as the pathophysiological characteristics of the patient. And then there is the impact of modern technology, the role of computers in data generation, sorting and transfer. And finally, there is the impact of pharmacoeconomics. How effective is the provision of radiopharmaceutical services? Outcome studies will need to be performed. And how well can we control radioactive waste and other ecological related problems?

The area of using noninvasive methods for monitoring what happens to drugs in patients, and what happens in patients when they are undergoing diagnosis or treatment with pharmaceutical drug products, can be illustrated by Figure II.

While data are available from the amount, rate and route of drug administration, and then of the clinical outcome, little information is generally available of the fate of the drug *in vivo*, of the patient’s individual characteristics, etc. In a word, there is currently an urgent need to develop effective methods for monitoring the individual patient in order to optimize any form of treatment. And it is our postulate that radiopharmaceuticals can play a major role in this process.

Let me conclude here with another issue: that of the relation of nuclear medicine and other areas of radiology, which will impact the role that the radiopharmacist/ radiopharmaceutical scientist should have in the use of the correlative imaging modalities (MRI, ultrasound, etc). Let me postulate that the radiopharmacist/ radiopharmaceutical scientist should be as heavily involved in the use of these correlative-imaging modalities as he/she is now in radiolabeled drug products. These areas are **complementary**, not **competitive**. The same principles of drug biodistribution, of pharmacokinetic analysis, and of pharmacokinetic/pharmacodynamic considerations apply.

Conclusion

There is much to be proud of what has been achieved in the last 25 years. But there is much more that needs to be done in the future. This represents a challenge - and an opportunity.

Appendix

CLASS SPECIFICATIONS FOR RADIOPHARMACIST, COUNTY OF LOS ANGELES, AS DEVELOPED IN THE 1970'S

TITLE: RADIOPHARMACIST

DEFINITION: Provides professional radiopharmacy services to the nuclear medicine and other medical services of a hospital and has immediate charge of the hospital's isotope laboratory

CLASSIFICATION STANDARDS: Positions allocable to this class apply the established professional principles and practices of pharmacology and nuclear radiation in the preparation, dispensing, and control of radiopharmaceuticals used either in producing scans used for imaging, metabolic, or functional studies of internal parts of the body for diagnostic purposes or in the treatment of patients with cancer or other diseases. Positions in this class also perform applied research in developing and testing new or modified radiopharmaceuticals; instruct students, interns, residents and others in the pharmacology of radionuclides; and provide training and supervision to radiopharmacy technicians and technician trainees. Under the general administrative supervision of the head of nuclear medicine services or the head of pharmacy services and the technical direction of a nuclear medicine physician, positions in this class have full responsibility for the quality and quantity of radiopharmaceuticals produced and for the purchase and control of radionuclides used by the hospital. In addition to established hospital policies and procedures, they work within the framework of the regulations of the Atomic Energy Commission¹, Food and Drug Administration, and State Department of Public Health governing the use and control of radionuclides.

EXAMPLES OF DUTIES:

Subjects radioactive substances and reagents to a variety of laboratory procedures involving chemical and electrolytic interactions for the purpose of labeling, or chemically fusing, radioisotopes onto chemical or biological substances and purifying the resulting radiopharmaceutical to eliminate wastes and impurities

Assays radiopharmaceuticals at the time of receipt or preparation and again at the time to be dispensed; using mathematics, including calculus, calculates the amount of individual doses based on the half-life of the radiopharmaceutical in relation to the type of procedure required, time of administration, and amount of radioactivity prescribed by the physician.

Using the above procedures, prepares and dispenses or

supervises the preparation of radiopharmaceuticals either for use in radiation detection procedures designed to produce images (known as scans) and metabolic or functional studies of the heart, lungs, liver, kidneys, bone structure, etc., for diagnostic scrutiny by the nuclear medicine physician or for the treatment of cancer and other diseases.

Coordinates the production and dispensing of radiopharmaceuticals with the scheduling of patients for diagnostic or therapeutic procedures so as to maximize the efficient use of time, equipment and materials and to enhance the quality of scans produced

¹ The functions of the old Atomic Energy Commission were divided among ERDA and the NRC (Nuclear Regulatory Commission), ERDA has since been merged into the Department of Energy (DOE). The lifting of the exemption, in 1975, further modified the relative responsibility of these various Agencies.

Reviews and evaluates the quality and value of the radiopharmaceuticals prepared by studying the resulting scans in consultation with nuclear medicine physicians, adjusts procedures to improve quality when possible

Performs pharmacological research in connection with the development of new radiopharmaceutical compounds or new methods for formulating existing compounds for the purpose of exposing the patient to less radiation, expediting the elimination of radioactivity by the patient producing higher quality scans, or saving time and money; reads professional literature and performs experimental laboratory techniques, including animal studies, to determine the chemical interaction of the proposed components of the drug and to calculate the likely effects of radiation dosimetry on the patient

Prepares formal reports for inclusion in proposals to the Atomic Energy Commission, Food and Drug Administration, and the State Department of Public Health required to obtain approval for testing and use of new radiopharmaceuticals, reports on research procedures and findings, calculation of dosimetry and serum levels, and procedures to be used in preparing the radiopharmaceutical, including quality control procedures.

Requisitions and supervises the ordering of radionuclides, reagents, laboratory equipment and supplies and of other pharmaceuticals used by the nuclear medicine section

Develops and supervises the maintenance of written controls on the receipt, storage, preparation, administration, and disposal of radionuclides and prepares reports of the circumstances and disposition of radioactive materials when spills or other accidents occur in the isotope laboratory

Lectures students, residents, interns, nuclear medicine technicians, and technician trainees on radiopharmaceutical characteristics and compounding procedures, provides general orientation to physicians and others on the policy, procedures, operations and purpose of the isotope laboratory

Attends meetings, conferences and seminars for the purpose of upgrading professional skills and knowledges and sharing information on the pharmacological aspects of the field of nuclear medicine

Develops operating, technical, reporting, and safety procedures for the conduct of an isotope laboratory, and closely monitors their implementation by technicians and trainees.

Supervises technicians and trainees in ordering, preparing assaying, and measuring doses of radiopharmaceuticals and in the maintenance of inventory controls on their receipt, preparation, administration, and disposal.

Participates in the selection of radiopharmacy technicians orients and trains new technicians and trains or supervises the training of technician trainees, plans, assigns and evaluates the work of subordinates including trainees

MINIMUM REQUIREMENTS

TRAINING AND EXPERIENCE Completion of a combination of postgraduate courses and professionally supervised experience, such as an internship or residency, in the pharmacology of nuclear medicine equivalent to a Master's degree in radiopharmacy in an accredited college of pharmacy

LICENSE A licence to practice as a Registered Pharmacist issued by the California State Board of Pharmacy

PHYSICAL CLASS "2" - Light.

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BEYOND OPERATIONAL CONSIDERATION IN A HOSPITAL RADIOPHARMACY



XA9848028

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Abstract

Hospital radiopharmacies have progressed from the chemistry laboratory in the basement where radioactive solutions were mixed to state of the art pharmaceutical facilities adjacent to nuclear medicine imaging suites. This progress can be attributed to new types of radiopharmaceuticals, advancements in nuclear instrumentation and equipment and competencies of individuals trained in Radiopharmacy practices. We are now entering another phase in the development of radiopharmacies which emphasizes both health care delivery and patient outcomes. Using a pharmaceutical care approach combined with expert pharmaceutical services, the direct and responsible provision of medication-related care for the purpose of achieving definite outcomes that improve a patient's quality of life can be accomplished in radiopharmacies. At a time when the health care system is undergoing momentous change, hospital radiopharmacies are challenged to aggressively pursue a clinically oriented role and to look at how the continued development of radiopharmacies can and should evolve beyond their operational characteristics.

1. INTRODUCTION

From the late 1960s to the mid 1990s, hospital based radiopharmacies have progressed from the "chemistry laboratory in the basement where radioactive solutions were mixed" to modern state-of-the-art, task-specific radiopharmacies adjacent to or immediately within clinical nuclear medicine imaging suites. This progression can be attributed to a variety of changes in the types, quantities and availability of primary radionuclides; the growing sophistication of radiopharmaceuticals; a constantly advancing technology in nuclear instrumentation and equipment with computer assisted operations; and a resource of specialty trained individuals with pharmaceutical expertise and radiopharmaceutical experience.

Historically, radiotracers (radio-medications) were mixed (compounded) on chemistry laboratory benches often times by personnel who were apprenticed by radiochemists and physicians or who acquired their skills by learning on-the-job. A number of highly competent radiochemists started the development of novel classes of radiopharmaceuticals, specifically those prepared with Technetium 99m. In 1969, the concept that a pharmacist whose basic pharmacy knowledge and pharmaceutical technical skills can be applied in processing radioactive materials was formalized into the Radiopharmacy Program at the University of Southern California, School of Pharmacy, Los Angeles, California USA. Additionally, in the

1970s, amendments to the United States Food, Drug and Cosmetic Act defined radiotracers as radioactive drugs and Nuclear Pharmacy was established as a specialty practice in pharmacy by the American Pharmaceutical Association and American Society of Hospital Pharmacists (now called American Society of Health-System Pharmacists). The combination of these important events promoted the recognition of the radioactive drug (radiopharmaceutical), the radiopharmacist and a specialized facility and practice site, the radiopharmacy.

Until the 1990s, many of the daily tasks that were performed in the hospital radiopharmacy by radiopharmacists often mirrored the tasks that were usually performed by nuclear medicine physicians, scientists, technologists and physicists. Product preparations were simple with one class of radiopharmaceutical which was prepared using one single method of manufacture and usually for one type of nuclear imaging procedure. Purity checks on these preparations were performed in a timely manner often only to confirm a recommended labeling effectiveness. Nuclear instruments such as well detector counters and isotope dose calibrators had push button technology but still required operator presence for the instruments' quality control checks. Dispensation of most radiopharmaceutical injectables required syringes to be manually filled to a calculated volume and manually checked in an isotope dose calibrator to validate the amount of radioactivity. Radiopharmaceuticals were dispensed in a manner to insure that the correct dosages were to be administered to the right patients at the right times with all the appropriate documentation required by regulatory agencies. While radiopharmacists were highly trained and competent to be the drug specialists on the nuclear medicine team, these operational tasks and product oriented services remained the main focus throughout the 1970s and 1980s.

2. PROCEDURAL CHANGES

The 1990s has seen an unprecedented development of new radiopharmaceuticals, increased professional expectations, new regulatory demands and diversification of radiopharmaceutical preparations. Several different products can have the same radionuclide and clinical indications, thus "poly-radiopharmacy" emerges and parallels traditional and evolving pharmacy practices. The same product may also be available from several radiopharmaceutical corporations. Brand name or proprietary radiopharmaceutical products are distributed by several radiopharmaceutical distributors which may have their manufacturing origin from one manufacturer. The parent radionuclide molybdenum 99 is supplied by a single source (Nordion, Canada) to several manufacturers of Technetium 99m Generators. One of these manufacturers may supply their trademark Tc99m generators to another manufacturer with only a label change indicating "Mfg by ... for ...". Reagent kits for the preparation of Tc99m labeled products such as Technescan® Sulfur Colloid (Mallinckrodt) and TECHNETIUM Tc99m Sulfur Colloid (Amersham) are manufactured by CIS-US, Inc., Bedford, MA.

Products are offered by various manufacturers that have the same United States Adopted Name (USAN) name and may be generically and biologically equivalent; yet are not pharmaceutically equivalent since they may contain different amounts of drug and excipients. Technescan® DTPA (Mallinckrodt), DTPA (Chelate) Multidose (Amersham) and DTPA (Dupont Pharma) have different milligram amounts of DTPA and different salt forms. Original formulations may also change to improve stability by changing pH or adding a stabilizer such as para-aminobenzoic acid as in the case of DTPA (Dupont Pharma). Technescan® MAA

(Mallinckrodt), Pulmolite® (DuPont Pharma) and MPI MAA KIT (Amersham) reagent kit vials contain different milligram amounts of albumin and different numbers of aggregated albumin particles. These particles also have different shapes, sizes and fragility. A different method of preparation of these generic products is also recommended by each of the manufacturers and becomes an important issue when regulatory agencies require "product preparation following the manufacturers' guidelines". A single brand name or generic product may now have different methods of preparation which result in a different type of preparation with its own unique biodistribution and/or use for a specific indication. Tc99m Succimer (dimercaptosuccinate) has a different biodistribution and organ/cellular localization depending upon the pH during preparation. Tc99m Exametazime may be stabilized with 5mg Methylene Blue Injection USP to extend the expiry period from thirty minutes to four hours for use as an adjunct in the detection of altered regional cerebral perfusion in stroke. Tc99m Exametazime without methylene blue is recommended for labeling autologous leukocytes for use as an adjunct in the localization of intra-abdominal infection and inflammatory bowel disease. The use of radiopharmaceuticals by different medical specialties and subspecialties is evolving rapidly. Once thought to be strictly for diagnostic use in a few medical specialties, radiopharmaceutical therapeutic treatment dosages are making rapid strides, especially using monoclonal antibodies. The use of radioactive forms of drugs in monitoring drug pharmacokinetics, biodistribution and pharmacokinetics of chemotherapeutic agents and human pathophysiology are within this decade.

The preparation of some radiopharmaceuticals have come full circle from the time-consuming chemical synthesis of the 1960s to the "instant kit compounding" of the 1970s to return again in the 1990s to labor-intensive, multi-step synthesis reagent kits. Some monoclonal antibody reagent kits require multiple additives, heating and cooling at specific temperatures, and filtration of the final product. Tc99m nofetumomab merpetan (Dupont Pharma) requires the following steps: (1) formulation of a ligand, (2) formation of a Tc99m ligand ester, (3) incubation at hot and cold temperatures, (4) Tc99m labeling with incubation and (5) purification with anion exchange filtration. Purity tests on radiopharmaceuticals prepared in the hospital continue to be performed on a routine basis. Previously, radiochemical purity testing was only a suggestion by the product manufacturer and the recommended radiochemical purity limit was often not stated. Recently revised product information supplied with reagent kits now include in the preparation instructions that "the radiochemical purity of the prepared radiopharmaceutical should be checked prior to patient administration". A few product information brochures include the manufacturer's recommended radiochemical purity limits and add the statement that "if radiochemical purity is not adequate, discard the finished drug". New product information not only include labeling effectiveness information but now emphasize the identification of the predominant labeled species of the drug. Tc99m labeled exametazime, mertiatide, teboroxime and sestamibi require that the predominant species be in a lipophilic chemical form. A three part chromatography system is used to identify the percentages of lipophilic, hydrophilic, pertechnetate and reduced-hydrolyzed technetium species of Tc99m Exametazime. A two part solvent-chromatographic filter system is used to define the percentage of Tc99m Mertiatide, hydrophilic impurities and non-elutable impurities. The lipophilic species of Tc99m Teboroxime and Sestamibi are identified from the soluble contaminants and reduced-hydrolyzed Tc99m using a dual chromatographic system. Although practice experience shows that batch testing for radiochemical purity rather than individual preparation testing is sufficient in most instances, this part of purity tests for quality control of radiopharmaceuticals are routinely performed since the manufacturers emphasize that the final formulation be tested for radiochemical purity.

3. OPERATIONAL CHANGES

Analytical measurements for radioactivity are now performed with computer-assisted instrumentation found in isotope dose calibrators, spectrometers and P-10 gas-operated imaging scanners. A menu driven dose calibrator can automate tasks such as inventory control, dosage calculation, radiochemical purity data, dose calibrator quality control tests and print hard copy information to satisfy regulatory requirements for record keeping. A system of independent dose calibrator ion chambers can be networked to a single console or operate with remote displays. The newer displays list the radionuclide and radioactivity; a welcomed feature which eliminates accepting a measurement on a wrong radionuclide setting. Dose calibrator ion chambers surrounded with additional auxiliary shielding and placed into the countertop or containment hood at surface level reduce radiation exposure through increased shielding, and decreased handling distance and time. A pneumatically driven vial/syringe plastic lift for the ion chamber can replace the "hands on" lift technique further reducing radiation exposure to fingers and hands. With a special lead or tungsten elution canister fitted to a mechanical canister separator/lift device (Cap-Mac, Capintec, NJ) that is inserted into the dose calibrator ion chamber, a Tc99m Generator can be eluted, the Tc99m measured, and the Mo99 contaminant assayed without removing the device from the dose calibrator. Thus several manual transfers of the elution vial are eliminated thereby further reducing radiation exposure to extremities. Dose calibrators are now specifically designed to measure large quantities of radioactivity (up to 800 curies), beta nuclides, and brachytherapy sources (Capintec). Spectrometers for well counters and multi-channel analyzers can be menu-driven instruments (Capintec) or operated by a computer (EG&G Ortec, TN). These instruments are bundled with specific procedure menus to analyze wipe tests which can identify a Kev energy range and convert from cpm to dpm or nCi and prompt entries for nuclear medicine Red Cell Volume and Schilling Tests. An imaging scanner allows rapid analysis of chromatography sheets/strips with automatic printed data and graphics. An imaging scanner is currently in use that provides high sensitivity digital data in one measurement from an entire chromatogram without mechanical stepped scanning (Bioscan, Inc., Washington, DC).

Preparation of radiopharmaceuticals that are free of particulate and microbial contamination is maintained using standard in-process aseptic techniques and a biohazard clean air cabinet that protects both the preparation and the preparer. Several small biohazard hoods are advantageous when several types of preparations need to be prepared or started at the same time. Thus Tc99m labeled reagent kits can be prepared simultaneously with Tc99m red blood cells and In111 white blood cells without potential cross-contamination or interrupting the preparation time sequence requirements. Chemical exhaust hoods have been designed specifically to process potentially volatile and gas radiopharmaceuticals. These hoods are now called radiation fume hoods and feature lead glass sashes and single piece interiors to simplify clean up and decontamination procedures.

Quality control tests for purity on routine hospital prepared radiopharmaceuticals continue to be performed prior to patient use with the exception of tests for sterility and pyrogens. The tests for sterility and apyrogenicity remain standard quality testing procedures but may be batch or composite samples collected for random testing of "processing methods". The use of the Bacterial Endotoxin Test (BET, United States Pharmacopeia) continues with LAL (Limulus Amebocyte Lysate) testing for end-product evaluation for the presence of gram-negative endotoxin. In 1997, the standards for endotoxin have been harmonized among the United States Pharmacopeia, European Pharmacopeia, and the World Health Organization.

The common standard for endotoxin biological activity or potency is now the International Unit (IU) where 1 IU is the equivalent of 1 Endotoxin Unit (EU, United States Pharmacopeia). The time required to perform a Bacterial Endotoxin Test in some radiopharmacies is shortened, under controlled conditions, to reduce product activity loss of selected products with short lived radionuclides and provide a result prior to product administration. Dispensation of radiopharmaceuticals is being performed with robotic techniques for the preparation of reagent kits and filling of syringes (Advanced Manufacturing and Mechatronics Centre, Middlesex University, London, U.K.). A drug monitoring computer software system shows promise in not only improving dosing accuracy, but also in performing calculations quickly, and dramatically reducing the risk of human error. Computers with software dedicated to record product and patient information as well as routine radiopharmacy tasks allow for specific printed information for labels to be attached to unit of use patient dosages (Nuclear Pharmacy Manager™, Dupont Pharma). A properly labeled patient dosage provides information verification should a medication misadventure (misadministration) occur or when a patient has an adverse drug experience. Hospital radiopharmacies that service remote sites and other health care facilities will soon be able to use telecommunications to access these individual sites to monitor most activities related to the radiopharmaceutical.

No discussion about Radiopharmacy is complete without mentioning the tools of the trade. Over the last decade, the amount of radioactivity handled in a hospital radiopharmacy has increased to curie quantities in a single day. With multiple elutions of large generators and the preparation of multiple products per day and combined with the trend towards higher patient dosages, radiation safety issues are major concerns for personnel. Fortunately, devices for personnel radiation protection continue to improve with "L" body shields of varying thickness of lead without open seams and with the highest density (6.2 gm/cc) lead glass. Traditional lead shields for vials and syringes are being replaced by high density tungsten which is more durable than lead alloy or lead and acrylic glass. The same products are modified for high-energy radiopharmaceuticals and for the 511 keV products. Eyeglasses and light weight lead vinyl aprons are accessory items which offer additional radiation protection. Storage of patient bodily fluids contained in needles, syringes, tubing and absorbents require special handling as biohazardous materials. Plastic Biohazard Sharps Containers are mandatory storage containers for sharp items in patient areas. Syringe and needle combinations and related devices are disposed into these containers immediately after patient administration. This practice helps avoid contact with the patients' bodily fluids and eliminates the chance that the syringe and needle may be inadvertently used on another patient. Lead-lined Sharps Shields have been designed for exact fit to various types of plastic sharps containers and comes with a key lock safety feature which meet certain regulatory occupational safety conditions. These container combinations are also used as direct depositories for syringes and needles used in compounding radiopharmaceuticals in the radiopharmacy. Newly designed or remodeled storage areas with leaded-lined walls and lead-lined storage cabinets allow for storage of brachytherapy sources, dose calibrator reference standards, camera flood sources and organ phantoms. Since many nuclear medicine departments may have standard, LFOV, Dual and Triple Head cameras, several flood source phantoms of different sizes and shapes need to be safely stored when not in use. Special rooms for short and long term storage are now being specially designed and equipped with sinks, body fluid waste sinks, food garbage disposal units, and a high-rate ventilatory exhaust system that continuously removes odors from long-term storage waste items of I131, I125, Sm153, Sr89 and other long-lived radionuclides.

4. PHARMACEUTICAL CARE

In the Hospital Radiopharmacy, the need for manufacturing, compounding, dispensation and distribution functions remain and continue to grow in spite of the upheaval in health care delivery, and the re-engineering of the pharmaceutical industry and hospital systems. Increasingly, the use of nuclear medicine continues to shift to an outpatient service as inpatient services become restrictive. This change to outpatient services requires delivery of superior service at a reasonable and competitive cost and in a timely manner. In addition, all the changes, in the demands for health care, the changing nature of diseases that need to be treated, an aging population with age-specific problems, the impact of recent health care legislation, managed care mandates and a changing pharmacy profession, have challenged the status of both the radiopharmacist and the operational considerations of the radiopharmacy. Many Radiopharmacy programs have responded by aggressively pursuing their distributive, managerial, and especially their clinical role. Radiopharmacies updated current practices, encouraged facility and equipment upgrades, and refined unit of use dosage distribution, drug information resources and computerized patient drug profiles. However, the often unrecognized feature of radiopharmacy services that needs to be reassessed is the potential impact on health outcomes. Using a pharmaceutical care approach combined with expert pharmaceutical services, radiopharmacy services can have a real, positive outcome on patient care. Pharmaceutical care is the direct, responsible provision of medication-related care for the purpose of achieving definite outcomes that improve a patient's quality of life. It is the goal of pharmaceutical care to improve an individual patient's quality of life through achievement of definite (predefined), medication-related outcomes. Already in the clinical arena, the radiopharmacist is readily available to interact and prospectively consult with other health care professionals to achieve these outcomes. Specifically valuable would be proactive interventions, rather than retrospective evaluations, that would influence outcomes in diagnostic imaging studies and radionuclide therapy. Since the expertise in radiopharmacy practice is the drug component, it is reasonable then that the drug use/patient outcome focus on drug related events. Early interventions prevent potential suboptimal tests which affect patient outcomes through postponement or incorrect study results. Examples are potential drug related problems associated with thyroid, coronary vessel and neuroendocrine nuclear medicine procedures. A myriad of iodine containing drugs affect the thyroid uptake of iodine radiopharmaceuticals, caffeine containing drugs affect a cardiac-pharmacological stress test induced with dipyridamol, and norepinephrine related drugs affect adrenal gland uptake of iodinated iobenguane (MIBG). Albeit, anecdotal, health outcomes are preceded by increased knowledge assessments from diagnostic nuclear medicine studies and the use of the appropriate radiopharmaceuticals; safety and effectiveness of the radiopharmaceutical/drug regimen; cost control, minimization and effectiveness; and most importantly patients' needs, expectations and satisfaction. Patient education and preparation become extremely important since many patients are taking medications and want to know "what is a radiopharmaceutical" and how it may affect the medications they are taking. Patients as consumers demand detailed information on their conditions and diagnoses and on the quality and outcomes data of the facilities, health care providers and services. Radiopharmacists frequently offer drug consultation and consumer information for these kinds of inquiries. There remains a need to review the patient's medication history, identify and prioritize items which are necessary to insure optimal radiopharmaceutical usage, and most appropriately to recognize and take appropriate intervention and monitoring actions. These features go beyond the operational characteristics of a radiopharmacy and surely contribute to the improvement in health care outcomes.

5. SUMMARY

Radiopharmacy continues to endorse the concept of pharmaceutical care, which basically involves the processes by which patient outcomes and quality of life are optimized, and proactively continues to determine the best way to use radiopharmaceuticals and clinical services for the benefit of the patient. Furthermore, a more sophisticated, integrated system needs to evolve that will make the most efficient use of modern technologies to provide the best in pharmaceutical (radiopharmaceutical) care. Such a system will make extensive use of computerization and telecommunications for guiding and monitoring productivity, for integrating drug and patient information, for analyzing pharmacoeconomics and socioeconomic elements, and for allowing novel applications of radiopharmaceuticals and radiopharmacy practices. While many of the conceptual changes discussed apply mostly to the United States, they could and should be the harbinger of things to come in hospital radiopharmacies in all countries throughout the world.

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**NUCLEAR PHARMACY CERTIFICATE PROGRAM:
DISTANCE LEARNING**



XA9848029

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Abstract

The Nuclear Pharmacy Certificate Program (NPCP) was developed to meet the need for licensed pharmacists wishing to change career paths and enter the practice of nuclear pharmacy. Additionally, the NPCP benefits employers that wish to employ a nuclear pharmacist in lieu of waiting for graduates that are available only at one time yearly from a college of pharmacy. The NPCP is not intended to replace traditional nuclear pharmacy education in academic institutions, but to offer an another option to pharmacists and potential employers.

The NPCP is divided into two components. One component involves over 130 hours of instruction through videotapes and accompanying workbooks. This component is completed while working in a nuclear pharmacy and with the assistance of a nuclear pharmacist serving as a supervisor. The nuclear pharmacist is available to answer questions and to administer examinations over the videotape material. Examinations are prepared by Purdue faculty and returned for grading. Scores on exams must reflect learning to the same degree as in an academic environment.

In the second component of the NPCP, the trainee attends a two-week session in the School of Pharmacy at Purdue University. The trainee must complete a significant portion of the videotape material before the on-campus session. In the on-campus component, videotape material is reinforced and expanded by laboratory exercises and lectures in dedicated, fully-equipped laboratories employed in the School of Pharmacy undergraduate program in nuclear pharmacy. Nuclear pharmacy faculty and consultants provide individualized instruction to each trainee. Assimilation of lecture and laboratory material is determined through several examinations. A comprehensive examination is administered which includes content from the videotape-workbook component of the NPCP. Certification is awarded to trainees who have completed the program and demonstrated their knowledge and competence by examination. Almost 200 individuals have successfully completed the NPCP over the past nine years.

1. Introduction

The Nuclear Pharmacy Certificate Program (NPCP) was developed to meet the need for training for licensed pharmacists wishing to change career paths and enter the practice of nuclear pharmacy. Guidelines for Nuclear Pharmacy Training prepared by the American Pharmaceutical Association, Academy of Pharmacy Practice and Management, Section on Nuclear Pharmacy and requirements established by the United States Nuclear Regulatory Commission were followed in designing the NPCP. Also, lecture and laboratory material utilized in the nuclear pharmacy career electives for undergraduate pharmacy students in the School of Pharmacy at Purdue University were employed in the development of the NPCP.

The NPCP is presented as two components. One component provides over 130 hours of instruction through videotapes and accompanying workbooks. This component is completed under the supervision of a nuclear pharmacist located in a nuclear pharmacy. The nuclear pharmacist serves as a preceptor for the trainee. The trainee is expected to work in the nuclear pharmacy with time available to view the videotape material.

The second component of the NPCP requires the trainee to attend a two-week session at the Purdue University campus after completion of a significant portion of the videotape material. In the on-site component, videotape material is reinforced and expanded through laboratories and lectures presented by Purdue University Nuclear Pharmacy faculty and outside experts. On-campus instruction is provided in dedicated, fully-equipped laboratories employed in the Purdue University undergraduate nuclear pharmacy and health physics programs.

2. Methodology: Videotape.

The videotape lecture material was prepared in a professional television studio. Graphics and demonstrations were incorporated as well as lecture material. Outstanding practitioners and educators were employed to develop videotape lectures and accompanying workbook material. Professionals involved in the preparation of the videotape-workbook component of the NPCP are listed in Table I.

When a trainee enrolls in the NPCP the complete workbook and one-half of the videotape material is shipped to the nuclear pharmacy or directly to the trainee. The remaining videotapes are shipped following completion of the first portion of the self-paced study material. The trainee

Table I. Instructional Staff

Videocassette - Workbook

Dr. Stanley M. Shaw, Ph.D.
Professor of Nuclear Pharmacy
Purdue University
West Lafayette, Indiana 47907

Dr. Robert Landolt, Ph.D., B.C.H.P.
Professor of Health Physics
Purdue University
West Lafayette, Indiana 47907

Dr. Wayne V. Kessler, Ph.D.
Professor of Bionucleonics
Purdue University
West Lafayette, Indiana 47907

Dr. Paul C. Simms, Ph.D.
Professor of Physics
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Dr. Richard J. Kowalsky, PharmD,
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On-Site

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Dr. Gordon Born, Ph.D.
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West Lafayette, Indiana 47907

Ms. C. Anne Smith, M.S., B.C.N.P.
Nuclear Pharmacy Program Director
Purdue University
West Lafayette, Indiana 47907

Table II. Videocassette and Workbook Self-Study Portion

Instructor**Material****Dr. Stan Shaw****Physics and Overview**

Radiation Energy
Atomic Structure
Nuclides
Radioactive Decay and Half-Life
Ideal Radionuclide for Imaging
Modes of Radioactive Decay
Interaction of Ionizing Radiation with Matter
Radiation Detection Methods
Radiopharmaceuticals: Characteristics and Chemistry

Dr. Robert Landolt**Radiation Protection**

Terms and Units
Protection from External Exposure
Portable Survey Instruments
Personnel Monitoring
Internal Dose Calculations
Contamination Control
Waste Management
Packaging, Labels and Placards
10 CFR Standards for Protection Against Radiation
10 CFR Notices, Instructions, and Reports to Workers

Mr. Jim Ponto**Drugs and Radiopharmaceuticals**

Drugs and Radiopharmaceuticals:
*Interactions and their effect on diagnostic accuracy of
nuclear medicine procedures*
Drugs and Radiopharmaceuticals:
*Interventions used to improve differential diagnosis in
nuclear medicine imaging*
Criteria for Product Selection
Instrument Quality Assurance
Technetium Chemistry; Radiolytic Decomposition
Pediatric Dosage Calculations
Adverse Reactions to Radiopharmaceuticals
Record Keeping
Preparation and Dispensing of Radiopharmaceuticals
Formulation Problems

Ms. Anne Smith

Radionuclide Generator: Mo-99/Tc-99m Generator
Quality Control Testing of Radiopharmaceuticals

Table II. Videocassette and Workbook Self-Study Portion (continued)

<u>Instructor</u>	<u>Material</u>
Dr. Richard Kowalsky	Radiopharmaceuticals for Brain Imaging Radiopharmaceuticals for Heart Imaging Radiopharmaceuticals for Kidney Imaging Radiopharmaceuticals for Treatment of Bone Pain Monoclonal Antibodies for Radioimmunodiagnosis Somostatin Receptor Imaging
Dr. Wayne Kessler	<u>Instrumentation</u> Spectrometry Counting Efficiency Coincidence Loss Background Liquid Scintillation Counting Statistics of Radioactivity
Dr. Paul Simms	Radionuclide Production, Part 1 Radionuclide Production, Part 2
Dr. Stan Shaw	<u>Radiation Biology and Protection</u> Energy Transfer Mechanisms of Change Aqueous Radiation Chemistry Target Theory and Dose-Response Radiation Effects on Macromolecules Radiation Effects on Cells Acute Effects Delayed Effects Genetic Effects
Dr. William Widmer	Late Effects of Ionizing Radiation

retains the workbook for review and as a resource while the videotapes are returned to Purdue University. The trainee is expected to devote about two hours daily viewing videotapes and taking notes. It is recommended that this be accomplished in the nuclear pharmacy so that questions can be addressed to the nuclear pharmacy preceptor. When the NPCP was reviewed by the United States Nuclear Regulator Commission, the agency recommended that the trainee should receive practical experience in a nuclear pharmacy under the supervision of a nuclear pharmacist during the videotape-workbook component of the program. Indeed, the availability of a nuclear pharmacy preceptor and practical experience while learning basic concepts and knowledge provides an excellent educational environment for the trainee.

Examinations are prepared by Purdue University nuclear pharmacy staff to ascertain learning of the self-paced videotape material. The examinations are designed to be in sequence with the videotape lessons and cover a reasonable amount of material in order to encourage in-depth learning. The exams vary from multiple choice to short answer as well as problem solving. Examinations are administered by the nuclear pharmacy preceptor in the same manner as in a formal classroom. Examinations are returned to Purdue University for grading. A minimum of 70% must be attained on each exam. Failure to do so results in a retake of a second exam over the same material. While the highest ethics are expected, knowledge of the content in the videotape material is assessed again in a comprehensive exam administered during the on-campus session at Purdue University.

The knowledge and concepts presented in the videotape-workbook material encompasses a broad spectrum of aspects basic to preparation for entry into nuclear pharmacy practice. Instructors and areas presented by videotape-workbook instruction are presented in Table II. The trainee is expected to complete the greater amount of the videotape material and pass appropriate examinations prior to attending an on-campus session. Radiation biology may be completed after the on-campus session. It is essential to have the trainee properly prepared in basic concepts and practice experience prior to attending an on-campus session in order to allow for an optimal learning experience while at Purdue University. The nuclear pharmacy preceptor is encouraged to allow the trainee to develop certain skills and concepts while working in the nuclear pharmacy prior to attending an on-campus session. A listing of areas suggested is presented in Table III.

Table III. Skills and Concepts^a

Description

Elution of Tc-99m Generator

Radionuclidic Purity Testing (Mo-99)

Chemical Purity Testing (Al ion)

Preparation of Radiopharmaceuticals

Dispensing of Calibration Sources and Doses

Radiochemical Purity Testing (Free, H/R, Bound)

Sterility Testing (if routinely performed)

Package Receipt

Package Shipment

Decontamination Procedures

Air Monitoring (I-131) Procedures

Blood Cell Labeling

Dose Calibrator Constancy

Dose Calibrator Accuracy

Dose Calibrator Linearity (when regularly scheduled)

SCA/MCA Calibration

SCA/MCA Efficiency Check (when regularly scheduled)

SCA/MCA Constancy

Area Surveys and Wipe Smear Testing

^aExperiences encouraged prior to attending the on-campus session

4. Methodology: On-campus Session

An on-campus session is offered on a regular basis three times per year. This allows trainees to master the videotape material at a reasonable pace and complete the total NPCP in a timely manner. Trainees must assume financial responsibility for travel to Purdue University plus meals and housing while on campus. Housing is available in a hotel located on the campus and within easy walking distance of laboratory facilities. The tuition charged for the NPCP includes everything except the above mentioned items. Upon arrival each trainee receives a complete set of laboratory exercises and lecture material for the on-campus session. As for the videotape-workbooks, the on-campus material is retained by the trainee for future reference. Since the level of radioactivity used in laboratories is minimal, personnel monitoring devices are not required. However, radiation safety procedures are strictly followed to promote the development of good habits when working in a nuclear pharmacy.

In the on-campus component of the NPCP, videotape material is reinforced and expanded by laboratories and lectures presented by Purdue University faculty and outside experts. On-campus instruction is provided in dedicated, fully-equipped laboratories employed in the undergraduate nuclear pharmacy and health physics programs at Purdue University. Instrumentation is available to allow individuals or a team of two trainees to work on laboratory exercises. Individualized attention is given to identify and correct any deficiencies occurring during training in the self-paced videotape component. Several examinations, including a comprehensive exam, are given during the on-campus session. Faculty involved in the on-campus training component are listed in Table I. Laboratories and lectures presented are listed in Table IV.

5. Discussion

The NPCP provides an economical and flexible mechanism for providing fundamental knowledge and laboratory experience to pharmacists that serves as a foundation for attaining competency as a practitioner in nuclear pharmacy. A considerable amount of knowledge can be gained while working and remaining at home. A trainee is only required to travel one time and spend 13-14 days away from home. Thus, living expenses are kept at a minimum.

Table IV. On-Site Laboratory Schedule

<u>Instructor</u>	<u>Laboratory</u>
Dr. Stan Shaw	Contamination and Decontamination Basic Radiation Safety G.M. Counting
Dr. Brigitte McGhee	I-131 Handling Techniques
Ms. Anne Smith	Gamma Ray Scintillation Spectrometry I Gamma Ray Scintillation Spectrometry II Multichannel Analyzer Dose Calibrator Shipping and Receiving Elution of the Tc-99m Generator and Quality Control of the Eluate Radiochemical Purity Testing Preparation and Dispensing of Selected Radiopharmaceuticals Aseptic Technique and Sterility Testing Gamma Camera Instrumentation Review of Math Used in Nuclear Pharmacy Counting Statistics in Nuclear Pharmacy Practice
Dr. Mark Green	Chemistry of Metal-Labeled Radiopharmaceuticals PET Radiopharmaceutical Chemistry PET Imaging and Concept Radionuclide Generator for PET
Dr. Stan Shaw	Regulatory Agencies 10 CFR Parts 19 and 20
Mr. Mack Richards	10 CFR Part 35 Medical Regulations Performance Criteria for Radiobioassay
Ms. Anne Smith	Formed Element Labeling and Aids Safety Procedures
Dr. Gordon Born	DOT Hazardous Materials Handling
Dr. Robert Landolt	Film Badge Dosimetry

The NPCP is educationally effective. It provides a sound, reasonable-paced instructional experience. Trainees proceed through videotape material at a self-paced rate. Progress is monitored by both the nuclear pharmacy preceptor and the Nuclear Pharmacy Program Director. Assimilation of material is facilitated by concurrent work in a nuclear pharmacy. The on-campus session reinforces the self-paced videotape material as well as expands knowledge and skills.

The NPCP was developed by faculty with years of experience and demonstrated excellence in education in nuclear pharmacy. Outside experts were solicited to broaden the background of the instructional faculty at Purdue University. Equipment and facilities are dedicated to nuclear pharmacy education as a result of an on-going undergraduate educational program in nuclear pharmacy within the School of Pharmacy at Purdue University.

Upon completion of the NPCP a trainee receives a formal certificate, a letter indicating successful completion and descriptive material useful for submission to a regulatory agency. Also, the trainee receives considerable continuing education credits that are useful in maintaining a pharmacy license. The NPCP is approved for continuing education by the American Council on Pharmaceutical Education.

The NPCP has been available since late in 1988. Almost 200 pharmacists has completed the program to date. Over 20 of the trainees are Board Certified in Nuclear Pharmacy. The NPCP has served the specialty by the provision of pharmacists with the basic knowledge and skills necessary to enter the practice of nuclear pharmacy. The NPCP does not replace the need for nuclear pharmacists educated by traditional academic programs, but does provide a mechanism to obtain properly prepared pharmacists to meet the needs of the nuclear pharmacy field at times when graduates from a college are not available.

NUCLEAR PHARMACY EDUCATION: INTERNATIONAL HARMONIZATION



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Abstract

Education of nuclear pharmacists exists in many countries around the world. The approach and level of education varies between countries depending upon the expectations of the nuclear pharmacist, the work site and the economic environment. In Australia, training is provided through distance learning. In Europe and Canada, nuclear pharmacists and radiochemists receive postgraduate education in order to engage in the small-scale preparation and quality control of radiopharmaceuticals as well as research and development. In the U.S.A., nuclear pharmacy practitioners obtain basic knowledge primarily through undergraduate programs taken when pursuing the first professional degree in pharmacy. Licensed practitioners in pharmacy enter the practice of nuclear pharmacy through distance learning programs or short courses.

While different approaches to education exist, there is a basic core of knowledge and a level of competence required of all nuclear pharmacists and radiochemists providing radiopharmaceutical products and services. It was with this realization that efforts were initiated to develop harmonization concepts and documents pertaining to education in nuclear pharmacy. The benefits of international harmonization in nuclear pharmacy education are numerous. Assurance of the availability of quality professionals to provide optimal products and care to the patient is a principle benefit. Spanning national barriers through the demonstration of self governance and unification in education will enhance the goal of increased freedom of employment between countries. Harmonization endeavors will improve existing education programs through sharing of innovative concepts and knowledge between educators. Documents generated will benefit new educational programs especially in developing nations.

A committee on harmonization in nuclear pharmacy education was formed consisting of educators and practitioners from the international community. A working document on education was developed and each knowledge area considered as either essential, familiar or unimportant to training in nuclear pharmacy. The committee considered a majority of the knowledge areas as important based upon the results indicating that the knowledge was either essential or when combining designations of essential and familiar. Results are summarized in the text with examples of the knowledge areas reviewed presented in tables.

The committee on nuclear pharmacy education will revise the working document and reconsider each knowledge area. The second working document will be sent to additional educators and practitioners for input. The document will be presented for discussion at a symposium on radiopharmacy, revised appropriately and sent to individuals in nuclear pharmacy throughout the international community. The finalized document on nuclear pharmacy education will be made available through an international pharmacy organization.

1. Introduction

Education of nuclear pharmacists exists in many countries around the world. The approach and level of education varies between countries depending upon the expectations of the nuclear pharmacist, the work site and the economic environment. In Australia, training is provided through a distance learning approach. In Europe and Canada, nuclear pharmacists and radiochemists receive postgraduate education in order to engage in the small-scale preparation and quality control of radiopharmaceuticals as well as research and development. In Europe an educational approach has been developing under the direction of committees and individuals in the European Association of Nuclear Medicine [1-3] ultimately resulting in the recommendation for the establishment of a postgraduate program within the framework of the European School of Nuclear Medicine [4]. In the U.S.A., nuclear pharmacy education was first formalized as a M.S. degree in Radiopharmacy in 1969 at the University of Southern California. Initially, in the U.S.A., nuclear pharmacists were located in larger hospitals, universities and medical centers. Within a few years the concept of a commercial centralized nuclear pharmacy (CCNP) was established. The CCNP operates as a business in a manner similar to a retail pharmacy. Unit doses are provided to hospitals within a city and surrounding regions by automobile. The CCNP

concept plus the availability of non-radioactive kits and the technetium-99m generator increased the need for nuclear pharmacists. The type of practice conducted in a CCNP allowed the utilization of a quality pharmacist with a first professional degree. Thus, several schools of pharmacy developed programs that allow specialized knowledge in nuclear pharmacy to be obtained through elective courses while attaining the first professional degree. Approaches to education in the U.S.A. have been discussed in a recent article [5].

While different approaches to education exist, there is a basic core of knowledge and a level of competence required of all nuclear pharmacists and radiochemists providing radiopharmaceutical products and services. Knowledge of nuclear physics, instrumentation, radiochemistry, radiation protection, radiation risks, radiopharmaceutical preparation and quality assurance, products and their clinical utility as well as regulatory rules are common to all. It was with this realization that efforts were initiated to develop harmonization concepts and documents pertaining to education in nuclear pharmacy. The objective of the endeavor being to develop guidelines satisfactory to the international community in nuclear pharmacy that would allow flexibility for individual nations yet reflecting areas of general agreement basic to all educational programs.

2. Methodology

A committee on harmonization in nuclear pharmacy education was established with members from several countries. Members are: Professor Dr. Peter Cox, Chair (University Hospital, Rotterdam, NL); Mr. Peter Eu (Peter MacCallum Cancer Institute, Victoria, Australia); Dr. M.C. Gil (Chilean Nuclear Energy Commission, Santiago, Chile); Dr. Stuart Hesselwood (Birmingham Regional Radioisotope Center, Birmingham, England); Mr. William Hladik, III, (University of New Mexico, Albuquerque, U.S.A.); Professor Dr. H.J. Machulla, (Eberhard Karis University Tübingen, Tübingen, Germany); Dr. J. Mallol (Technologia Farmaceutica, Tenerife, Spain); Dr. Stephen Mather (St. Bartholomews Hospital, London, England), Dr. Charles Sampson (Addenbrookes Hospital, Cambridge, England); Dr. Stanley Shaw (Purdue University, West Lafayette, U.S.A.) and Professor Dr. Len Wiebe (University of Alberta, Edmonton, Canada). The committee acts under the direction of a steering committee composed of the chairs from two other committees on harmonization, the chair of the committee on education, and two other members.

The committee on harmonization in nuclear pharmacy developed a working document utilizing the "Syllabus for Nuclear Pharmacy Training" prepared by the American Pharmaceutical Association, Academy of Pharmacy Practice and Management, Section on Nuclear Pharmacy Practice (APhA, APPM, SNPP). The syllabus lists in detail knowledge areas of importance to training nuclear pharmacists in the U.S.A. The document was modified to allow committee members to designate knowledge areas as essential, familiar or unimportant. The document did not indicate a specific method by which the training would be conducted. An example of a detailed knowledge area in the document is presented in Table I, while major headings for the entire document are listed in Table II. Each committee member reviewed the working document. Results were tabulated and returned to committee members for further input. Also, the results were shared with individuals attending the Special Interest Group (SIG) on Radiologic Pharmacy at the FIP World Congress of Pharmacy and Pharmaceutical Sciences '97 held in Vancouver, Canada, 31 August - 5 September, 1997.

3. Results and Discussion

Preliminary results indicate considerable agreement regarding the importance of a large body of knowledge for education in nuclear pharmacy. This is especially true if the knowledge areas of importance are considered based upon summation of the endorsement as either essential or familiar. Certain knowledge areas received almost unanimous agreement as essential while a limited number of areas were considered as unimportant by the majority of the committee.

As might be expected, knowledge areas in radiation physics and instrumentation considered as important by the committee included: 1) structure and properties of atoms; 2) concepts of radiation and radioactive decay including radioactive decay processes; 3) decay schemes for radionuclides used in nuclear medicine; 4) the production of radionuclides, especially by radionuclide generators; 5) interactions of alpha, beta and gamma radiation with matter; and 6) concepts and principles of operation for ionization and crystal scintillation detectors.

Under mathematics of radioactivity use and measurement, practical calculations involved with radioactivity measurements and counting statistics were viewed as important as well as calculations associated with the operation and use of a radionuclide generator. Radiopharmaceutical preparation and dispensing calculations were considered as essential by

Table I. Example of a Detailed Knowledge Area

<u>Knowledge and Concept</u>	<u>Committee Response^a</u>		
I. Structure and Properties of Atoms			
A. Atomic Models, Nomenclature	7-E	3-F	U
B. Nuclides and Radionuclides			
1. isotope, isobars, isotones, isomers	8-E	2-F	U
2. chart of the nuclides	3-E	6-F	1-U
C. Orbital Energy Levels			
1. energy units: eV, keV, MeV	7-E	3-F	U
2. electron shell binding energy, excitation and de-excitation	3-E	7-F	U
3. characteristic x-rays, Auger electrons, visible radiation	6-E	4-F	U
D. Nuclear Energy Levels			
1. nuclear excitation and de-excitation processes	3-E	7-F	U
2. gamma rays	7-E	3-F	U
E. Mass and Energy Intraconversion	3-E	6-F	1-U
F. Nuclear Forces and Binding Energy	1-E	8-F	1-U
G. Nuclear Fission and Fusion	2-E	8-F	U
II. Radiation and Radioactive Decay			
A. Radiation			
1. defined	10-E	F	U
2. principal forms	10-E	F	U
B. Nuclear Stability and Radioactive Decay			
1. decay constant	10-E	F	U
2. half life	9-E	1-F	U
3. average life	9-E	F	1-U
4. effective half life	10-E	F	U
C. Types of Decay			
1. alpha	8-E	2-F	U
2. negatron (beta minus)	10-E	F	U
3. positron (beta plus)	10-E	F	U
4. electron capture decay	10-E	F	U
5. isomeric transition	10-E	F	U
D. Consideration of Radioactive Decay Processes			
1. disposition of decay energy	9-E	1-F	U
2. beta energy spectrum	7-E	3-F	U
3. neutrinos	4-E	5-F	1-U
4. beta only vs. beta-gamma emission	8-E	2-F	U
5. annihilation reaction	9-E	1-F	U
6. characteristic x-rays	6-E	4-F	U
7. Auger electrons	8-E	2-F	U
8. isomeric transition	10-E	F	U
9. metastable states	10-E	F	U
10. internal conversion	10-E	F	U

^aKnowledge and Concept Classification: E = essential ; F = familiar; U = unimportant

Table II. Major Knowledge Areas

RADIATION PHYSICS AND INSTRUMENTATION

- I. Structure and Properties of Atoms
- II. Radiation and Radioactive Decay
- III. Decay Schemes of Radionuclides used in Nuclear Medicine
- IV. Production of Radionuclides
- V. Interactions of Radiation with Matter
- VI. Instruments for Radiation Detection and Measurement

MATHEMATICS OF RADIOACTIVITY USE AND MEASUREMENT

- I. Radioactivity
- II. Nuclear Counting Statistics and Measurement
- III. Health Physics Equations and Use
- IV. Radiopharmaceutical Preparation and Dispensing Calculations
- V. Generator Operation and Use
- VI. Calculations Involved with Radioactivity Measurement and Counting Statistics
- VII. Quality Assurance Calculations
- VIII. Calculations Associated with the Quantitative Assessment of Radiopharmaceutical Absorption, Distribution, Metabolism and Excretion
- IX. Calculations Involved with Medical Decisions
- X. Radiation Dosimetry Calculations

RADIATION PROTECTION AND REGULATIONS

- I. Interactions of Radiation with Matter
- II. Units of Radiation Measurement
- III. Occupational and Non-Occupational Exposure Radiation Protection Guides
- IV. Principles of Radiation Protection
- V. Personnel Monitoring and Precautions
- VI. Area Monitoring (Personnel and Work Environment)
- VII. Radioactive Packages and Sources
- VIII. Radioactive and Biohazardous Waste Disposal Methods
- IX. Radiation Safety
- X. Radiation Accidents

RADIATION BIOLOGY

- I. Interaction of Ionizing Radiation with Matter
- II. Radiation Chemistry
- III. Cellular Response
- IV. Effects of Nucleic Acids
- V. Radiation Genetics (Hereditary Effects)
- VI. Effects of Ionizing Radiation on the Embryo and Fetus
- VII. Whole-Body Effects of Ionizing Radiation
- VIII. Acute Effects of Ionizing Radiation
- IX. Delayed Effects of Ionizing Radiation
- X. Low Level (Low Dose Exposure to Ionizing Radiation)
- XI. Radiotherapy

Table II. Major Knowledge Areas (continued)

RADIOPHARMACEUTICAL CHEMISTRY

- I. Production of Radionuclides
- II. General Physicochemical Properties of Radioactive Compounds
- III. Properties of Radiopharmaceuticals
- IV. Quality Control of Radiopharmaceuticals
- V. Technetium Radiopharmaceuticals
- VI. Iodine Radiopharmaceuticals
- VII. Radiolabeled Blood Cells
- VIII. Prepared Radiopharmaceuticals (*i.e.* Quality Control, Physicochemical and Kinetic Properties, and Dosage Forms, etc.)
- IX. Positron Emitting Nuclides (*i.e.* Preparation, Quality Control, Physicochemical and Kinetic Properties, and Dosage Forms, etc.)
- X. Receptor Specific Radiopharmaceuticals (*i.e.* Preparation, Quality Control, Physicochemical and Kinetic Properties, and Dosage Forms, etc.)

THE CLINICAL USE OF RADIOPHARMACEUTICALS

- I. *In Vivo* Kinetics of Radiopharmaceuticals
- II. Specific Procedures Which Employ Radiopharmaceuticals
- III. Preparation and Monitoring of Patients Who Receive Radiopharmaceuticals

every member of the committee. This was true, also, for quality assurance calculations for radionuclidic, radiochemical and chemical purity of radiopharmaceuticals. The importance of inverse square law and half-value layer was noted while calculations involved with radiation dosimetry and medical decisions (sensitivity and specificity) were not considered as highly important to the training of a nuclear pharmacist.

Under the heading radiation protection and regulations, certain areas did not receive a consensus agreement partly due to the variations in regulations and terminology between different countries. Additional effort must be made to generalize aspects associated with regulations and allow for specifics to be added by individual countries. However, there were many knowledge areas considered important by the majority of the members of the committee. The basic principles of radiation protection (time, distance and shielding), personnel monitoring devices and precautions, units of radiation measurement, area monitoring procedures (surveys, wipe tests) and limits of contamination were all viewed as important. So also were waste disposal methods, procedures for opening and shipping radioactive packages as well as laboratory design. Emergency procedures received endorsement from all members of the committee.

Interestingly, radiobiology (radiation effects) as presented in the working document, did not receive strong support for training. There was a greater number of designations as familiar as compared to essential for many areas of knowledge listed. This may reflect a problem with the detailed listing of knowledge associated with radiation effects or, indeed, that nuclear pharmacists need only to have some general familiarity with radiation effects *vs.* this knowledge being essential to the practice of nuclear pharmacy.

Under radiopharmaceutical chemistry there were several areas that were considered as essential by all or almost all of the committee members. Areas and concepts included:

1) distinction between radionuclide, radiochemicals, and radiopharmaceuticals; 2) carrier and concepts such as carrier-free and carrier-added; 3) physical properties of radioactive compounds and radiopharmaceuticals; 4) biological properties of radiopharmaceuticals; 5) substrate specific and substrate nonspecific radiopharmaceutical localization; 6) the quality control of radiopharmaceuticals; 7) all aspects of the molybdenum-99/technetium-99m generator; and 8) technetium chemistry. The preparation and composition of technetium kits, and specific kits were rated as essential. Radiolabeling of blood cells was considered as essential knowledge. Safety techniques for handling radioiodide and solution chemistry for radioiodide were noted as essential while procedures for labeling various individual products with iodide were considered as less important. The quality control, physicochemical and kinetic properties of prepared radiopharmaceuticals from radionuclides such as gallium, indium, and thallium received endorsement as either essential or familiar depending upon the radionuclide.

For the three major areas listed under the heading of the clinical use of radiopharmaceuticals, the members of the committee strongly supported education in the preparation and monitoring of patients who receive radiopharmaceuticals. *In vivo* kinetics of radiopharmaceuticals such as normal and abnormal kinetics, the absorption, distribution, metabolism and elimination as well as factors that may affect the kinetics of radiopharmaceuticals received support as important to the education of a nuclear pharmacist. Interpretation of the procedure outcome, its' effect on patient management and economic implications were considered to be as familiar knowledge. So also, was sensitivity, specificity and the predictive value of diagnostic procedures as well as expected benefits of therapeutic procedures.

4. Conclusions

The process of developing harmonization and documentation in nuclear pharmacy education has only begun. The original working document must be modified to reflect input from committee members and then be reviewed again for content and format. Methodology for instruction could be added to the document. Also, methodology to allow the addition of knowledge areas specific to the practice of nuclear pharmacy in individual countries. For example, radioimmunoassay is considered of greater importance in some countries than others.

The committee on harmonization in nuclear pharmacy will prepare a second draft of the working document. The document will be distributed to a number of educators and practitioners for review. Additional guidance will be obtained at the 8th European Symposium on Radiopharmacy and Radiopharmaceuticals in Lillehammer early in 1999. Final validation will be obtained through solicitation of comments from a large number of educators and practitioners in nuclear pharmacy throughout the international community. It is anticipated that the finalized document would be available from a professional organization such as the International Pharmaceutical Federation (FIP) located at the Hague, The Netherlands.

The benefits of international harmonization in nuclear pharmacy education are numerous. Assurance of the availability of quality professionals to provide optimal products and care to the patient is a principle benefit. Spanning national barriers through the demonstration of self governance and unification in education will enhance the goal of increased freedom of employment between countries. Harmonization endeavors will improve existing educational programs through sharing of innovative concepts and knowledge between educators. Documents generated will benefit new educational programs especially in developing nations.

Indeed, harmonization is a worthy endeavor in order to bring together the strengths and experience of the international community for the benefit of all nations.

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TECHNOLOGY TRANSFER AND COUNTRY REPORTS

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**LOW-COST INDIGENOUS RADIOPHARMACEUTICAL KITS
MANUFACTURING CAPABILITY:
A SUCCESSFUL WORK ACCOMPLISHED IN ETHIOPIA**



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Abstract

Nuclear Medicine Unit at Black Lion Hospital is the only Nuclear Medicine service giving center in the country. We have been importing Radiopharmaceutical-kits for 10 subsequent years costly, with frequent irregularities, only limited Numbers of kits mainly for Liver, Brain, Thyroid & Kidney imagings. Most of the Nuclear Medicine (NM) diagnostic procedures were not undertaken at our unit, because of unavailability of vital Radiopharmaceutical-kits (Rp-kits) in the country since they were not manufactured in the country. In order to solve this long standing problem of the country persistent efforts were made. The success in Rp-kits manufacturing indigenously has the advantage of disseminating the NM Technology with in the country also. With the continuous efforts made 7 Aqueous-Rp-kits were manufactured successfully in our unit viz:- 1) ^{99m}Tc - s - colloid - for Liver imaging, 2) ^{99m}Tc -DTPA - for Brain + Renal imaging 3) ^{99m}Tc - MDP - for Bone imaging, 4) ^{99m}Tc -Tin (11) pyrophosphate for in-vivo R B C labelling (For the study of Blood-Pool and Myocardial Infarction), 5) ^{99m}Tc - Tin (11) Gluconate for Brain + Kidney Static imaging 6) ^{99m}Tc - Tin (11) Phytate for Liver imaging, 7) ^{99m}Tc - TBI for Myocardial perfusion study. Their physico-chemical behaving patterns were studied and the chemical and biological quality control procedures were conducted upon the indigenously produced kits at the National Drug Quality Control center and they were found to be sterile, apyrogenic & non-toxic. The efficiency of the kits was tested in many patients in our unit and found to be effective and reliable. Aqueous kits produced were observed to be as effective and reliable as their lyophilized counterparts with respect to their physico-chemical properties and biospecificity (organ specificity) but possessing short shelf lives unlike lyophilized kits.

1. Introduction

Nuclear Medicine is a standardized diagnostic medical technique nowadays applied significantly in most countries of the world. Radiopharmaceuticals are decisive tools of Nuclear Medicine. Cold kits or carriers are decisive on influencing the organ specificity of a particular radiopharmaceutical. After being prepared successfully employing different techniques the carriers are labelled with appropriate Radioisotopes obtained from radio-nuclide generator system produced by nuclear-reactor or cyclotron. When the pharmaceutical carriers are tagged with the radioisotope, they are called Radiopharmaceuticals.

We can also define the unlabeled Rp-kit administered into humans in the analogy as a moving car in the complete dark without its front or back light. It is an obvious fact that such a car cannot be traced or identified whether it is moving or not, and in which direction it is moving or where it has stopped. With this analogy we can also consider a radio-nuclide of a labelled Rp as the front or back light of a moving vehicle in the dark. We can easily identify such vehicle from a distance at night. We can trace in which direction it is moving because of its light, otherwise impossible.

Rp-kits are manufactured using various techniques in different countries optimizing the ratio of reacting substances, pH, amount of reducing agent, stabilizing agent, preservative, oxidation state of the main chemical entity, etc. by chemical manipulation. Once the kits are manufactured successfully in the solution form they can be lyophilized (freeze dried) to increase their shelf-lives or can be left as aqueous preparations to be kept at freezing temperature as low as possible. Otherwise their physico-chemical in vitro stability (e.g. labelling efficiency) is affected even with low room temperature which in turn adversely affects its biospecificity. Consequently aqueous kits do have short shelf-lives compared to their lyophilized counterparts. However, owning the lyophilizer is expensive and difficult to acquire for Nuclear Medicine Centers of developing countries like Ethiopia with low economic status and too many constraints in which the meagre resources are prioritized for worse problems.

In order to overcome such problems and establish reliable Nuclear Medicine services to patients in developing countries like Ethiopia, producing aqueous kits is a possible option to freeze dried kits.

Besides, storing the aqueous kits at very low freezing temperatures (e.g. deep freezes of -50°C or lower) until they are labeled for patient use is believed to increase their shelf-lives significantly (i.e. by several months) versus their counterparts kept at -10 to -20°C or higher temperatures. Hence, the aqueous kits are recommended to be stored in deep freezes of -70°C or lower, before they are used unlike most lyophilized kits which are kept at room temperature. Once the cold kits are manufactured successfully they can be stored properly in the shelf until they are employed for the patient being formulated as a radiopharmaceutical.

2. Objectives of the study

The objectives of the study are

- 1) to disseminate N M Technology in the country via producing Rp-kits locally
- 2) to enable the country to carry out various N M diagnostic procedures without sending the patients abroad
- 3) to decrease the operational costs of the Rps used in Nuclear Medicine Unit of the country and
- 4) to promote Radiopharmaceutical + Nuclear Medicine Research & teaching in the country

3. Materials and Methods

The following materials and methods were used

- A) Instruments: laminar air flow cabinet, Auto-claving machine, High sensitivity electronic balance, Bi-water distiller, pH-meter, Computerized Gamma-scintillation counter, Dose calibrators, Cappers & Decappers, etc.

- B) Chemicals commonly used $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$, HCl , NaOH , Ascorbic acid, distilled water, Sterile physiologic saline. etc and Related ligand (each carrier)

METHODOLOGY (COMMON)

- A) 1 Sterilize all the necessary materials used for kit preparation by autoclaving
- 2 Weigh the required ligand and dissolve it in physiologic saline
 - 3 Weigh their required quantity of $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ and dissolve in 0.5M HCl
 - 4 Filter the solution using 0.22 micro meter membrane filter & add some amount of this to the ligand solution of normal saline and make up the volume with physiologic saline to your requirement
 - 5 Again filter this solution with 0.22 micro-meter membrane filter
 - 6 Dispense the required volume to each vial in laminar air flow cabinet
 - 7 Put rubber bands and aluminum caps to each vial and cap the vials
 - 8 Label the vials

B) QUALITY ASSURANCE

One of the kits (vials) was taken and labeled with Tc-99m and In-vitro stability test of the Rp was conducted using ITLC & ascending paper chromatography in acetone, Normal saline, 85% Methanol and Acetate Buffer Daily. The percentage labelling and R_f values in the indicated solvent system were determined on the kits at each time. The follow up of the shelf-life of the manufactured kits continued in this manner for more than one month in similar method. After physico-chemical quality study was completed the product was sent for biological QC study. Then the products produced indigenously were tested for tissue specificity.

4. Results and Discussion

The In-vitro physico-chemical stability of locally manufactured kits were as good as those produced in technologically advanced countries in Nuclear Medicine, but slowly went on decreasing. For instance, R_f values and radiochemical purities are as good as those indicated in the literature [2,4,6-10,11]. However, the radiochemical purity (RCP) of the lyophilized commercial Tc-99m-DMSA in normal saline (ITLC and ascending paper chromatography - Whatmann No-1) showed lower values than indicated in the literature (Tables I). This might be due to the in-vitro instability of the commercial kit as a result of its long storage (i.e. over four years). It has also been observed from the chromatograms developed that the low radiochemical purity of Tc-99m-DMSA using ascending paper chromatography and ITLC in normal saline was due to the significant scattering of this kit in this solvent. However, there was less scattering of this Radiopharmaceutical in Acetone (ITLC) and 85% Methanol (ascending paper chromatography). Consequently, higher RCP values of the Radiopharmaceutical were observed in the above two solvent system (i.e. >99% & 96.5% respectively) Vs the very low RCP values in Normal Saline in ITLC & ascending paper

chromatography (i.e. 65% & 66.5% respectively). Similarly, the RCP Values of the locally produced aqueous $^{99m}\text{Tc-MDP}$ in the same solvent (normal saline) was also relatively low (i.e. ITLC-91% and paper chromatography - 85%). However, it is obvious that these values are much greater than that of the RCP values of Tc-99m-DMSA in the same solvent (Table-I). This may give us the clue that Acetone and 85% methanol might be more appropriate solvents than normal saline for the in-vitro QC evaluation of Tc-99m-DMSA. Nevertheless, more & statistically significant work should be continued on freshly prepared DMSA with the indicated solvents to substantiate the clue. Similar work can also be initiated on other radiopharmaceuticals to evaluate their relative in-vitro stability through time & determine the more appropriate solvent system for the individual radiopharmaceutical. Similarly, ascending paper chromatography in normal saline and 85% methanol & ITLC in acetone and normal saline were effectively used in the determination of RCP & Rf values of locally produced aqueous kits of DTPA, MDP, gluconate & sulfur colloid labeled with Tc-99m (Table I & II). The locally produced aqueous kits stored at -10 to -20°C were found to be more stable and possessed longer shelf-lives compared to those stored at 0°C or greater. It is presumed that if the aqueous kits are stored at very low temperature like -50°C or even lower, their in-vitro instability can be reduced & their shelf-lives can be elongated significantly. The RCP of locally produced aqueous kits were found to be greater when the determination was carried out immediately after labelling than when it was done in the later few hours. The kits were found to be free from any microbial contamination (i.e. sterile & apyrogenic) and non-toxic. The organ specificity of our kits were as good as kits from advanced countries in the beginning but went on decreasing through time (Tables I & II). The clinical imaging with those kits were found to be effective.

5. Conclusion and Recommendations

The aqueous Rp-kits manufactured in our unit are equally effective as kits from advanced nations. However, they are found to have short shelf-lives versus the lyophilized kits. We have stopped importing kits from abroad hence the cost of Radiopharmaceuticals operations has decreased. When we get an automated lyophilizer we will go one step ahead to produce lyophilized kits which are with long shelf-lives and will be able to supply Rp-kits to other centers in the country which are likely to be established. In addition, if the aqueous kits are produced at larger quantities they can expire before they are used unlike the lyophilized kits. On the basis of our experience on aqueous kits we would like to recommend the following:

- 1) The **Aqueous-kits** can also apply for other developing countries with limited resources and technology transfer.
- 2) Aqueous kits should be stored at very low temperature until they are labelled & used for patients to prevent their fast in-vitro physico-chemical degradation. Deep freeze of very low temperature like -50°C or even lower is required for this purpose.
- 3) Their possible in-vitro stability at various temperature ranges should be thoroughly studied.
- 4) More appropriate and additionally useful solvent system should be investigated for the study of physico-chemical properties of individual kits.

Table I QC OF LOCALLY PRODUCED AQUEOUS MDP and lyophilized Commercial DMSA* kits using ITLC (SG) and Ascending paper Chromatography (whatmann No.1) in different solvent system

Rf Values & Radiochemical purity (RCP) of ^{99m}Tc -MDP Vs Commercial ^{99m}Tc -DMSA as determined in Acetone, Normal saline and 85% Methanol (MeOH)

^{99m}Tc -MDP (ITLC - 7.5 cm)			^{99m}Tc -MDP (paper Chromat. 13.2cm)	
Solvent used	Acetone	Normal Saline	85% MeOH	Normal saline
QC parameters used				
Rf Value	0.00	0.85-1.00	0.00	0.85-1.00
RCP	100%	91%	99.5%	85%
^{99m}Tc -DMSA* (ITLC - 7.5 cm)			^{99m}Tc - DMSA* (paper Chromat. 13.2cm)	
Rf value	0.00	0.9-1.00	0.00	0.9-1.00
RCP	> 99%	65%	96.5%	66.5%

* Lyophilized Commercial DMSA kits are included here to evaluate the validity of the similar solvent system used in both cases as comparison to our **locally produced Aqueous-MDP**

Table II QC of locally produced Aqueous DTPA and sulfur Colloid (SC) kits using ITLC (SG) and ascending (Whatmann No.1) paper chromatography as determined in Acetone, Normal Saline and 85% MeOH

^{99m}Tc -DTPA (ITLC-7cm)			^{99m}Tc -DTPA (paper Chromat. 14 cm)	
Solvent used	Acetone	Normal Saline	85% MeOH	Normal saline
QC parameters used				
Rf Value	0.00	0.9-1.00	0.2-0.4	0.9-1.00
RCP	99.95%	98.9%	95%	98.6%
^{99m}Tc -SC (ITLC-7cm)			^{99m}Tc -SC (paper Chromat. 13.2cm)	
Rf value	0.00	0.00	0.00	0.00
RCP *	86%	86%	87%	87%

*The RCP OF Locally produced Aqueous sulfur Colloid is very low here due to its in -vitro instability through time.

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RADIOPHARMACEUTICALS PRODUCTION ACTIVITIES IN EGYPT

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Abstract

Applications of radiopharmaceuticals and labelled compounds in the field of nuclear medicine in Egypt have increased so rapidly in the last few years. At present, a large number of hospitals are utilizing these radioisotopic techniques for both diagnosis and treatment. The following production activities are taking place in the Egyptian Radioisotope Production Laboratories. By utilizing the research reactor a large number of radioisotopes which find wide applications in nuclear medicine were produced, such as iodine-131, phosphorus-32, sodium-24, potassium-42 and molybdenum-99 / technetium-99m generators. Gallium-67, indium-111 and iodine-123 will be produced locally after installation of the cyclotron at the end of 1998. A large number of Tc-99m based kits for diagnostic medical applications have been produced. Also, many radiopharmaceuticals labelled with iodine-131 were produced. The radioisotope production laboratory is able to supply many hospitals with the radioimmunoassay kits of the thyroid related hormones (T₄, T₃ and TSH). Research and development activities are taking place in the field of monoclonal antibodies and tumor markers with special consideration of AFP, CEA, PSA and β hCG.

Introduction:

Developments in the field of nuclear medicine increased extremely rapidly in the last few years. This included radiopharmaceuticals for diagnostic and therapeutic applications. A national radiopharmaceutical production programme has been laid down and consequently went into implementation with full cooperation and technical assistance by the International Atomic Energy Agency. The role of IAEA in the Egyptian radiopharmaceutical production programme may be considered a successful model for implementation in developing countries.

The programme involves the following fields of production activities

- Reactor-Produced isotopes
- Cyclotron-Produced isotopes
- Radiopharmaceuticals.
- Radioimmunoassay kits for thyroid-related hormones
- Monoclonal antibodies production
- Tumor markers

1- Production of radioisotopes:

a- Reactor-produced Isotopes:

Egypt has at present two research reactors, the first is a 2 Mw, $2 \times 10^{13} \text{ n cm}^{-2} \text{ s}^{-1}$ reactor which was commissioned in 1961. The second is a 22 Mw, $3 \times 10^{14} \text{ n cm}^{-2} \text{ s}^{-1}$ multipurpose type, which is capable among other functions to produce various types of radioisotopes

Egypt was since the early sixties utilizing the the Russian 2Mw reactor and a Norwegian-built radioisotope production plant to produce the following radioisotopes: Iodine-131, sodium-24, potassium-42, chromium-51, phosphorus-32 and colloidal gold-198. At present, only I-131 and P-32 are routinely produced while other isotopes were being replaced by the ideal and widely used Tc-99m for diagnostic nuclear medicine applications. As for the production of I-131, the Norwegian dry distillation method has been replaced in 1995, by the Hungarian molten tellurium dioxide method.

The commissioning of Egypt's Second Research Reactor in January 1998, with higher energy and flux, radioisotope production capabilities will be greatly enlarged in terms of types and quantities of isotopes. This includes Co-60 sources (upto 50,000 Curie), Ir-192 needles, and molybdenum-99 in the gel form for Tc-99m generator production. Moreover, the reactor is equipped with a boron neutron capture therapy facilities where boron is normally injected and localized in the brain, where neutrons are captured by boron with the consequence emission of alpha particles which destroy the tumor cells in the brain.

Mo-99 - Tc-99m chromatographic generator production cells have been installed where imported fission Mo-99 has to be loaded on a small alumina chromatographic columns. Up to 50 generators could be produced per batch .

From the economical point of view the column generator technology using fission Mo-99 was found to be expensive. This is due to the high costs of the imported Mo-99 which is mainly attributed to the fact that fission Mo-99 producers all over the world are limited.

In the last years considerable efforts have been directed towards the gel generator system for Tc-99m which combine the advantage of column generators and using the inexpensive (n, γ) produced Mo-99 using the research reactor. In this respect research activities were devoted towards the use of insoluble and radiation resistant radioactive molybdate gels as chromatographic column matrices.

A project for the production of Mo-99 - Tc-99m gel generators (gel in the form of zirconium molybdate) has started through an IAEA TC project EGY/2/008 and a contract has been signed with the Chinese Nuclear Power Institute for installation of cells for the production of Mo-99 - Tc-99m gel generators which will utilize the irradiation capabilities of Egypt's Second Research Reactor. The capacity of the production cells is up to 200 Curie per batch. The production procedure will be according to the guidelines of the GRP for radiopharmaceutical production.

b- Cyclotron-produced Isotopes.

A 20 MeV cyclotron, which is capable to accelerate charged hydrogen, deuterons helium-3 and Helium-4 will be commissioned late 1998. This will represent a flexible tool to produce a group of short lived isotopes for diagnostic medical applications.

Three production units are being installed for the production of iodine-123, gallium-67 and indium-111. These units are kindly implemented through an IAEA TC project. The cyclotron facility will also accommodate a unit for fast neutron therapy.

2- Production of Freeze Dried Technetium-99m Based Kits:

Research and development activities have been directed towards the production of Tc-99m radiopharmaceuticals for imaging of brain, liver, spleen, lungs, heart, kidneys and skeleton. The Tc-99m based kits listed in Table (1) had been locally produced. The products are prepared under aseptic conditions.

Table (1) : Locally produced Tc-99m-based kits

Kit	Application
Tc-99m-EHDP	Bone imaging agent
Tc-99m-MDP	Bone imaging agent
Tc-99m-DTPA	Dynamic kidney scanning agent
Tc-99m-Citrate	kidney scanning agent
Tc-99m-GHA	Dynamic kidney scanning agent
Tc-99m-DMSA	Static kidney imaging agent
Tc-99m-Phytate (precolloid)	Liver and spleen imaging agent
Tc-99m-HSAM	Lung imaging agent
Tc-99m-Sulfur colloid	Liver imaging agent
Tc-99m-PYP	Bone imaging agent

The freeze dried kits for Tc-99m labelling are subjected to quality control tests and measurements to ensure safety, efficacy, sterility, apyrogenicity, radiochemical purity, stability and suitability for the intended purpose.

R and D activities are going on to design new organic entities suitable for labelling with Tc-99m to be used as new radiodiagnostic agents and application in defined organ imaging. Also, much more work on Tc-99m complexes in solution appears to be necessary. In Table (2) are listed the Tc-99m based kits under study and those that will be studied in future.

Table (2): Tc-99m-Base kits under study

Kit	Application
Tc-99m-HIDA derivative	Hepatobiliary imaging agent
Tc-99m-MAG ₃	For renal function study
Tc-99m-ECD	For brain perfusion
Tc-99m-HM-PAO	For brain perfusion
Tc-99m-MIBI	For myocardial perfusion

- 1- Tc-99m-anti-CEA monoclonal Ab.
- 2- Tc-99m-receptor binding agents.

A well equipped laboratory for organic synthesis was installed in order to synthesize most of the compounds listed in Table (2).

3- Production of Radiopharmaceuticals Labelled with Radioiodine:

Under sterile conditions o-iodohippuric acid (OIH) is produced, for labelling with iodine-131. I-131-OIHA is used in the nuclear clinics as a renal function measuring agent. Also, α -methyl tyrosine is produced and labelled with I-131 as a diagnostic agent in parkenson's disease. Experiments were carried out on the labelling of some long chain fatty acids such as 16-bromohexadecanoic acid and 17-iodoheptadecanoic acid with iodine-131 as imaging agent for heart muscle.

4- Production of Radioimmunoassay (RIA) Kits:

Radioimmunoassay (RIA) is considered now in Egypt as the most common and main microanalytical technique for determination of minute quantities of biological substances such as hormones, enzymes, serum proteins, vitamins and drugs. Application of RIA techniques for "in-vitro" diagnosis has grown so rapidly in the last few years in Egypt, that now about 38 laboratories are performing RIA activities using various RIA kits which are imported from different companies in Europe and USA. The most currently used RIA kits in these laboratories are those for thyroid related hormones and sex hormones.

The enzyme linked immuno sorbent assay(ELISA) technique has been introduced in some laboratories in Egypt, however, its consumption is rather limited compared to the RIA techniques.

Difficulties concerning the use of RIA kits are mainly encountered from the importation from different manufacturers. Many problems arise from importation namely, increase of costs, expenditure of foreign currency, transportation problems, delay of delivery and finally changing from one supplier to another will seriously affect reliability of the data obtained from the same laboratory or from different laboratories using different brand for the same analyte. These facts revealed the necessity of adopting a project with a fundamental objective of local production of bulk reagents for various analytes which will make locally produced RIA kits available in the Egyptian market with lower costs and ease of delivery.

Now a very well equipped RIA laboratory has been established and which is routinely producing RIA kits for thyroid related hormones (T4 & T3 RIA kits and TSH IRMA kits) which is regularly distributed to many RIA laboratories and hospitals. All the components and reagents of the kits are prepared locally. R&D activities have taken place to develop new and improved techniques for the preparation of coated polystyrene tubes, polystyrene beads, cellulose particles and magnetizable particles. According to these R and D activities the RIA production laboratory is able to produce coated tubes either by using first antibody or by double antibody technique.

5- Tumor Markers:

Due to increased consumption of tumor markers in Egypt it has been found necessary to start a national project aiming to produce bulk reagents for RIA tumor markers specially those which find wide applications in our country. This of course will effectively minimize the cost/test for patients and will allow overcoming the logistics of importation of tumor marker kits which is very expensive and costs a lot of foreign currency.

A project for tumor markers production using the isotopic techniques has been adopted in cooperation with the IAEA (Tc project EGY/2/007) and four tumor markers which are commonly used in Egypt have been chosen to start with. These are AFP, CEA, PSA and β hCG. Installation of laboratories and their procurement with the essential equipments have started and five young scientists were sent abroad for training in this field.

The consumption per year of these tumor markers in some specialized hospitals utilizing either the isotopic on non-isotopic techniques are listed in Table (3).

Table (3): The approximate consumption rate/year (Assay tubes/year) of some tumor markers in some specialized laboratories in Egypt during 1996.

AFP		CEA		β hCG		PSA	
Isotopic	Non Isotopic	Isotopic	Non Isotopic	Isotopic	Non Isotopic	Isotopic	Non Isotopic
6100	8000	700	8600	6200	6100	600	6600

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QUALITY CONTROL AND ASSURANCE OF Tc-99m GENERATORS AND KITS APPLIED IN SAEC LABORATORIES

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Abstract

A brief description of quality assurance and quality control system applied in SAEC laboratories for production of ^{99m}Tc - radiopharmaceuticals, is provided. The system includes documentation, procedures, releasing of the products and responsibilities. The system described here undergoes a continuous development.

INTRODUCTION

Syrian Atomic Energy Commission had established a laboratory for production of Tc-99m Kits and generators in order to cover the demands of local hospitals. The products of this laboratory such as MDP, DTPA and PHYTATE kits and the eluate of the produced generators, are used in human being for diagnostic purposes, therefore any impurities or undesired forms of complexes which might affect the human health, image quality or localization of the radionuclides in the target organs should be controlled. In other words, in order to insure the efficacy and safety of products, several factors affecting the quality should be controlled during preparation process and several properties should be tested after preparation. Precaution is taken to insure that all steps of process are carried out in accordance with the rules of GMP and GRP.

In order to achieve the requirements of the quality, a severe quality assurance system should be established and implemented. Such system should be written and revised by responsible persons and all the procedures to be applied and records of all relevant data must be documented.

THE ESTABLISHED QUALITY ASSURANCE SYSTEM

DESCRIPTION OF THE SITUATION

The radiopharmaceutical laboratory in SAEC has been designed and established in Co-operation with IAEA, where the rules for GRP were considered in the design and installation process. The area of the laboratory is about 100m² which is divided in to three major parts which are: kit preparation, generator production and quality control laboratories.

The kit preparation lab consists of: clean room with LAF cabinet insuring class 1 environment, freeze drier, balances and filtration devices. laboratory for preparation of glassware and chemicals which contains equipments such as autoclave, pH meters, ultrasonic cleaners...., All spaces related to this lab . are considered controlled area.

Tc-99m generator laboratory contains: two hot cells which are provided with accessories and UV lamps for sterilization, in order to ensure higenity of internal environment , radio chemical hoods, isotope calibrator, working bench's, LAF for preparation of cold generator before loading it with Mo-99 in hot cell.

The quality control laboratory consists of radiochemical purity, sterility and pyrogenity tests, chemical purity test labs which contains gamma spectroscopy isotope calibrator, LAF cabinet, incubator and equibments for chromatography.

Quality is defined in different ways, but here we consider the quality as that products should satisfy the requirements specified by international standards. Quality assurance is defined in the guide of GMP as: ' 'the sum total of the organized arrangements made by the object of ensuring that products will be of the quality required by their intended use.' ' Quality control therefore can be defined in this context as" the verification of the quality of products to satisfy the predefined standard specification" ' then the products can be accepted or refused according to these verifications.

Hence the quality assurance system specified in our laboratories consider xxII USP as standard specifications, and the quality control programme applied is a conformity control. The system of quality assurance consider all procedures from receiving raw materials to releasing final products, with emphasis on the qualifications and safety of the staff involved in the preparation and quality control process as, in accordance with guides of GMP and GRP, in order to ensure good quality products. The most important steps involve:

- Disinfection of premises, glassware. And equipment's.
- Calibration and verification of instrument in use.
- Testing of chemicals and raw materials.
- Documentation of procedures and records.
- Determination of responsibilities.

DOCUMENTATION

The Standard procedures for all aspects starting with cleaning and disinfecting processes , preparation, QC.. ending and releasing of final products are described in written forms and documented with related records. The Records for all procedures should be signed by the persons involved. All QC Mecords should be signed by QC manager and the certificate of releasing the product should be signed by quality control manager and the head of the laboratory.

About 10 procedures and about 30 records are specified for the preparation and quality control of the Kits. Also similar numbers of procedures and records are expected for generators production.

THE QUALITY CONTROL SCHEME FOLLOWED IN THE LABORATORY

As mentioned before the radio pharmaceutical production laboratory in SAEC has been designed and established in co-operation with IAEA guide lines of GRP considered in the design and installation process. Although all procedures are carried out according to predescribed

procedures and all necessary precautions are considered during preparation process, products are undergone tests for quality with accordance to specified procedure and the results of these tests are compared with defined limits(Standards) in order to confirm the validity of the products (conformity tests).

Both prepared Kits and generators are undergone specified tests where they share some of them and here we will review each of them. Freeze-dried Kits are sealed under vacuum and them they are tested for physical chemical and radio chemical purities. Then the investigations are continue for sterility, pyrogenity and biodistribution in rats. In very rare cases bioscan investigations are carried out on rabbit. The main tests applied for quality control for generators are: the performance, the elution efficiency, the Mo-99 break through, the radionuclidic purity, the chemical purity, the radiochemical purity, physical purity, as well as sterility and pyrogenity.

Physical properities include appearance, color and pH value. Chemical purity in the Kits concentrate on the stannious content and in the eluate of generators concentrate on the alumina and (sodium chloride) contents. Stanious is determined iodometrically while aluminum is determiened by complexing with chromazurol-S.

Radiochemical purity for both Kits and generators eluates. are investigated using ascending chromatography, where the proportions of pertechnetate, hydrolyzed reduced technetium and technetium complexes are determined and compared with the allowed limits. Biodistribution of the Tc-99m labeled complexes in rat were investigated by intravenous injection of about technetium complexes are determined and compared with the allowed limits. Biodistribution of the Tc-99m labeled complexes in rat were investigated by intravenous injection of about 0.1-0.5 mci of Tc-99m in the tail. After about two hours from the injection the rat is scarified and the relevant organs are counted in fixed geometry. The biodistribution is calculated as the ratio of the activity in the organ to the total activity. In the case of DTPA Kits, the rat is counted after one hour from the injection and after one day, then the rat is scarified and the relevant organs are counted.

Sterility measures are considered for all steps of preparation by sterilization of all equipments and reagents in use, but also further tests are carried out on the final products.

The sterility tests were carried out using two media which are fluid thioglycolate and Soya bean casein. Incubation is carried out for two weeks at 35C° for FTM and at 25C° for SCD. Negative results indicate the sterility of the products.

Test for pyrogen is carried out using limulus amebocyte lysate (LAL) test where positive and negative standards are used for comparison .

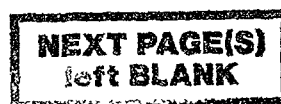
Radionuclidic purity of generators eluate and the Mo-99 breakthrough are assayed using a precalibrated gamma spectroscopy while the efficiency of the generator is determined using isotope calibrator.

CONCLUSION

As mentioned above quality of control Tc-99m radiopharmaceuticals and generators produced in SAEC laboratories undergoes long series of tests to ensure their validity for human use. The overall system of quality assurance applied also undergoes continuous development via research and training in order to keep our products competitive with others.

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THE PRODUCTION OF CYCLOTRON RADIOISOTOPES AND RADIOPHARMACEUTICALS AT THE NATIONAL ACCELERATOR CENTRE IN SOUTH AFRICA

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Abstract

Accelerator radioisotopes have been manufactured in South Africa since 1965 with the 30 MeV cyclotron at the Council for Scientific and Industrial Research (CSIR) in Pretoria. After its closure in 1988, the radioisotope production programme was continued at the National Accelerator Centre (NAC) with the 200 MeV separated-sector cyclotron (SCC) utilizing the 66 MeV proton beam, which is shared with the neutron therapy programme during part of the week. A variety of radiopharmaceuticals, such as ^{18}F -FDG, ^{67}Ga -citrate, a ^{67}Ga -labelled resin, ^{111}In -chloride, ^{111}In -oxine, an ^{111}In -labelled resin, ^{123}I -sodium iodide and ^{123}I -labelled compounds, ^{201}Tl -chloride, as well as the $^{81}\text{Rb}/^{81\text{m}}\text{Kr}$ gas generator, are prepared for use in the nuclear medicine departments of 12 State hospitals and about 28 private nuclear medicine clinics in South Africa. A few longer-lived radioisotopes, such as ^{22}Na , ^{55}Fe and ^{139}Ce , are also produced for research or industrial use. A research and development programme is running to develop new production procedures to produce radioisotopes and radiopharmaceuticals, or to improve existing production procedures. As part of a programme to utilize the beam time optimally, the production of some other radioisotopes is investigated.

1. INTRODUCTION

The National Accelerator Centre is a multidisciplinary research centre and is operated by the Foundation for Research Development (FRD) as a national facility. It provides accelerator and ancillary facilities for use on a national basis for :

- (a) Research and training in the physical, chemical and biomedical sciences.
- (b) Research in and the treatment of cancer with neutrons and charged particles.
- (c) The development of new or improved production procedures for radioisotopes for use in research, industry and in radiopharmaceuticals.
- (d) The development of new radiopharmaceuticals for use in nuclear medicine for diagnostic studies.

The NAC is the only particle therapy facility in the Southern Hemisphere and the only one in the world where both high energy neutrons and high energy protons are used for patient treatment. One of the treatment vaults contains the isocentric neutron therapy unit (66 MeV p/Be), while the 200 MeV horizontal proton beam therapy facility occupies a second vault. A second vertical proton therapy line is foreseen for a third vault.

2. RADIOISOTOPE PRODUCTION PROGRAMME

The 66 MeV beam is utilized for radioisotope production. Proton therapy takes place on Mondays, Tuesdays, Wednesdays and Thursdays, and neutron therapy on Tuesdays, Wednesdays and Fridays. The 200 MeV proton beam is used for nuclear physics research over the weekend. However, because of the tight time schedule, radioisotope production can only be done during night time (from about 20h00 until 06h00 the next morning) on Mondays, Wednesdays and Thursdays. The production of ^{67}Ga usually starts Monday evening and the bombardment of the zinc target continues (during night time), with a few interruptions of the bombardment, until Thursday morning. During these interruptions other radioisotopes, such as ^{18}F , ^{22}Na , ^{81}Rb , ^{111}In , ^{123}I , ^{139}Ce and ^{201}Tl , are produced. The chemical separation procedure to recover the appropriate radioisotope from the target material usually starts immediately after the end of the bombardment.

Table I shows the production details for ^{18}F , ^{22}Na , ^{55}Fe , ^{67}Ga , ^{81}Rb , ^{111}In , ^{123}I , ^{139}Ce and ^{201}Tl . The bombardment takes place in a bombardment station and the target usually consists of a metal or salt disc which is encapsulated in an aluminium can. At the end of the bombardment the target is transported to a hot-cell and the aluminium can cut open. The target is removed and transported to the processing hot-cell to recover and purify the appropriate radioisotope. The highly-pure radioisotope is then converted to the required radiopharmaceutical form, which is dispensed and dispatched to the nuclear medicine centres by road or by air.

Table I. Radioisotope production details.

Radionuclide	Production Reaction(s)	Target Material	Bombardment Energy (MeV)	Beam Current (μA)
^{18}F	$\text{Ne}(\text{p},\text{X})^{18}\text{F}$	Ne	63.0 – 58.3	20
^{22}Na	$\text{Mg}(\text{p},\text{X})^{22}\text{Na}$	Mg	61.5 – 40.0	80
^{55}Fe	$\text{Mn}(\text{p},\text{n})^{55}\text{Fe}$	Mn	35.2 – 11.0	80
^{67}Ga	$\text{Zn}(\text{p},\text{xn})^{67}\text{Ga}$	Zn	36.7 – 21.9	80
	$\text{Ge}(\text{p},\text{X})^{67}\text{Ga}$	Ge	61.5 – 38.5	80
$^{81}\text{Rb}/^{81\text{m}}\text{Kr}$	$\text{Kr}(\text{p},\text{xn})^{81}\text{Rb}$	Kr	52.5 – 45.0	30
	$\text{Rb}(\text{p},\text{X})^{81}\text{Rb}$	RbCl	62.9 – 57.7	65
^{111}In	$\text{In}(\text{p},\text{xn})^{111}\text{Sn} \rightarrow ^{111}\text{In}$	In/ In_2O_3 (55/45)	62.6 – 54.2; 53.0 – 43.4	80
^{123}I	$\text{I}(\text{p},5\text{n})^{123}\text{Xe} \rightarrow ^{123}\text{I}$	NaI	62.9 – 47.9	65
^{139}Ce	$\text{Pr}(\text{p},\text{X})^{139}\text{Ce}$	Pr	61.5 – 20.0	80
^{201}Tl	$\text{Tl}(\text{p},\text{xn})^{201}\text{Pb} \rightarrow ^{201}\text{Tl}$	Tl	28.6 – 21.0	30

Table II. Radioisotopes for medical and non-medical use produced and supplied to users during the period 1 April 1996 to 31 March 1997 (with corresponding values for the previous 12 months in brackets).

MEDICAL RADIOISOTOPES	Radiopharmaceutical	Consignments		Activity (MBq)	
	^{18}F -solution	1	(5)	876	(10849)
	^{18}F -FDG	3	(5)	936	(2233)
	^{67}Ga -citrate	493	(503)	257520	(283312)
	$^{81}\text{Rb}/^{81\text{m}}\text{Kr}$ -generators	187	(250)	103785	(138138)
	^{123}I -NaI-solutions	52	(50)	11789	(9364)
	^{123}I -NaI-capsules	47	(38)	15179	(12286)
	^{123}I -mIBG	203	(183)	65858	(58336)
	^{123}I -IPPA	3	(13)	765	(2273)
	^{123}I -BMIPP	15	(16)	3419	(3110)
	^{123}I -VIP	9	(8)	1010	(1768)
	^{123}I -epidepride	4	(0)	1057	(0)
	^{123}I -epidepride deriv.	2	(0)	549	(0)
	^{201}Tl -chloride	32	(24)	5220	(3434)
NON-MEDICAL RADIOISOTOPES	Radioisotope	Consignments		Activity (MBq)	
	^{22}Na	7	(8)	10854.6	(13673)
	^{139}Ce	1	(0)	2800	(0)
	^{111}In	11	(10)	1710	(1716)

The ^{18}F , ^{67}Ga , ^{81}Rb , ^{111}In , ^{123}I and ^{201}Tl are used to prepare the following radiopharmaceuticals for diagnostic studies at nuclear medicine centres :

^{18}F -FDG, ^{67}Ga -citrate, a ^{67}Ga -labelled resin, the $^{81}\text{Rb}/^{81\text{m}}\text{Kr}$ gas generator, ^{111}In -chloride, ^{111}In -oxine, an ^{111}In -labelled resin, ^{123}I -sodium iodide and ^{123}I -labelled organic compounds and ^{201}Tl -chloride (Table II).

Table II shows the medical radioisotopes delivered to nuclear medicine centres in South Africa and the non-medical radioisotopes produced during the 1996/1997 financial year.

3. RESEARCH AND DEVELOPMENT PROGRAMME

Chemical procedures have been developed for the recovery and purification of various radioisotopes from the bombarded targets, the preparation of labelled compounds, as well as quality control methods [1-6]. Nuclear data measurements have also been done at the NAC [7-10].

Research is continuously carried out to develop new methods, or to improve existing methods, for the recovery and purification of radioisotopes from the target materials. As part of a programme to utilize the beam time optimally, the production of some other radioisotopes is investigated. The labelling of organic compounds with ^{123}I to prepare new radiopharmaceuticals is also being investigated. New studies, which will begin soon, are those for the production of ^{75}Se , $^{82,83}\text{Sr}$, ^{103}Pd , ^{124}I and ^{124}I -labelled compounds. In a joint research and development project with the Forschungszentrum Jülich in Germany the excitation functions of $^{125}\text{Te}(p,2n)^{124}\text{I}$, $^{126}\text{Te}(p,3n)^{124}\text{I}$ and $^{85}\text{Rb}(p,3n)^{83}\text{Sr}$ reactions from thresholds up to 70 MeV will be measured. Therefrom, the optimum conditions for the production of ^{124}I and ^{83}Sr will be determined.

4. THE ACQUISITION OF A DEDICATED 70 MeV CYCLOTRON FOR THE PRODUCTION OF RADIOISOTOPES AND NEUTRON THERAPY

The two solid-pole injector cyclotrons and the SCC accelerate beams of charged particles to various energies for (a) nuclear physics research, (b) radioisotope production and (c) proton and neutron therapy. The cyclotrons have been operated since July 1996 according to an extremely tight schedule based on nine energy changes per week, required by the internationally-accepted daily treatment schedule for patients with protons (200 MeV) and neutrons (using 66 MeV protons). As the main components of the SSC and the injector

cyclotrons have now been in operation for 14 years, the rate of unexpected failures have increased significantly. This is partly due to ageing, wear-and-tear, corrosion, radiation damage, etc., but mainly because of the repeated electrical and mechanical cycling of the magnets, radiofrequency systems and other components of the cyclotrons and beam lines during energy changes. As a consequence of the much tighter time schedule and increased rate of failure, unplanned emergency repairs often have to be carried out during the already limited beam time available for the production of radioisotopes and neutron therapy. Since the various radioisotopes are only produced once per week no additional production run can be carried out. This causes a lot of discomfort to the nuclear medicine community and the patients, and causes a loss of income to the NAC. The continuous supply of cyclotron-produced radioisotopes is vital to the Health Sector in South Africa since short-lived radioisotopes cannot be imported economically. The rescheduling of diagnostic studies or neutron therapeutic treatments to other days is detrimental to patients, and wasting valuable time of radiographers and radiotherapists who travel to the NAC to supervise treatments. Therefore, the NAC plans to purchase a 70 MeV cyclotron in the future if it can obtain money from the government or elsewhere.

5. CONCLUSION

The NAC produces highly-pure radioisotopes which are used for the preparation of important radiopharmaceuticals for diagnostic nuclear medicine. It also produces longer-lived radioisotopes, mainly for the export market. The NAC is a centre of excellence for research in the physical, chemical and biomedical sciences and a lot of students (diploma, degree and post-graduate) benefit from the training and research done at the NAC.

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DEVELOPMENT OF RADIOISOTOPE PRODUCTION ON THE ACCELERATOR INSTALLATION TESLA



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Abstract

This paper describes several aspects of the development of radioisotope production on the VINCY Cyclotron of the Accelerator Installation Tesla, in the Vinča Institute of Nuclear Sciences, Belgrade, Yugoslavia.

The concept of radioisotope and radiopharmaceutical production is shown as well as the cross section of the part of the building where the channel for the radioisotope production H4 will be situated. The chemical processing of irradiated targets will be performed in the part of the Laboratory of Radioisotopes which is near (about 200 m) the cyclotron. Total area of the "hot" Laboratory is 144 m² with all necessary connections (ventilation, power, pneumo post, etc).

Based both on the cyclotron characteristics and on nuclear medicine needs in Yugoslavia we plan to produce ²⁰¹Tl, ¹¹¹In, ⁶⁷Ga, ¹²³I and ¹⁸F.

The radioisotope production will start with ²⁰¹Tl and the radiopharmaceutical ²⁰¹TlCl, which is currently imported.

The first "cold" experiments simulating the separation of ²⁰¹Tl from irradiated target consisting of enriched ²⁰³Tl were performed using the extraction method with butyl acetate.

Our experiments with ¹²³I confirmed that all labelling procedures applied for ¹³¹I-radiopharmaceuticals currently produced in the Laboratory of Radioisotopes: ortho-iodo-hippuric acid-¹³¹I (¹³¹I-IHA) and meta-iodo-benzyl-guanidine-¹³¹I (¹³¹I-MIBG) could be applied for ¹²³I-labelled radiopharmaceuticals.

1. INTRODUCTION

Nuclear medicine in Yugoslavia is well established medical speciality representing a recognized and important part of the overall health care system.

Nowadays, there are thirteen nuclear medicine centers in Yugoslavia dealing with in-vivo diagnostics, and over thirty centers performing in-vitro diagnostics. They can provide some 100,000 diagnostic, and around 1,000 therapeutic procedures yearly, using ^{99m}Tc , ^{125}I , ^{131}I and ^{201}Tl , mainly [1]

These centers are equipped with twenty five computerized gamma cameras and other related equipment. Presently, all of the common radioisotopes used in nuclear medicine can be applied, except the positron emitters. Nuclear medicine could readily accept new radiopharmaceuticals for modern diagnostic procedures based on ^{123}I , ^{111}In , ^{67}Ga , and with special collimators for 511 keV, even ^{18}F .

Due to its favorable geographic location, the VINCY Cyclotron is planned to be a regional center for the production of radioisotopes and radiopharmaceuticals for the Balkan region as well as for some other countries.

2. CHANNEL FOR THE PRODUCTION OF RADIOISOTOPES ON THE TESLA ACCELERATOR INSTALLATION (H4)

The engineering design of the shielding vault of the channel H4 for the production of radioisotopes of the TESLA Accelerator Installation has been finished.

Fig.1. presents the cross section of the channel H4 dedicated to the radioisotope production.

Table I: Radiopharmaceuticals labelled with ^{201}Tl , ^{123}I and ^{18}F

RADIOPHARMACEUTICALS	APPLICATION
^{201}Tl -chloride	Assesment of myocardium perfusion and tissue viability
^{123}I - iodine	Assesment of myocardium perfusion and tissue viability
- iodo amphetamine	Measurement of cerebral blood flow, identification of viable tissue
- fatty acids	Measurement of myocardium metabolism
- monoclonal antibodies	Cancer detection and monitoring of tumour progress
- hippurate	Assesment of renal function
- fibrinogen	Detection of thrombi
- MIBG	Diagnosis of pheochromocitoma and neuroblastoma
^{18}F - deoxyglucose	Glucose metabolism in central nervous system and cardiovascular system, investigation of epilepsy, stroke, ischaemia, etc
- receptor agents	Assesment of neuroreceptor pharmacology

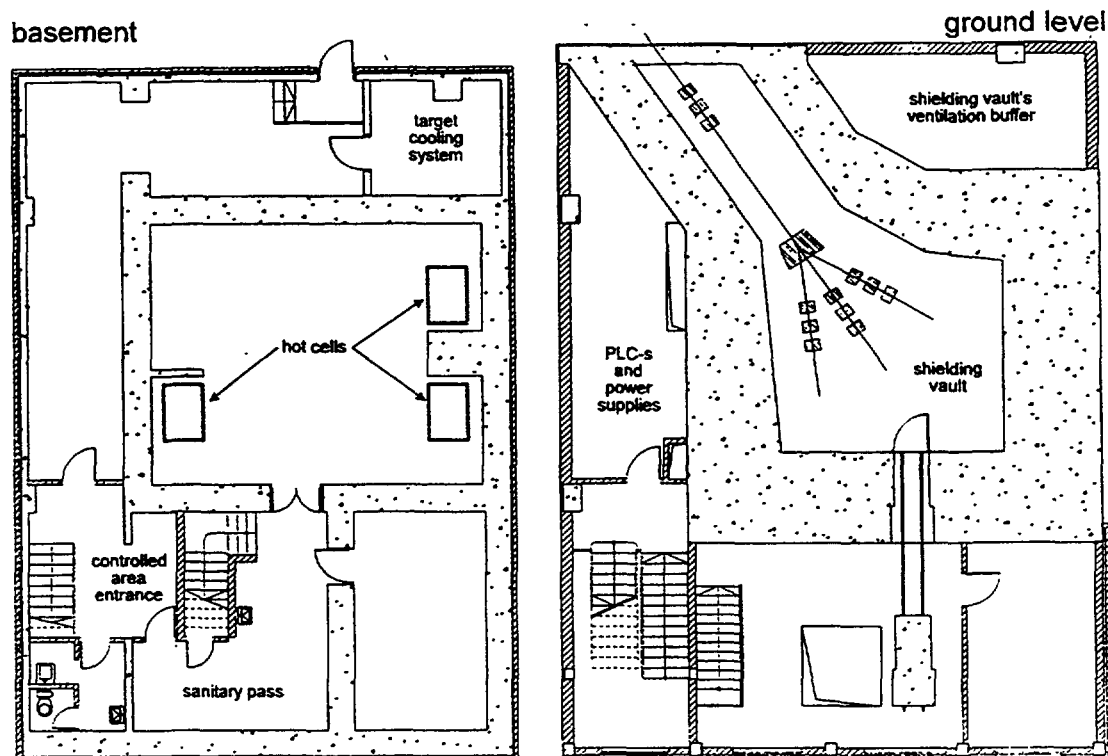


Fig. 1 Channel for radioisotope production

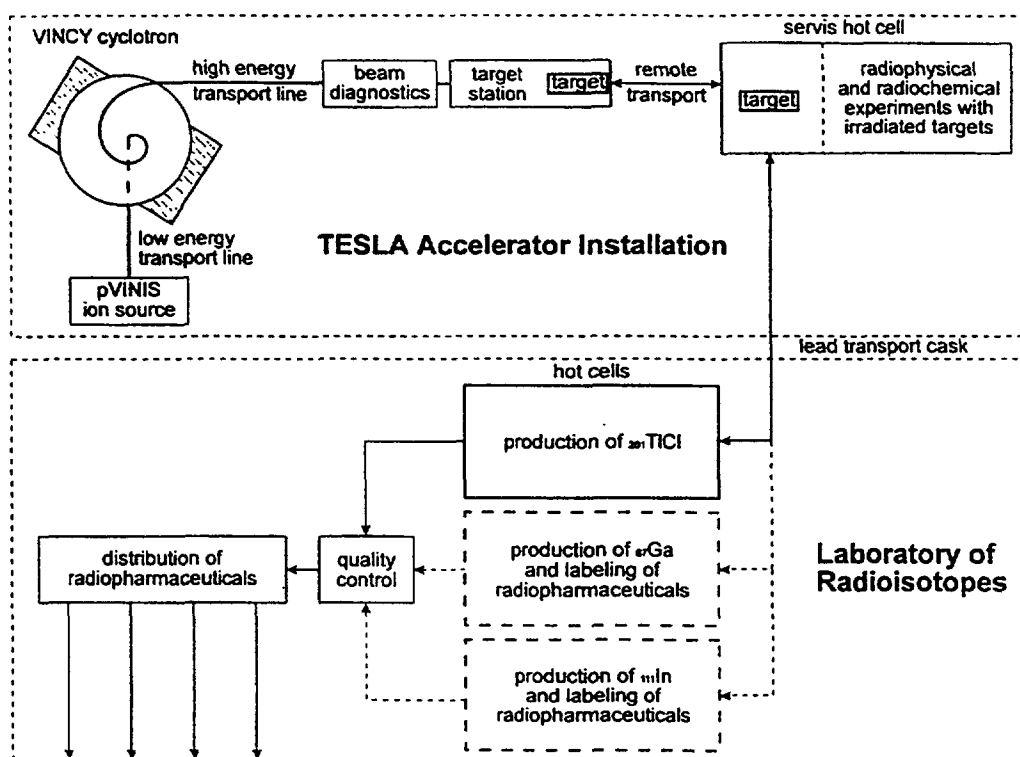


Fig.2 Concept of radioisotope and radiopharmaceutical production

Three target stations – for the irradiation of solid, liquid, and gaseous targets, are placed in the same room. The experimental channel H4 has a ventilation system which is independent on the ventilation system of the rest of the TESLA Accelerator Installation. This system has three zones, and a special regime of operation in the case of accident. A sanitary pass separates the radiation controlled area, the shielding vault and the area with the hot cells, from the rest of the experimental channel H4.

The beam transport line of the experimental channel H4 has been designed and the ion beam envelopes have been calculated in accordance with the engineering design of the shielding vault. The ion beam will reach the solid target without switching. A switching magnet with the radius of 1.5 m will bend the ion beam for $\pm 27^\circ$ and, thus, it will make possible the irradiation of the liquid, and gaseous targets.

The engineering design of the target station for the irradiation of solid targets has begun. The solid target will be transported to the target station and from it by a monorail transport system, connecting the shielding vault and a service hot cell, which is placed below it. The angle of incidence of the ion beam at the solid target will be 7° . The target will have its own cooling system.

Irradiated target will be placed in lead transport cask and forwarded to Laboratory of Radioisotopes for further chemical processing (Fig 2).

3. PARAMETERS OF THE BEAMS OF THE VINCY CYCLOTRON

The VINCY cyclotron is expected to deliver the following beams:

1. Proton beams:

70 MeV; current $2\ \mu\text{A}$

22-36 MeV; current $20\text{-}40\ \mu\text{A}$

7-16 MeV; current $60\ \mu\text{A}$

2. Deuteron beam:

60-73 MeV; current $10\text{-}20\ \mu\text{A}$

4. PLAN FOR THE ROUTINE PRODUCTION OF RADIOISOTOPES

The concept of radioisotopes and radiopharmaceuticals production is shown in Fig. 2.

The plan for the production of radioisotopes is based on several aspects:

- a) Clinical applicability (diagnostics and therapy)
- b) Costs of the production

c) Possibilities of the distribution of the produced radioisotopes and radiopharmaceuticals

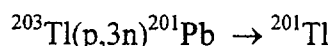
The preliminary list of radioisotopes for the production of radioisotopes on the VINCY Cyclotron includes ^{201}Tl , ^{111}In , ^{67}Ga and ^{123}I (γ -emitters) and ^{18}F (positron emitter).

Some other radioisotopes were also considered. There are γ -emitter $^{195\text{m}}\text{Au}$ and two positron emitters ^{15}O and ^{13}N . The generator systems $^{81}\text{Rb}/^{81}\text{Kr}$ and $^{82}\text{Sr}/^{82}\text{Rb}$ giving γ -emitting daughters, will be probably included.

The first radioisotopes which will be produced on the the VINCY Cyclotron are:

Thallium-201

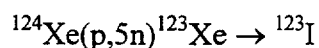
The suggested production route will be the irradiation of the solid target (metallic thallium with 98.5% enrichment in ^{203}Tl) by the proton beam of the energy of about 30 MeV by using the following nuclear reaction:



The method of separation will probably be extraction.

Iodine-123

For the production of this radioisotope the gaseous target consisting of ^{124}Xe (enrichment 99.3%) irradiated with the proton beam of about 30 MeV will be used. The radiochemical separation is rather simple as in the nuclear reaction noble gas ^{123}Xe is formed. The nuclear reaction of choice is:



Fluorine-18

The suggested production route is the nuclear reaction on water target (enriched in ^{18}O) by the protons of about 12 MeV via nuclear reaction $^{18}\text{O}(p,n)^{18}\text{F}$. The chemical form of fluorine is $^{18}\text{F}^-$ (aq).

Some of the most important radiopharmaceuticals labelled with these radioisotopes are given in Table I.

5. RESULTS OF THE PRELIMINARY EXPERIMENTS

5.1 Thallium-201

The chemical processing of the irradiated thallium target is divided into two steps. In the first step the produced radioactive lead is separated from the unreacted thallium. The separation procedure applied is extraction with buthyl acetate [2,3]. In the second step ^{201}Tl is separated from ^{201}Pb .

The first experiments were performed by using extraction as the separation method. Tl_2SO_4 was dissolved in sulphuric acid and Tl(I) oxidized to Tl(III) with KBrO_3 in the presence of NaCl . Thallium was then extracted with butyl acetate by performing two successive extractions.

It was found that the extraction depends on the concentration of sulphuric acid and NaCl . So 5M H_2SO_4 and 1.5M NaCl were chosen.

Distribution coefficient K_D in the first extraction was found to be up to 10^3 and depends on the concentration of Tl(III) and the efficiency of the separation of the organic and water phase.

The experiments were performed mostly with the inactive compounds. The concentrations of thallium and lead were determined by atomic emission spectroscopy. Some experiments were performed also by using the imported $^{201}\text{TlCl}$ (CIS bio international)

5.2 Iodine-123

Labelling of ortho-iodo-hyppuric acid (IHA) was performed by using two methods. The first is routinely used in our Laboratory for labelling IHA with ^{131}I [4]. It includes the isotopic exchange at 130°C during three hours. ^{123}I -IHA was separated by two successive crystallizations. The second method is also based on isotopic exchange, which is performed at 1 bar overpressure and 130°C during 30 minutes in an autoclave [5]. The separation of the labelled product is achieved by using the difference in solubilities of the reaction products.

Meta-iodo-benzyl-guanidine (MIBG) was labelled also by two methods. The first, called hydrothermal melting point method [6], is based on the chemical reaction occurring at the temperature between the solvent boiling point and the compound melting point. The second method [7] is called the catalytic method. The catalyst is Cu(I) ion. Sn(II) is used as the reducing agent and ascorbic acid as an antioxidant.

First method was found to be more advantageous.

Our experiments confirmed that all performed ^{131}I -labelling procedures could be applied for ^{123}I -labelled radiopharmaceuticals.

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