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Report of a co-ordinated research project
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DEVELOPMENT OF KITS FOR ^{99M}Tc RADIOPHARMACEUTICALS FOR INFECTION IMAGING

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FOREWORD

Infectious diseases remain a major health problem and cause of death worldwide, particularly in developing countries. Nuclear medicine imaging, because of its sensitivity, offers an attractive option for diagnosis of focal infections. This needs a reliable radiopharmaceutical that can selectively concentrate in sites of infection. Over the years ^{67}Ga and other radiopharmaceuticals that localize in inflammation associated with infection sites, also known as "non-specific agents" have been used for infection imaging. However, experience has shown that an "infection specific agent" that concentrates selectively at sites of infection and not inflammation would have several advantages. The first such agent developed more than two decades ago was ^{111}In-leucocytes which is still considered a "gold standard". Considerations of cost, availability, and superior properties for imaging make ^{99m}Tc a better label than ^{111}In. ^{99m}Tc white blood cell (WBC) was developed subsequently and used for infection imaging. However, both ^{111}In and ^{99m}Tc WBCs have a number of drawbacks, in particular: each patient's blood sample has to be collected and individually radiolabelled; well-trained staff and suitable facilities for separating and labelling the patient's blood are needed; the risk of infection and cross-contamination associated with potential blood-borne microorganisms; and cost of materials. Because of these, considerable efforts have been continuously made towards developing convenient replacements for ^{99m}Tc WBCs with limited success, ^{99m}Tc antigranulocyte antibody being a good example. However, these radiopharmaceuticals still have many disadvantages, related to either their cost and availability or their performance. In view of the large potential for applications in patients, the development of new and improved ^{99m}Tc labelled infection specific imaging agents was considered as a very worthwhile aim for scientific research in general and, in particular, for the establishment of a Co-ordinated Research Project (CRP) by the IAEA. The CRP could investigate alternate biochemical pathways, promising recent advances in ^{99m}Tc labelling methodologies and recent progress in evaluation methods. Based on recommendations of two consultants meetings, the IAEA initiated a CRP entitled Development of Kits for ^{99m}Tc Radiopharmaceuticals for Infection Imaging in 2000. Twelve laboratories from Asia, Europe, North America, and South America participated in the CRP, which was concluded in 2003.

Among the objectives of this CRP was the development of different ^{99m}Tc labelling strategies in participating laboratories that would be useful in the development of ^{99m}Tc labelled infection imaging agents. In addition, techniques were to be developed for the in vitro and in vivo testing of label stability. Finally, it was hoped that one or more of the identified agents would prove to localize in infection by a specific mechanism. The CRP may be said to be successful in all three measures. Finally, with the identification of ^{99m}Tc ubiquicidine fragment (UBI 29-41) as a radiolabelled agent with potential clinical utility, this CRP can be considered to have made a major contribution by providing the first validated specific ^{99m}Tc labelled infection imaging agent.

The IAEA wishes to thank all participants in the CRP and, in particular, E. Pauwels of Leiden University, Netherlands and D. Hnatowich of Massachusetts University, USA for providing material support and guidance to the participants.

The IAEA officer responsible for this publication was D.V.S. Narasimhan of the Division of Physical and Chemical Sciences.

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SUMMARY

1. INTRODUCTION

The most significant advancement in public health in the last century is undoubtedly the introduction of vaccines and antibiotics that have effectively eradicated or controlled most of the infectious diseases. In spite of the great strides in management of infectious diseases, infections remain among the most frequently encountered and costly causes of death and disease worldwide, particularly in developing countries. Since most of the infectious diseases can be diagnosed by simple laboratory tests and effectively treated with drugs, it can be perceived that a significant proportion of those resulting in mortality could be due to conditions difficult to diagnose. Timely diagnosis in such cases could help in instituting effective treatment and reduce morbidity and mortality.

Nuclear medicine imaging, because of its sensitivity, offers an attractive option for imaging focal infections. Some examples of conditions which can benefit from imaging are deep-seated muscular or orthopaedic infections especially those resulting from previous surgery; acute life-threatening infections which require immediate effective treatment such as acute appendicitis, severe chronic infections arising from drug-resistance; and opportunistic infections in immune-compromised individuals. The questions often answered by imaging are presence or absence of infection and its location, severity and probable cause. In order to answer these questions, the most important requirement is a reliable radiopharmaceutical that can selectively concentrate in sites of infection. The ideal radiopharmaceutical for imaging should be able to answer the clinical questions posed above, but in addition be non-toxic, of low radiation burden to the patient, showing minimal uptake in non target tissues, be inexpensive, easy to prepare and widely available.

Radiopharmaceuticals for imaging infection have generally been divided into two types — ‘specific’ and ‘non-specific’. The distinction between them relates to the mechanism of their action. ‘Non-specific’ agents work solely by their ability to localize at the site of inflammation that is often accompanied with infection. ‘Specific’ agents, while also exhibiting some degree of non-specific localization show an additional interaction with either the host immune system or the agent causing the inflammation that increases the efficiency of delivery. Both ‘specific’ and ‘non-specific’ radiopharmaceuticals may, depending on the nature of the condition under study is equally effective. For example, a ‘non-specific’ agent, ^{111}In-hIgG has shown high accuracy in diagnosis of number of infections by localizing in associated inflammations. However, in some cases, particularly with regard to identifying the cause of an inflammation, a ‘specific’ product may be more appropriate.

The most well established ‘specific’ agent that is still regarded as the ‘gold-standard’ for infection imaging is ^{111}In labelled WBCs. In view of the cost, limited availability, and not so favourable nuclear properties for imaging of ^{111}In, techniques for ^{99m}Tc labelling of WBCs have also been developed and used. However, both these products have a number of drawbacks, in particular: the need for labelling individual patient’s blood sample and reinjection; need for well-trained staff and suitable facilities; the risk of infection and cross-contamination; and the considerable cost of the materials required for cell labelling. Because of these, WBC labelling procedure has not become so extensively used commensurate with its potential and considerable efforts have gone towards developing convenient replacements. They include ^{99m}Tc labelled anti neutrophil antibodies, chemotactic peptides, and platelet factor. However, these radiopharmaceuticals retain many disadvantages related to either their cost and availability or their performance.

To achieve a more desirable ^{99m}Tc radiopharmaceutical for infection imaging, one displaying a faster blood clearance, less non- target uptake and better concentration in infection sites, it will be necessary to exploit alternate biochemical pathways. Some of these pathways like binding of anti microbial peptides for example ubiquicidine, have been recently explored with promising results. Recent years have also witnessed significant advances in ^{99m}Tc radiochemistry. Rational approaches to ^{99m}Tc labelling of different molecules have also been introduced. These can be fruitfully exploited for incorporating ^{99m}Tc into biomolecules of promise for infection imaging without altering the biological

properties. Considerable progress has also been made in methodologies for in vitro and in vivo evaluation and characterization of the ^{99m}Tc labelled agents. The labelling of various receptor specific peptides with ^{99m}Tc is a good example of such advances. These also can be very useful in objective evaluation of potential infection specific agents.

In view of the large potential for applications in patients, the development of new and improved infection imaging agents labelled with the most commonly used radioisotope for imaging, namely ^{99m}Tc, is still considered a very worthwhile aim for scientific research and development particularly in developing Member States. Development of ^{99m}Tc infection imaging agents was also identified as a relevant topic for a Co-ordinated Research Project (CRP) of the IAEA by two consultants meetings organized in 1995 and 1998. Based on their recommendations and considering the need for a superior ^{99m}Tc labelled infection imaging agent and advances in ^{99m}Tc radiopharmaceutical chemistry, a Coordinated Research Project entitled Development of kits for ^{99m}Tc radiopharmaceuticals for infection imaging was initiated in 2000.

Twelve laboratories from Asia, Europe, North America, and South America participated in the CRP that was concluded in 2003. The first Research Co-ordination meeting (RCM) held in Budapest, Hungary, took stock of the recent advances in ^{99m}Tc infection imaging agents, and decided on the potential agents to work on. Among the several ^{99m}Tc agents considered, three were identified for further study in the CRP. These were ^{99m}Tc ubiquicidine (UBI) as a 'specific' agent and ^{99m}Tc-ethylenediaminetetraacetic acid biotin monomer (EB1) and ^{99m}Tc-human neutrophil elastase inhibitor (HNE2) as superior 'non specific' agents. The available literature on all three agents suggested that their further study and evaluation could be useful for arriving at a desirable infection agent. The second RCM held in Mexico City reviewed the progress and decided to focus further efforts on UBI and EB1 in view of the promising results obtained and uncertainty in future availability of HNE2. The third and final RCM was held in Vienna to consolidate and document the results of all participants.

2. ACHIEVEMENTS OF THE CRP

In the three years of this CRP, a strong collaboration among participants was established as is evident by the exchange of information among participants and especially in the distribution of reagents. These collaborations are expected to continue even beyond the CRP.

Among the objectives of the CRP was the development of ^{99m}Tc labelling strategies in participating laboratories that would be useful in the preparation of infection imaging agents. In addition, techniques were to be developed for the testing of label stability in vitro and in vivo. Finally, it was hoped that one or more of the identified agents would prove to localize in infection by what appears to be a specific mechanism. The CRP can be considered successful in all three measures. In general, each participating laboratory became proficient in labelling the chosen molecules with ^{99m}Tc using a variety of techniques. By the final RCM, in vivo and in vitro quality assurance measurements were standardized by all participants and applied such that the ^{99m}Tc agents were viewed as reliably labelled. Finally, with the identification of ^{99m}Tc UBI as a radiolabelled agent with potential clinical utility, this CRP can be considered to have made a major contribution to nuclear medicine by providing the first convenient ^{99m}Tc labelled 'specific' infection imaging agent.

3. SUMMARY OF RESULTS

The results obtained in the different laboratories with each of the identified compound are summarized below

3.1. ^{99m}Tc human neutrophil elastase inhibitor (HNE2)

Source: HNE2 is available from a single source (provided from Dynax Corporation, USA) and its binding protein, human neutrophil elastase, is not available. The later makes quality assurance of radiolabelled HNE2 difficult. However, HNE2 is very similar in size and structure to BPTI (Aprotinin)

and BPTI binds effectively to trypsin. Both BPTI and trypsin are readily available. Therefore, BPT1 was used to standardize the conjugation and radiolabelling of HNE2. BPTI in sufficient quantities was provided by Dr. Hnatowich, USA, to be used as a model for the radiolabelling of the much less available HNE2

Labelling: HNE2 and BPTI were both radiolabelled using HYNIC/tricine, MAG3, and DTPA. Purification after labelling was necessary and was accomplished on either P4 columns or using a Sep-pak.

Quality assurance: Adequate quantities of trypsin were also supplied by Dr. Hnatowich, USA, to use in a shift assay with BPTI. Shift assays performed in Argentina and India with open column gel chromatography (Sephadex G75-100) showed the expected shift and, therefore, provided evidence that the BPTI was properly labelled.

Stability studies: Argentina showed by cysteine challenge assay of ^{99m}Tc DTPA-BPTI and ^{99m}Tc HYNIC–BPTI, that both were very stable. Both were also stable in PBS but both also showed binding to serum proteins, especially in the case of ^{99m}Tc HYNIC/tricine. Serum stability studies in India of ^{99m}Tc MAG_3-HNE2 also showed stability.

Animal studies: In Argentina, labelled BPTI showed high uptake in kidneys at 2 h post injection in mice. In India, MAG_3-BPTI provided high kidney uptake in normal mice. Monkey studies in Hungary with DTPA-HNE2 showed high kidney uptake, low liver, and a somewhat less than optimum T/NT ratio between 1.2-1.7 for inflammation vs. bacterial infection.

Summary: The ^{99m}Tc labelling of HNE2 or BPTI was accomplished satisfactorily. Due to the difficulty in procuring HNE2 after the first year, further studies to evaluate its usefulness in infection imaging had to be postponed.

3.2. ^{99m}Tc ethylenediaminetetraacetic acid biotin monomer (EB1)

Source: EB1 was provided by Dr. Hnatowich of USA initially while, later in the CRP, solid EB1 was synthesized and distributed from Dr. M. Jehangir of Pakistan and Dr. N. Ramamoorthy of India.

Labelling: ^{99m}Tc labelling of EB1 could be achieved with specific activities as high as 40 GBq/mgm.

Quality Assurance: ITLC with acetone was used to determine pertechnetate. Sep-pak could be used to measure ^{99m}Tc colloids. In this, ^{99m}Tc EDTA is eluted in the first wash with 0.001 M HCl and ^{99m}Tc EB1 in the second wash with 50% ethanol/saline. ^{99m}Tc colloids remain on the column. Typical labelling efficiencies were greater than 95%. The shift assay using sephadex G25 open column chromatography or HPLC were both used: the molar ratio of avidin was shown to be critical to achieve a complete shift. Kits containing tin for preparing ^{99m}Tc EB1 were produced in Pakistan, India, and Poland. The kit preparation from Poland was freeze dried using mannitol. There was general agreement that the tin content was not critical in these kits.

Stability studies: Analysis of ^{99m}Tc EB1 incubated for 24 h in human serum at 37^{o}C showed no evidence of instabilities.

Animal studies: ^{99m}Tc EB1 cleared rapidly through the kidneys showing some accumulation in this organ but otherwise very low backgrounds everywhere. The infection/inflammation ratio in mice was 1.4 and T/NT was about 2-3. However, uniform numbers of bacteria were not used to prepare the infection sites making comparison difficult. For example, an excellent T/NT ratio of five was achieved in Thailand using high concentrations of bacteria.

Summary: ^{99m}Tc EB1, regardless of source of EB1or whether labelled from a kit or instantly, is easily radiolabelled at high specific activity and the radiolabel is stable in vitro and in vivo. The

participants agreed that ^{99m}Tc EB1 probably provides superior T/NT ratios compared to ^{99m}Tc UBI but unlike the later is non-specific.

3.3. ^{99m}Tc ubiquicidine (UBI)

Source: The 29-41 amino acid fragment of Ubiquicidine (UBI 29-41) was used for ^{99m}Tc labelling in this CRP. Scrambled peptide of the same amino acids but different sequencing was used as control. Both were synthesized and distributed to participants by Dr. Pauwels of Netherlands. The peptide was found to be stable to transport. In addition, the peptide was also synthesized in Poland for HYNIC conjugation and for kit preparations in that country.

Labelling: ^{99m}Tc labelling of UBI 29-41 was carried out both by direct and indirect methods.

Direct method: As demonstrated in several participant laboratories, UBI 29-41 may be radiolabelled directly without the need for an exogenous chelator. The mechanism of direct labelling was investigated by molecular mechanics and quantum mechanical calculations by Mexico. The arginine and lysines appear to provide a cage for the reduced technetium. While the initial method developed in The Netherlands involved potassium borohydride and pyrophosphate, investigators in Mexico showed that both were not essential and might have functioned only to raise pH above 8. Thus, the final method excludes these reagents avoiding concerns regarding the toxicity of borohydride. Kits for the direct labelling of ^{99m}Tc UBI 29-41 have been developed in Poland, India, and Pakistan. The compositions of the three different formulations are listed in the relevant reports and all appear to work well.

Indirect method: MAG_3, DTPA, and HYNIC were considered for conjugation of UBI 29-41 and indirect labelling with ^{99m}Tc, in part, to determine their influence on the pharmacokinetic properties of the labelled agent. While DTPA conjugation was shown in Poland to provide a useful method of labelling UBI 29-41 with ^{111}In, this chelator gave too low a specific activity with ^{99m}Tc. In Argentina, ^{99m}Tc MAG_3-UBI 29-41 and ^{99m}Tc HYNIC/tricine UBI 29-41 were found to be similar in stability but the former gave lower binding to S. aureus. However, the difference in pharmacokinetics was dramatic in that kidney accumulations were much higher with the latter. While using HYNIC, between tricine and EDDA as coligands, labelling efficiencies at room temperature were better (>90%) with tricine and lower with EDDA. In the later case heating in boiling water bath improved labelling but the stability of the peptide under these conditions is uncertain. As shown in Poland, combined EDDA/tricine provides a poor label compared to tricine alone but the label is more stable in serum and is expected to show improved pharmacokinetics. UBI 29-41 was synthesized in Poland with the HYNIC attached but the properties appear to be exactly the same with the conjugated UBI 29-41. In all cases, the coupling ratio was 3:1 but groups attached per molecule were not measured. Two UBI 29-41 labelling kits were made in Poland, one with tricine and another with tricine/EDDA and kits were made in Thailand and Pakistan differing in the amount of tricine. However there appears to be no significant differences in pharmacokinetics, labelling yield (approx. 95%), stability of either the kit or the end product. The in vitro binding to S. aureus varied between 25–40%.

Quality assurance: Labelling efficiency was established using reverse phase HPLC, C18-Sep-pak, ITLC, and paper chromatography. Sep-pak was judged the method of choice for the analysis of ^{99m}Tc UBI 29-41. Labelled peptide is eluted with acidic methanol, pertechnetate with 0.001 M HCl and ^{99m}Tc colloids remain on the column. Nevertheless, HPLC analysis is essential to look for non-radioactive impurities such as tricine. Serum stabilities under physiological conditions were measured along with cysteine challenge as a further measure of quality with what can be considered as satisfactory results. The only quality assurance procedure of integrity of the peptide is bacterial binding.

Animal studies: Biodistribution in normal mice showed that the labelled peptide is predominantly excreted by the kidneys. ^{99m}Tc HYNIC/tricine UBI 29-41 exhibited much more liver radioactivity than ^{99m}Tc UBI 29-41 prepared by direct labelling which exhibited much higher kidney

activity. Stability in vivo of the peptide was shown in The Netherlands in that intact UBI 29-41 was identified in urine. The T/NT ratio was approximately 1.5–1.8 for indirectly labelled peptide compared to 2.5-3.5 for the direct labelled one, both after 2 h

Patients: Even though patient studies were not a primary objective of the CRP, one laboratory performed preliminary clinical trials. Seven patients in Mexico with suspected bone infections were studied with direct labelled ^{99m}Tc UBI 29-41, with the approval of the appropriate institutional and national regulatory committees. The T/NT ratio was about 2.2±0.7. Radioactivity levels were found at 4 h to be decreasing and at 24 hr no activity remained at the site of infection. Overall, the biodistributions look similar to that seen in mice. It appeared that the best imaging time is 1-2 h. Clinicians evaluated the images as "good" and "useful" and concluded that chronic infection showed slight uptake but acute infection showed intense accumulation. Several infections were confirmed by biopsy and there were no evidence of false positive results.

Summary: There seems to be no outstanding advantages to indirect labelling so that the participants agreed that direct labelling was preferred. There was also agreement that ^{99m}Tc UBI 29-41 appears to show specific binding to infection.

4. CONCLUSIONS AND RECOMMENDATIONS

The work done under the CRP has demonstrated that it is feasible to have ^{99m}Tc labelled compounds specific for infection imaging, as shown with ^{99m}Tc UBI 29-41. This can be considered to have paved the way for further development of more such ^{99m}Tc radiopharmaceuticals using other mechanisms as well. A novel approach to infection imaging using radiolabelled bacteriophage was proposed by Dr. Hnatowich of USA based on preliminary promising results obtained in his laboratory with a ^{99m}Tc labelled phage. The participants are expected to continue the collaboration established in the CRP to distribute M13 phage, study its labelling with ^{99m}Tc and compare its potential for infection imaging with that of ^{99m}Tc UBI.

The participants identified the absence of a uniform infection model that is available to all laboratories and that permits reliable cross comparisons. Further work in developing a reliable and reproducible animal infection model will be very useful in the pursuit of other ^{99m}Tc labelled infection imaging agents.

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REPORTS BY PARTICIPANTS IN THE CRP

IN VITRO AND IN VIVO EVALUATION OF ^{99m}Tc-LABELLED PEPTIDES FOR INFECTION IMAGING

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Abstract

The aim of the present work was to evaluate the different in vitro and in vivo behaviours of UBI 29-41 labelled with ^{99m}Tc by direct and indirect methods, in order to examine its specificity for detection of *S. aureus* infected sites. The UBI 29-41 was labelled with ^{99m}Tc by a direct method (98% labelling yield), using KBH_4 and stannous pyrophosphate in order to reduce the ^{99m}Tc. The conjugation of UBI 29-41 with NHS-MAG_3 and with NHS-HYNIC, for labelling with ^{99m}Tc was also studied. The conjugates were purified by Sephadex G-15 column and labelled with ^{99m}Tc using tricine as coligand for HYNIC conjugate and sodium tartrate for MAG_3 conjugate. Chromatographic studies were performed using ITLC and reverse phase and gel permeation HPLC. Radiochemical purities higher than 98% were obtained in all cases. Biodistribution studies and digital autoradiography in normal and *S. aureus* infected NIH mice were performed. Results were correlated with chromatographic and in vitro bacteria binding assays.

The purpose of this investigation was to select the best method for labelling UBI 29-41 in order to obtain images with the highest infected site / normal site ratio and a favourable biodistribution in mice.

1. INTRODUCTION

Radiolabelled peptides are an emerging class of radiopharmaceuticals that share chemical and biological properties. The study of peptides/receptor systems provides a novel means by which, using nuclear medicine imaging, one can characterize cellular structures and tissues. Although radiopeptides have potential for application across the whole range of nuclear medicine investigations, their intial focus was in oncology and the present interest has focussed especially on the field of inflammation and infection. Particular attention has been devoted to the use of cytokines to study acute and chronic inflammation [1], chemotactic peptides [2], platelet factors, monoclonal antibody derivatives, and non-specific tracers.

A radiolabelled human neutrophil elastase inhibitor has recently been investigated in a monkey model. It shows rapid plasma clearance and specifically binds to neutrophil elastase released at inflammatory sites by activated neutrophils providing early high-quality images [3]. Recently, radiolabelled antimicrobial peptides derived from ubiquidicin protein have been introduced in attempts to distinguish infection from sterile inflammation [4,5].

Direct or indirect labelling methods have been used to radiolabel these peptides with ^{99m}Tc. A widely used method for labelling small peptides is by conjugation of bifunctional chelators to the peptide and several attempts have been made using hydrazinonicotinamide (HYNIC) and N_3S compounds (S-benzoyl MAG3). The purpose of this investigation was to label different peptides (UBI 29-41, BPTI and HNE-2) with ^{99m}Tc and evaluate its suitability as agents for in vivo use. Labelling with ^{99m}Tc was carried out using a direct method and after conjugation of the peptides with different chelating agents, such as NHS-HYNIC, c-DTPA and NHS-acetyl-MAG_3.

2. MATERIALS

UBI 29-41 (TGRAKRRMQYNRR) and UBI 29-41 scrambled (KRNQRMARYRRGT) peptides were provided by E. Pauwels, NHS-HYNIC was provided by R. Mikolajczak and NHS-MAG_3 was provided by D. Hnatowich. Other chemicals were purchased from Sigma Chemical Co. *Staphylococcus aureus* (*S. aureus*) ATCC 25923 was provided by the Bacteriological Section of Instituto A. Roffo. Mice were provided by the Div. Bioterio of Ezeiza Atomic Center.

3. METHODS

3.1. Labelling of UBI 29-41 with ^{99m}Tc by direct method

3.1.1. Labelling procedure

10 µl of UBI peptide solution (1 mM in 0.01 M of acetic acid pH 4) was added to 2 µl of an aseptic solution of 0.5 mg/ml of stannous pyrophosphate. Immediately thereafter, 4 µl of a solution of 10 mg of KBH_4 per ml of 0.1 M NaOH was added. After addition of 0.1 ml of freshly eluted ^{99m}Tc-sodium pertechnetate, the mixture, having a final pH between 5 and 6, was gently stirred at room temperature for 15 minutes (min) and then used as such.

A similar radiollabelling was done with UBI 29-41 scrambled as a control. Likewise ^{99m}Tc labelled IgG was used as a control for inflammation.

3.1.2. Quality control

ITLC studies: Radiochemical purity was determined by instant thin-layer chromatography (ITLC) using saline or methyl ethyl ketone as solvent.

HPLC studies: The composition of the samples (1-15 µL) was analysed by Reverse Phase (RP) HPLC (Delta Pack column C18, Waters) and Gel Permeation (GP) HPLC (Protein Pack 60 column, Waters), and the recovery of the activity was checked in each case. The gradient for RP-HPLC was performed as follows: 0% B for 3 min, 0-100% B in 10 min, 100% B for 5 min, 100-0% B in 5 min, 0% B for 5 min; Solvent A (water/ TFA 0.1%) and solvent B (water/ TFA 0.1%- acetonitrile 40:60). Chromatography was carried out at a flow rate of 1.0 mL / min.

3.1.3. In vitro *assays*

Challenge with cysteine: ^{99m}Tc labelled peptide was tested for instability toward cysteine at different molar ratios of cysteine : compound (5:1, 50:1 and 500:1) in 0.2 M PBS (pH 7.2). Samples were incubated for 1 h at 37°C. After incubation, the percentage dissociation of ^{99m}Tc was measured by ITLC in saline.

Stability in serum and PBS: Approximately 15 µg of the labelled peptide were added to 200 µL of diluted human serum (1:20) and incubated at 37° C. The percentage of radioactivity bound to peptide was evaluated at 0, 2, and 24 h by GP-HPLC and ITLC.

Binding to bacteria: About 2 µg of ^{99m}Tc-labelled peptide each were transferred to Eppendorf vials. Next, 0.8 ml of 50% (v/v) of 0.01 M acetic acid in Na-PB containing 0.01% (v/v) Tween-80 was added to each. 0.1 ml of Na-PB containing 2.5×10^6, 5×10^6, 1×10^7 and 3×10^7 viable *S. aureus* respectively was added. The samples, with a final pH of 5, were incubated for 1 h at 4°C and thereafter, the vials were centrifuged in a pre-cooled centrifuge at 2000g for 5 min.

3.1.4. In vivo assays

Biodistribution studies were carried out following the general protocol described below:

Biodistribution in normal mice: The radiopharmaceutical (50 µL of labelled peptide) was administered by the tail vein of the mouse. Two hours (h) later, the animals were sacrificed by cervical dislocation and tissues of interest isolated. Percent of injected dose per gram in all major tissues were calculated.

Biodistribution in *S. aureus* infected mice: 50 and 80 µ L of *S. aureus.* suspension containing about 3×10^8 CFU/mL were injected i.m. in the mice thigh. 18 h later, the radiopharmaceutical was administered for the biodistribution study.

Biodistribution in mice injected with heat killed *S. aureus*: 1 mL of the above *S. aureus* suspension was heated at 100°C for 2 h to obtain killed *S. aureus.* 50 µL of this suspension were injected i.m. in the right mice thigh.

Biodistribution in mice injected with irradiated *S. aureus*: 1ml of *S.aureus* suspension containing about 3×10^8 CFU/mL was gamma irradiated with a 2.5 KGy dose to obtain the irradiated *S. aureus.* Non-viability was tested by cultivating bacteria in solid and liquid medium. 50 µL of *S. aureus* suspension were injected i.m. in the right thigh of mice.

Biodistribution in inflamed mice: Biodistribution studies were carried out in mice with a sterile turpentine-induced inflammation in the right thigh.

Digital autoradiographies: Accumulation of the tracers in normal, *S. aureus* infected or inflamed thighs of mice was assessed by digital autoradiography. Likewise, the thighs of mice injected with heat killed or irradiated bacteria were also assessed by this method. Data acquisitions were made on a digital equipment (Instant Imager, Packard) with a detector area of 20cm×24 cm (Oxford positron detector). Normal resolution was 0.5 mm pixels. Images of normal and injected thighs were acquired simultaneously at 2 h p.i. ROIs were generated over the injected site (target) and contralateral site (non-target) by drawing the region on the monitor. Target to non-target (T/NT) ratios were calculated without background correction.

3.2. Labelling of UBI 29-41 with ^{99m}Tc by indirect methods

3.2.1. Conjugation and labelling with NHS-MAG$_3$

A solution of NHS-MAG$_3$ (40 mg / mL in DMF) was added dropwise with agitation to a 5 mg/mL solution of UBI 29-41 in HEPES 0.1M pH 8.0. The final chelator/peptide molar ratio was 3: 1. After 30 min incubation, the conjugated peptide was purified on a Sephadex G-15 size-exclusion column eluted with 0.25 M ammonium acetate buffer, pH 5.2. Fractions were collected and analysed by UV absorbance at 280 nm (U-2001, UV/VIS spectrophotometer, Hitachi).

200 µl of MAG3-UBI 29-41 were mixed with 40 µl of a buffer consisting of 50 mg/ mL sodium tartrate in 0.5 M sodium bicarbonate, 0.25 M ammonium acetate and 0.18 M ammonium hydroxide at pH 9.2. Finally, 105 µl (89.9 MBq) of $^{99m}TcO_4^-$ and 5 µl of a fresh solution of $SnCl_2{\cdot}2H_2O$ (1mg/mL in 10 mM HCl) were added. The incubation time was 30 min at room temperature. A similar radiolabelling was done with UBI 29-41 without conjugation in order to use as a control.

3.2.2. Conjugation and labelling with NHS-HYNIC

The conjugation of UBI 29-41 with NHS-HYNIC was done at a chelator/peptide molar ratio of 3:1. Sephadex G-15 purification and analysis by UV spectrophotometry were carried out as in 3.2.1.

100 µL of a 10mg/mL of tricine solution in water was added to 25 µg of HYNIC-UBI dissolved in 25 µL of 0.25 M ammonium acetate, pH 5.2. After that 8.1 MBq of $^{99m}TcO_4^-$ and 10 µl of a fresh solution of $SnCl_2{\cdot}2H_2O$ (1mg/mL in 10 mM HCl) were added. The incubation time was 30 min at room temperature.

3.2.3. Quality control

See details in 3.1.2.

3.2.4. In vitro assays

See details in 3.1.3

3.2.5. In vivo assays

Biodistribution in *S. aureus* infected mice: The biodistribution studies were carried out in *S. aureus* infected mice according to the general protocol described in 3.1.4.

Digital autoradiographies: Accumulation of the tracers in *S. aureus* infected areas in mice was assessed by digital autoradiography according to the protocol described in 3.1.4.

3.3. Labelling of BPTI and HNE-2 with ^{99m}Tc by indirect methods

The methods used in this part of the research were taken directly from the literature (6). Only little changes on the protocol are mentioned below.

3.3.1. BPTI Conjugation and labelling with NHS-HYNIC and cDTPA

In the conjugation reaction, the quantities used were more than six times of BPTI, NHS-HYNIC, and cDTPA compared to literature (6). After Biogel P4 purification, the first four purified fractions of the HYNIC-BPTI and the first three of DTPA-BPTI were labelled with ^{99m}Tc. The labelled fraction with the best labelling efficiency was selected for each conjugated product.

DTPA-BPTI was labelled with ^{99m}Tc at pH 5.2 and HYNIC-BPTI was labelled with ^{99m}Tc using only tricine as coligand. ^{99m}Tc-DTPA-BPTI was purified by C18 Sep-Pack cartridge**.**

3.3.2. HNE-2 Conjugation and labelling with NHS- MAG_3

The chelator to peptide ratio was 20:1.

3.3.3. Quality control

See details in 3.1.2.

3.3.4. In vitro assays

See details in 3.1.3.

3.3.5. In vivo assays

See details in 3.1.4. Biodistributions in normal mice were carried out for ^{99m}Tc-[(Tricine)HYNIC-BPTI] and ^{99m}Tc-DTPA-BPTI (pH 5.2) after C18 Sep-Pack purification.

4. RESULTS

4.1. Labelling of UBI 29-41 with ^{99m}Tc by direct method

4.1.1. Labelling procedure

No particular difficulty was experienced in radiolabelling antimicrobial peptides with this method. Therefore, no post-labelling purification was required. The ranges of specific activity for ^{99m}Tc-UBI 29-41 and ^{99m}Tc-UBI 29-41 scrambled were 1-47.9 MBq/µg and 1.1–7.6 MBq/µg, respectively.

4.1.2. Quality control

ITLC studies: Radiochemical purity of ^{99m}Tc-UBI 29-41 and ^{99m}Tc-UBI 29-41 scrambled determined by ITLC was 95.5 ± 4.9% (n = 6) and 96.2 ± 3.1% (n = 3) respectively.

HPLC studies: The RP-HPLC profile of the ^{99m}Tc-UBI 29-41 showed one major peak (95.8 ± 2.9%, n = 22) at RT (retention time) = 10.87 min and a second peak (3.2%) at RT = 1.89 min (corresponding to pertechnetate). The percentage of recovery was 51.0–91.6% of the total activity and results were related to the number of HPLC injections.

Likewise, the RP-HPLC profile of the ^{99m}Tc-UBI 29-41 scrambled showed one major peak at RT = 10.35 min (96.2 ± 3.1%, n = 3) and the pertechnetate peak (2%). The percentage of recovery was 92% of the total activity. When these products were analysed by GP-HPLC the major peak was at RT=10.13 min and the pertechnetate peak at RT=16.93 min. The total recovery was 88.9%.

4.1.3. In vitro *assays*

Challenge with cysteine: Cysteine challenge showed that ^{99m}Tc-UBI 29-41 was less transchelated than ^{99m}Tc- UBI 29-41 scrambled (6.8% vs 25.2% of total activity at ratio 500:1).

TABLE I. STABILITY TO CYSTEINE CHALLENGE

	% of ^{99m}Tc transchelated to cysteine		
Molar ratio (cysteine to peptide)	5 to 1	50 to 1	500 to1
^{99m}Tc-UBI 29-41	1,2	3,3	6,8
^{99m}Tc-UBI 29-41 Scrambled	1,6	5,4	25,2

Stability in serum and PBS:

TABLE II. SERUM STABILITY (% OF TOTAL ACTIVITY BOUND TO PEPTIDE)

Time (h)	3	24
^{99m}Tc-UBI 29-41	26,7	21,42

Stability studies in PBS showed that ^{99m}Tc-UBI 29-41 was more stable than ^{99m}Tc- UBI 29-41 scrambled (92.4% vs 79.7% at 48 h).

TABLE III. PBS STABILITY % (OF TOTAL ACTIVITY BOUND TO PEPTIDE)

Time (h)	^{99m}Tc-UBI 29-41	^{99m}Tc-UBI 29-41 Scrambled
0	99,40	96,9
24	97,7	92,2
48	92,4	79,7

Binding to serum protein:

TABLE IV. PERCENTAGE OF TOTAL ACTIVITY BOUND TO SERUM PROTEIN

Time (h)	0	24
^{99m}Tc-UBI 29-41	49,6	73,7

Binding to bacteria: The results obtained by binding to *S. aureus* at different CFU per tube showed no significant differences between both labelled peptides.

TABLE V. PERCENTAGE OF TOTAL ACTIVITY BOUND TO *S. AUREUS*

Number of *S. aureus*(CFU)	^{99m}Tc-UBI 29-41	^{99m}Tc-UBI 29-41 Scrambled
2,50E+06	8,3	7,0
5,00E+06	28,9	30,6
1,00E+07	38,9	-
3,00E+07	41,4	40,9

4.1.4. In vivo *assays*

Biodistribution of ^{99m}Tc-UBI 29-41 in normal mice at 15min, 4 and 24 h p.i.: Biodistribution studies have shown main elimination via kidney for ^{99m}Tc-UBI 29-41.

TABLE VI. PERCENTAGE I.D./G FOR ^{99m}Tc -UBI29-41 AT 15 MIN, 4H AND 24 H

	15 min		4 h		24 h	
Organ	average	s.d.	average	s.d.	average	s.d.
Blood	2,13	0,82	0,16	0,03	0,03	0,01
Liver	7,70	0,01	4,35	1,67	1,22	0,42
Spleen	2,52	0,81	1,31	1,34	0,37	0,06
Kidney	19,13	1,14	12,72	3,57	1,17	0,14
Stomach	1,54	0,54	0,83	0,38	0,07	0,02
Intestine	0,63	0,08	0,84	0,40	0,25	0,35
Lungs	2,85	0,97	0,31	0,12	0,06	0,01
NT	0,35	0,23	0,08	0,06	0,00	0,00

Biodistribution of ^{99m}Tc-UBI 29-41 and ^{99m}Tc-UBI 29-41 scrambled (control agent) in S. aureus infected mice at 2 h p.i.: There were big differences between results corresponding at T/NT (Act/g) ratio of ^{99m}Tc-UBI 29-41 (2,56) and ^{99m}Tc-UBI 29-41 scrambled (0.99).

TABLE VII. PERCENTAGE I.D./G FOR ^{99m}Tc -UBI 29-41 AND ^{99m}Tc -UBI 29-41 SCRAMBLED AT 2H P.I.

	^{99m}Tc-UBI 50 µl *S.a.*		^{99m}Tc-UBI 80 µl *S.a.*		^{99m}Tc-UBI-scrambled	
Organ	average	s.d.	average	s.d.	average	s.d
NT	0,03	0,02	0,03	-	0,09	0,01
T	0,11	0,07	0,16	-	0,09	0,05
T/NT	3,03	0,70	5,83	-	0,99	0,53

Biodistribution of ^{99m}Tc-UBI 29-41 in mice injected with heat killed S. aureus. (2 h p.i.):

TABLE VIII. PERCENTAGE I.D./G. FOR ^{99m}Tc -UBI 29-41 AT 2H P.I.

Organ	average	s.d.
NT	0,04	0,02
T	0,05	0,00
T/NT	1,37	0,48

Biodistribution of ^{99m}Tc-UBI 29-41 in mice injected with irradiated S. aureus. (2 h p.i.):

TABLE IX. PERCENTAGE I.D./G. FOR ^{99m}Tc -UBI 29-41 AT 2H P.I.

	S.a. injected 2 days post irradiation		*S.a.* injected 2 weeks post irradiation	
Organ	average	s.d.	average	s.d.
Blood	0,16	0,04	-	-
Liver	3,56	0,50	-	-
Spleen	0,55	0,12	-	-
Kidney	19,75	1,23	-	-
Stomach	0,50	0,06	-	-
Intestine	0,57	0,18	-	-
Lungs	0,47	0,03	-	-
NT	0,18	0,23	0,24	0,02
IT	0,36	0,42	0,28	0,08
T/NT	2,32	0,83	1,16	0,35

Biodistribution of ^{99m}Tc-UBI 29-41 and ^{99m}Tc-IgG (as a control) in inflamed mice (2 h p.i.): Comparison of biodistribution results from both labelled radiopharmaceuticals in mice bearing an induced inflammation foci showed that T/NT for ^{99m}Tc-UBI 29-41 was 2 times lower than ^{99m}Tc-IgG at 4h p.i.

TABLE X. T/NT RATIOS FOR ^{99m}Tc -UBI 29-41 AT 2H P.I. AND ^{99m}Tc -IgG AT 4 H P.I.

	^{99m}Tc-UBI 29-41		^{99m}Tc-IgG	
Organ	average	s.d.	average	s.d.
NT	0,08	0,03	0,54	0,13
IT	0,11	0,03	1,28	0,16
T/NT	1,31	0,17	2,49	0,81

Digital autoradiography: Results obtained working over ROI of ^{99m}Tc-UBI 29-41 images provided by a digital autoradiography at 2h p.i had a good correlation in comparison with the biodistribution results.

TABLE XI. T/NT RATIOS FOR ^{99m}Tc-LABELLED MOLECULES CALCULATED FROM DIGITAL AUTORADIOGRAPHY

^{99m}Tc-labelled molecule		area (mm^2)	T / NT	s.d.
UBI 2 h	Inflammation	572	1,96	0,18
UBI 2 h	S.a. Infection	6,5–101	5,28	0,73
UBI 2 h	Irradiated S.a.	3-61	2,61	0,22
UBI 2 h	Heat killed S.a.	313	1,02	0,15
Scrambled (control) 2 h	S.a. Infection	17,5	3,38	-
IgG (control) 4 h	Inflammation	427–513	2.49	0,18

4.2. Labelling of UBI 29-41 with ^{99m}Tc by indirect methods

4.2.1. Conjugation and labelling procedure

The ranges of specific activity for ^{99m}Tc-MAG_3-UBI 29-41 and ^{99m}Tc-((Tricine)HYNIC-UBI 29-41) were 0.8–1.3 MBq/µg and 0.5-1.9 MBq/µg, respectively.

4.2.2. Quality control

ITLC studies: Radiochemical purity of ^{99m}Tc-[(Tricine)HYNIC-UBI 29-41] determined by ITLC was 96.2 ± 3.1% (n = 3).

HPLC studies: The RP-HPLC profile of the ^{99m}Tc -MAG3-UBI 29-41 showed one major peak (98.4 ± 0.9%, n = 5) at RT = 10.45 min and a second peak (1.6%) at 1.6 min corresponding to pertechnetate. Likewise, the RP-HPLC profile of the ^{99m}Tc - [(Tricine)HYNIC-UBI 29-41] showed one major peak (86.7% , n = 19) at RT = 10.47 min. The percentage of recovery was between 65–95% of the total activity for both radiopharmaceuticals. Due to its low radiochemical purity, ^{99m}Tc-[(Tricine) HYNIC-UBI 29-41] was purified by a Sep-Pack C18 cartridge before doing in vitro and in vivo assays. The final radiochemical purity after purification was 99%. The labelling yield of native UBI 29-41 used as a control in the MAG_3-UBI 29-41 labelling method was 42%, showing a high non-specific binding for this indirect method.

4.2.3. In vitro *assays*

Challenge with cysteine: It showed that ^{99m}Tc-MAG_3-UBI 29-41 was less transchelated than ^{99m}Tc-[(Tricine) HYNIC-UBI 29-41] (1.7% vs 6.1% of total activity at ratio 500:1).

TABLE XII. % OF ^{99m}TC TRANSCHELATED TO CYSTEINE

Molar ratio (cysteine to peptide)	5 to 1	50 to 1	500 to 1
^{99m}Tc-MAG_3-UBI 29-41	0,8	1,1	1,7
^{99m}Tc-[(Tricine)HYNIC-UBI 29-41]	8,8	9,4	6,1

Stability in serum:

TABLE XIII. SERUM STABILITY (% OF TOTAL ACTIVITY BOUND TO PEPTIDE)

Time (h)	3	24
^{99m}Tc-MAG_3-UBI 29-41	-	93.8
^{99m}Tc-[(Tricine)HYNIC-UBI 29-41]	91,2	80,6

Binding to serum protein:

TABLE XIV. PERCENTAGE OF TOTAL ACTIVITY BOUND TO SERUM PROTEIN

Time (h)	0	24
^{99m}Tc-MAG_3-UBI 29-41	40	49,1
^{99m}Tc-[(Tricine)HYNIC-UBI 29-41]	32	43

Binding to bacteria: The results of binding to bacteria of ^{99m}Tc-[(Tricine)HYNIC-UBI 29-41] were better than those corresponding to ^{99m}Tc-MAG_3-UBI 29-41 and were worse than those of UBI 29-41 labelled by direct method.(see 4.1.3.4.)

TABLE XV. PERCENTAGE OF TOTAL ACTIVITY BOUND TO *S. AUREUS*

Number of *S. aureus* (CFU)	^{99m}Tc-MAG$_3$-UBI 29-41	^{99m}Tc-[(Tricine)HYNIC-UBI 29-41]
2,50E+06	6,6	3.9
5,00E+06	6,7	11.9
1,00E+07	12,5	31.4
3,00E+07	15,9	-

4.2.4. In vivo *assays*

Biodistribution of ^{99m}Tc-MAG$_3$-UBI 29-41 and ^{99m}Tc-[(Tricine)HYNIC-UBI 29-41] in S. aureus infected mice (2 h p.i.):

TABLE XVI. % I.D./G FOR ^{99m}Tc -MAG3-UBI 29-41 AND ^{99m}Tc-((TRICINE)HYNIC-UBI 29-41) AT 2H P.I.

	^{99m}Tc-MAG$_3$-UBI 29-41		^{99m}Tc-[(Tricine)HYNIC-UBI 29-41]	
Organ	average	s.d.	average	s.d.
Blood	0,20	0,03	1,71	0,18
Liver	0,63	0,17	16,89	1,50
Spleen	0,08	0,01	0,90	0,12
Kidney	0,77	0,13	45,46	7,45
Stomach	0,37	0,49	4,08	1,81
Intestine	3,43	0,77	6,22	2,76
Lungs	0,16	0,05	2,36	0,23
NT	0,04	0,01	0,26	0,07
IT	0,06	0,02	0,45	0,09
T/NT	1,60	0,56	1,76	0,10

Digital autoradiography:

TABLE XVII. T/NT RATIOS FOR ^{99m}TC -LABELLED MOLECULES CALCULATED FROM DIGITAL AUTORADIOGRAPHIES

Biomolecule		n	T/NT	error	area (mm^2)
^{99m}Tc-MAG$_3$-UBI 29-41.	*S.a.* Infection	3	3,08	1,11	184-237
^{99m}Tc-[(Tricine)HYNIC-UBI 29-41]	*S.a.* Infection	3	2,15	0,53	186-247

4.3. Labelling of BPTI and HNE-2 with ^{99m}Tc by indirect methods

4.3.1. BPTI Conjugation and labelling with NHS-HYNIC and cDTPA

Radiochemical purity of ^{99m}Tc-[(Tricine) HYNIC-BPTI] and ^{99m}Tc-DTPA-BPTI determined by RP-HPLC was higher than 95% and 55%, respectively. The range of specific activity of these products was between 0.07-0.6-MBq/µg. Fraction 2 had the highest labelling yield for HYNIC-BPTI with tricine. Fraction 1 had the highest labelling yield for DTPA-BPTI at pH 5.2. The radiochemical purity of the C18 Sep-Pack purified ^{99m}Tc-DTPA-BPTI was 77%.

TABLE XVIII. LABELLING EFFICIENCY AND % OF RECOVERY FOR ^{99m}Tc-LABELLED BPTI AND HNE-2

Fraction	HYNIC-BPTI	DTPA-BPTI	MAG_3-HNE-2	HYNIC-BPTI	DTPA-BPTI	MAG_3-HNE-2
1	86,1	60,9	0			-
2	94,6 ± 4,2	57,1 ± 5,0	99.0	95,8%	88%	95.7%
3	38,7	2,4				-
4	54					-

4.3.2. HNE-2 Conjugation and labelling with NHS- MAG_3

Radiochemical purity of ^{99m}Tc-MAG_3-HNE-2 determined by RP-HPLC was 99.0%. The specific activity was range of 1.4 MBq/µg. Fraction 2 had the highest labelling yield.

4.3.3. Quality control

HPLC studies:

TABLE XIX. RETENTION TIMES AND RADIOCHEMICAL PURITIES OF ^{99m}Tc-LABELLED BPTI AND HNE-2

RT(min)	^{99m}Tc-[(Tricine) HYNIC-BPTI]	^{99m}Tc-DTPA-BPTI	Sep-Pack purified ^{99m}Tc-DTPA-BPTI	^{99m}Tc-MAG_3-HNE-2
2.0	1.8%	37.7%	2.4%	1.0%
10.0				99.0%
11.7	98.2%			
11.9			77.0%	
12.3		62.3%		
13.4			20.6%	

4.3.4. In vitro assays

Challenge with cysteine: The results of cysteine challenge assay of labelled BPTI are similar to labelled HNE2 and different from HNE4. The anomalous behaviour of HYNIC-BPTI was very similar to HYNIC-HNE2. (6)

TABLE XX. % OF ^{99m}Tc TRANSCHELATED TO CYSTEINE

Molar ratio (cysteine to peptide)	0.5	5	50
^{99m}Tc-DTPA-BPTI	0	0.2	1.0
^{99m}Tc-[(Tricine) HYNIC-BPTI]	5.7	21.2	5.3

Stability in PBS: Both radiollabelled products show very good stability in PBS. The dissociation of ^{99m}Tc-[(Tricine) HYNIC-BPTI] was less than 10% at 24 h. (analysed by RP-HPLC)

TABLE XXI. PBS STABILITY (% OF TOTAL ACTIVITY NOT BOUND TO PEPTIDE)

Time (h)	0	24
^{99m}Tc-DTPA-BPTI	48.0	48.6
^{99m}Tc-((tricine)HYNIC-BPTI)	1.0	6.2

Binding to serum protein: The activity bound to serum protein of ^{99m}Tc-[(Tricine) HYNIC-BPTI] was 20% higher than the value of ^{99m}Tc-DTPA-BPTI.

TABLE XXII. % OF TOTAL ACTIVITY BOUND TO SERUM PROTEIN

Time (h)	0
^{99m}Tc-DTPA-BPTI	78.3
^{99m}Tc-[(Tricine) HYNIC-BPTI]	97.9

Binding to trypsin: Sephadex G75 radiometric elution profile of ^{99m}Tc-[(Tricine) HYNIC-BPTI]: trypsin (molar ratio 1:10) showed the biggest maximum activity in the fraction number 8 corresponding to the complex and other maximum in fraction number 12 corresponding at ^{99m}Tc-[(Tricine) HYNIC-BPTI] (Fig. 1).

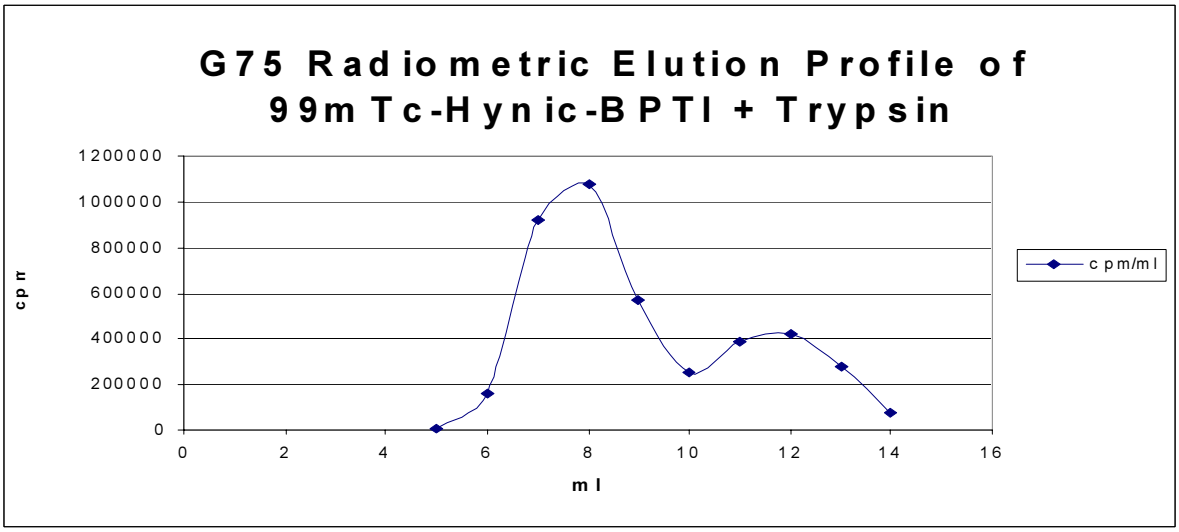

FIG. 1. Shift in radioactivity profile of ^{99m}Tc-[(Tricine) HYNIC-BPTI] on binding with trypsin.

4.3.5. In vivo assays

Biodistribution of ^{99m}Tc-[(Tricine) HYNIC-BPTI] and ^{99m}Tc-DTPA-BPTI in normal mice: The highest radioactivity levels were found in liver and spleen following administration of ^{99m}Tc-DTPA-BPTI at pH 5.2. The highest radioactivity levels were found in kidneys following administration of ^{99m}Tc-[(Tricine) HYNIC-BPTI]. The two compounds showed different patterns of biodistribution. First biodistribution pattern was similar to HYNIC-HNE2 and HYNIC-HNE4 and the second was similar to DTPA-HNE2 and DTPA-HNE4 (pH 5.2) (6).

TABLE XXIII. % I.D./G FOR ^{99m}Tc-[(TRICINE) HYNIC-BPTI] AND ^{99m}Tc -DTPA-BPTI (PH 5.2) AT 2H P.I.

	^{99m}Tc-[(Tricine) HYNIC-BPTI]		^{99m}Tc-DTPA-BPTI (pH 5.2)	
Organ	average	s.d.	average	s.d.
Blood	1,54	0,66	1,35	0,14
Liver	14,05	5,22	6,25	2,31
Spleen	7,02	4,96	2,22	0,66
Kidney	72,50	23,71	171,70	13,02
Stomach	1,31	0,72	0,88	0,20
Intestine	2,33	1,04	0,53	0,09
Lungs	4,32	2,28	2,81	0,67

5. CONCLUSIONS

Labelling UBI 29-41 by direct method showed a stable product with high yield and good in vitro properties.

The labelled UBI 29-41 showed the highest target to non target ratio when injected in *S. aureus.* infected mice compared with the labelled UBI 29-41 scrambled peptide and labelled IgG. On the other hand, ^{99m}Tc-IgG showed the highest T / NT ratio in inflamed mice.

The T/NT ratio for the ^{99m}Tc-UBI 29-41 increased from 3.0 to 5.8 when using 80 μL of bacteria suspension instead of 50 μL.

In the experiments with viable, irradiated and heat killed bacteria, the results showed a decreasing T/NT ratio (3.0, 2.3 and 1.4). These data could suggest that the envelope of the non-viable bacteria (irradiated) has not suffered modifications 48 h post irradiation since the electrostatic binding still appear to be functioning. On the other hand, in the case bacteria two weeks post irradiation, the T/NT ratio had no significant differences (LC = 5%) with T/NT for heat killed bacteria.

Comparing the T/NT ratios of ^{99m}Tc UBI 29-41 labelled by direct and two indirect methods (HYNIC and MAG_3), a decreasing uptake at the infected site was observed (3.03 ± 0.70, 1.76 ± 0.10 and 1.60 ± 0.56 respectively), although the two indirect methods did not show significant differences (LC=5%) . These data are in agreement with the in vitro binding results. ^{99m}Tc-((tricine)HYNIC-UBI 29-41 showed a higher kidney elimination than the other ones. ^{99m}Tc-MAG_3-UBI 29-41 showed a different pattern of biodistribution.

The labelling procedures for BPTI and HNE-2 were carried out with good yields except in the case of using cDTPA. Different patterns of biodistribution were obtained for labelled BPTI conjugated with cDTPA and HYNIC respectively.

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^{99m}Tc-LABELLED LIGANDS FOR INFLAMMATION AND INFECTION IMAGING

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Abstract

Detection of inflammation and infection foci at an early phase, non-invasivelly, sensitively and specifically is a great challenge for nuclear medicine specialists even today. We investigated ^{99m}Tc-labelled EB1, UBI and HNE2 for imaging infections. All three agents showed high labelling efficiency. The label proved to be stable for 24 h in physiological saline and human serum. Animal examinations revealed a fast renal excretion, low background activity for all the three molecules; ^{99m}Tc EB1 showed the best T/NT ratio in inflammation models, ^{99m}Tc UBI proved to be specific for infection imaging and ^{99m}Tc HNE2 was found to be useful only in the primate model.

1. INTRODUCTION

To achieve a more desirable radiopharmaceutical for infection imaging, one displaying a faster blood clearance, less non-target uptake and better concentration in infection/inflammation sites, it will be necessary to exploit alternate biochemical pathways. Some of these pathways like antimicrobial peptides have been recently explored with promising results. The recent years have also witnessed significant advances in ^{99m}Tc radiochemistry and rational approaches to ^{99m}Tc labelling of different molecules have been introduced.

In our study different specific and non-specific agents were evaluated in normal and infection/inflammation induced animals following the protocols finalized in the 1st RCM.

2. MATERIALS AND METHODS

In this work we labelled and tested the following agents:

- ethylendiaminetetraacetic acid biotin monomer (EB1, kit form, Polatom, Poland))
- ubiquicidin (UBI, kit form, Polatom, Poland)
- DTPA– HNE2 conjugate
- HYNIC- UBI conjugate
- DTPA –UBI conjugate
- HNE2

2.1. Radiolabelling

All the radiolabelling reactions were performed as described in the reports of 1st and 2nd Research Coordination Meeting of the CRP and the Leiden Protocol.

2.1.1. ^{99m}Tc *EB1*

The kit obtained from POLATOM was labelled with ^{99m}Tc as per protocol recommended.

Quality control: The yield of kit containing EB1 was determined by ITLC using 0.9% saline (ITLC-SG will assess the percent of ^{99m}Tc present as colloid Rf=0.0) and acetone (ITLC-SG or Whatman No.1, will assess the percent as pertechnetate Rf=0.9) as eluent. Samples of 1-2 µL were applied at 3 cm from the bottom. After developing, the chromatography sheeet was sliced into 1 cm sections and radioactivity in each measured by gamma counter (Table I). Size exclusion HPLC (BIORAD 800) runs were performed as follow: linear gradient, Solvent: 0.1 M phosphate buffer, Flow rate: 1mL/min, UV:280 nm, Column: TSK-6-3000 SW

2.1.2. ^{99m}Tc *UBI*

To 10 µL 1mM peptide solution in 0.01M acetic acid pH 4, was added 4 µL of 0.5 mg/mL of stannous pyrophosphate; immediately thereafter 4µL of a solution of 10 mg of KBH_4 (SIGMA) per mL of 0.1M NaOH was added. Solution was freeze-dried and kit form was provided from Polatom, Poland.

After addition of 0.1 mL of ^{99m}Tc-sodium-pertechnetate (200-800 MBq/mL), the mixture stirred at room temperature for 60 min.

2.1.3. ^{99m}Tc *DTPA-HNE-2*

Conjugation: To 0.1 mL HNE-2 solution was added 0.1 mL 0.5 M $NaHCO_3$ solution. 0.1 mL suspension of 0.1 mg DTPA in DMF was then added with shaking. After 30 min. incubation at room temperature the coupled peptide was purified on a 0.7×20 cm G-25 column with 0.25 M ammonium acetate buffer, pH 5.2 as eluent. The elution was monitored at U.V. 280 nm. The conjugated peptide eluted in 2-5 mL range.

Labelling: The conjugated peptide concentration was 0.5 ug/uL. To the peptide 18 ul buffer consisting of 0.5 M sodium bicarbonate, 0,25 M ammonium acetate and 0,18 M ammonium hydroxide at pH 9.2 were added. Finally, 0.1 mL /500 MBq of ^{99m}Tc -pertechnetate and 40 µL fresh solution of $SnCl_2.2H_2O$ solution (1 mg/mL in 10 mM HCl) were added. The final pH was 7.6. After incubation at room temperature for 1h the labelled peptide was purified over Sephadex G-25 column.

Radiochemical analysis: ITLC-SG in saline-hydrolysed ^{99m}Tc activity remained at the origin; ITLC-SG in acetone-all the hydrolized and labelled ^{99m}Tc activity remain at origin; HPLC analysis with Chrompack-SS RP-18 column (250×4 mm) was used with elution programme as given in the Leiden Protocol.

2.1.4. ^{99m}Tc *HYNIC –UBI*

Conjugation: 1 mg UBI was dissolved in 0.4 mL bicarbonate buffer pH 8.5 and 1.2.mg NHS HYNIC in 0.1 mL dry DMF was added with shaking. After 2 h at room temperature the mixture was purified with Sephadex G-25 column 0.7× 20 cm with 0.25 M ammonium acetate buffer pH 5.2 . The peak fractions were collected and dispensed in 5 vials each containing ~0.2 mg peptide.

Radiolabelling employed tricine as the coligand. To 50 µg of conjugated peptide in 0.25 M ammonium acetate, pH 5.2 were added ^{99m}Tc pertechnetate (500 MBq / 0.1 mL) and 50 µL of tricine solution in water (100 µg/µL). Finally 20 µl of fresh $SnCl_2.2H_2O$ solution (1 mg/mL) was added. The final pH was 5.3. After incubation at room temperature for 60 min, the labelled peptide was purified over Sephadex G-25 column with 50 mM PBS, pH 7.2.

Radiochemical Analysis: ITLC-SG in saline, ITLC SG in acetone and HPLC as described above

2.1.5. ^{99m}Tc *DTPA–UBI*

Conjugation: 1.0 mg peptide was dissolved in 0.1 mL water, and then 0.1 mL 0.5 M $NaHCO_3$ solution was added. 3 .0 mg DTPA anhydride dissolved 0.1 mL dry DMF. From DTPA suspension 0.1 mL was added to protein with shaking. After 1 h incubation at room temperature, the coupled peptide was purified on a 0.7×20 cm G-25 column, with 0.25 M ammonium acetate buffer, pH 5.2.The elution was monitored at U.V. 280 nm. The eluted conjugated peptide was kept frozen in 1 mL lots

Labelling: To 0.1 mg DTPA–UBI in 20 µL buffer pH 9.2, 0.1 mL (500 MBq) of ^{99m}Tc-pertechnetate and 40 µL $SnCl_2 \times 2H_2O$ fresh solution (1 mg/mL in 10 mM HCl) were added. The final pH was 7.6. After incubation at room temperature for 2 h labelled peptide was purified over Sephadex G-25 column.

Radiochemical analysis by ITLC and HPLC was carried about as described earlier.

2.2. Pathological animal models

2.2.1. Sterile inflammation induction

Heat killed E. coli (0101-RG/W) bacteria (endotoxin, lipopolysaccharide – LPS) were used for inducing sterile muscle inflammation. Two hundred microgramm LPS in 100 µL physiological saline was deeply injected into the right thigh muscle in C57/Black mice 24 h before further examinations.

The injected LPS in the recommended dose did not cause detectable signs of inflammation in the animals. On that basis we choose a 4 times higher concentration of injected LPS. Even that model showed different (from mild to hard hemorrhagic) severity of muscle inflammation.

2.2.2. Bacterial infection induction

Staphylococcus aureus (OKI-110003) was chosen as microorganism for muscle infection induction. Frozen bacteria suspension was cultured by 24 h incubation at 37°C in shaking waterbath. Staphylococcus aureus bacterial suspension in 10^7 CFU / 100 µL was injected deeply into the right thigh muscle 24 h before further examinations.

2.2.3. Bioassay in mice

Animals were injected into the tail veins with labelled compounds. The same dose was injected in a 50 mL glass for estimating the injected dose. Fifteen minutes, 2 h and 24 h after injection, three mice were sacrificed in each group. Organs e.g.: tail, blood, muscle, bone, heart, larynx with thyroids, lungs, liver, spleen, kidneys, gastric, small intestines, large intestines, and muscle with induced infection/inflammation were removed, weights and activities were measured, induced / normal muscle ratios and standard biodistribution were calculated as I.D. / whole organ and I.D. / gram tissue.

2.2.4. Imaging of infectio/inflammation in dogs

Infection and inflammation were similarly induced in Beagle dogs. Static gamma camera imaging was done 20 mts post injection of 280 MBq/1.5 ml ^{99m}Tc EB1 and 15 min after 320 MBq/1.6 ml ^{99m}Tc UBI from kit, respectively

3. RESULTS AND DISCUSSIONS

The results of ITLC and HPLC are given in Tables I, II and III. All the examined agents were found to show good (>90%) labelling efficiency. ^{99m}Tc EB1 was stable up to 24 h after labelling using 700 MBq added activity.

Labelling of the kit form of UBI revealed high and stable labelling efficiency only up to 10 – 20 MBq activity. Higher (500 – 700 MBq) activities and greater labelling volume (over 1 mL) revealed not satisfactory labelling yield (app. 80%) and stability. The labelled componds were found to be stable also in human serum up to 24 h.

Basically all agents showed a rapid clearance via kidneys resulted low background activities in the blood and other organs. Selected results on animal distribution are given in Tables IV and V. Normal biodistribution studies may not be necessary, because induced animal model biodistributions show very similar to data of “other” organs. Induced muscle inflammation and infections vary on a wide scale from mild to severe. Other sterile inflammation models are necessary.

Fifteen (15) min, 2 h, 4 and/or 6 h, 24 h (12 h and 18 h also) would be recommended for biodistribution and imaging studies. This will also help in calculation of mean residency times and

internal dosimetry data. Labelled HNE-2 was investigated only by scintigraphy in Rhesus monkey. Images showed high thyroid and stomach activity which may be due to poor labelling efficiency or in vivo instability.

Kit form of EB1 and UBI (both from Polatom) showed very similar results on ^{99m}Tc labelling and in animal experiments than the earlier in-house forms. ^{99m}Tc EB1 imaging showed high uptake in inflammation site in dog (Fig.1). ^{99m}Tc UBI showed lower but specific uptake in infection site in dog (Fig. 2.)

TABLE I. PAPER CHROMATOGRAPHY RESULTS (N=3)

	^{99m}Tc - EB1	^{99m}Tc - UBI	Conjugation of HNE_2 with DTPA	Conjugation of UBI with Hynic	Conjugation of UBI with DTPA
			Labelling efficiency (in %)		
After labelling	97,8 ± 0,2	98,3 ± 0,2	96,3 ± 0,1	87,0 ± 0,2	88,7 ± 0,5
After 3 h	94,4 ± 0,9	97,1 ± 0,3	96,1 ± 0,1	83,2 ± 0,3	85,9 ± 0,9
After 6 h	94,2 ± 0,6	95,5 ± 0,1	95,3 ± 0,7	82,6 ± 0,6	86,8 ± 0,5

TABLE II. ITLC ANALYSIS RESULTS(N=3)

	^{99m}Tc - EB1	^{99m}Tc - UBI	Conjugation of HNE_2 with DTPA	Conjugation of UBI with Hynic	Conjugation of UBI with DTPA
			Labelling efficiency (in %)		
After labelling	93,6 ± 0,1	99,0 ± 0,1	97,5 ± 0,5	92,0 ± 0,9	92,1 ± 0,2
After 3 h	92,4 ± 0,3	97,9 ± 0,4	97,5 ± 0,4	91,7 ± 0,5	87,7 ± 0,2
After 6 h	90,7 ± 0,4	92,5 ± 0,5	94,4 ± 0,6	86,9 ± 0,2	82,9 ± 0,2

TABLE III. REVERSE-PHASE HPLC ANALYSIS RESULTS

	^{99m}Tc - EB1	^{99m}Tc – UBI	Conjugation of HNE_2 with DTPA	Conjugation of UBI with Hynic	Conjugation of UBI with DTPA
After labelling	99,0% ± 0,1	96,2% ± 0,1	97,3 ± 0,1	92,6 ± 0,3	93,6 ± 0,8
After 3 h	98,3% ± 0,1	94,6% ± 0,1	96,6 ± 0,7	91,5 ± 0,9	88,3 ± 0,1
After 6 h	93,2% ± 0,2	92,8% ± 0,2	93,9 ± 0,1	86,9 ± 06	84,2 ± 0,1

TABLE IV. ^{99m}Tc EB1 BIODISTRIBUTION IN INDUCED MICE

	No. 1		No. 2		No. 3			
	I.D.% / 1g tissue	I.D.% / w.organ	I.D.% / 1g tissue	I.D.% / w.organ	I.D.% / 1g tissue	I.D.% / w.organ	Average	SD±
15 min								
Tail	3,6	1,52	3,97	1,76	3,42	1,82	3,66	0,28
Blood	12,33	13,48	9,04	15,32	12,10	11,72	11,16	1,84
Control muscle	0,98	5,32	1,42	6,12	1,28	4,56	1,23	0,22
Induced muscle	4,01	0,48	2,99	0,28	2,76	0,32	3,25	0,67
Bone	1,02	1,98	0,96	1,98	1,06	2,34	1,01	0,05
Heart	1,52	0,33	1,54	0,24	1,62	0,22	1,56	0,05
Thyroid	5,26	0,23	5,52	0,32	4,83	0,23	5,20	0,35
Lungs	3,61	0,62	3,88	0,93	3,95	0,91	3,81	0,18
Liver	4,62	6,22	4,58	5,32	4,48	4,92	4,56	0,07
Spleen	1,58	0,21	1,99	0,20	1,71	0,23	1,76	0,21
Kidneys	14,13	4,49	12,10	4,01	13,01	4,36	13,08	1,02
Stomach	14,85	2,78	14,45	2,69	13,28	2,92	14,19	0,82
Small intestine	4,96	4,32	4,01	3,98	4,66	4,56	4,54	0,49
Large intestine	1,58	1,92	1,23	1,97	1,97	0,99	1,59	0,37
Activity %		43,9 %		45,12 %		40,10 %	43,04 %	2,62
Ind./Cont. M.	4,09	X	2,11	X	2,16	X	2,78 X	1,13
2 h								
Tail	2,6	1,58	1,87	1,23	2,56	1,84	2,34	0,41
Blood	3,59	3,2	1,86	2,22	2,92	2,88	2,79	0,87
Control muscle	1,12	1,96	0,67	2,98	0,34	1,24	0,71	0,39
Induced muscle	3,55	0,18	3,22	0,24	1,25	0,22	2,67	1,24
Bone	0,58	1,12	0,58	1,41	1,12	1,89	0,76	0,31
Heart	0,45	0,12	0,92	0,21	1,23	0,15	0,87	0,39
Thyroid	1,9	0,11	0,23	0,12	2,98	0,34	1,70	1,39
Lungs	2,33	0,54	2,43	0,75	4,23	0,65	3,00	1,07
Liver	4,78	4,99	4,22	4,96	5,12	5,33	4,71	0,45
Spleen	2,11	0,51	1,99	0,22	2,12	0,34	2,07	0,07
Kidneys	8,68	3,11	8,45	4,04	9,12	1,99	8,75	0,34
Stomach	7,22	0,98	16,56	1,54	8,45	2,01	10,74	5,07
Small intestine	6,54	5,99	7,02	5,45	9,34	6,76	7,63	1,50
Large intestine	3,32	1,67	3,45	2,81	3,65	1,88	3,47	0,17
Activity %		26,06 %		28,18 %		27,52 %	27,25 %	1,08
Ind./Cont. M.	3,17	X	4,81	X	3,68	X	3,88 X	0,84
24 h								
Tail	3	1,72	2,12	1,49	3,67	2,00	2,93	0,78
Blood	0,44	0,34	0,55	0,31	0,61	0,67	0,53	0,09
Control muscle	0,22	0,64	0,22	0,56	0,15	0,42	0,20	0,04
Induced muscle	0,78	0,21	0,87	0,31	0,87	0,25	0,84	0,05
Bone	0,44	0,86	0,67	0,71	0,56	0,91	0,56	0,12
Heart	0,23	0,02	0,65	0,09	0,67	0,08	0,52	0,25
Thyroid	1,78	0,05	1,45	0,04	1,24	0,05	1,49	0,27
Lungs	1,91	0,56	0,65	0,19	0,86	0,45	1,14	0,68
Liver	3,98	2,99	3,55	3,29	2,96	2,12	3,50	0,51
Spleen	0,67	0,06	0,88	0,06	0,81	0,06	0,79	0,11
Kidneys	4,56	1,41	5,34	1,88	4,01	1,96	4,64	0,67
Stomach	3,23	0,96	2,99	0,16	4,24	0,78	3,49	0,66
Small intestine	1	1,02	1,23	0,76	0,98	0,66	1,07	0,14
Large intestine	19,56	6,56	17,34	5,45	21,45	6,91	19,45	2,06
Activity %		17,4 %		15,30 %		17,32 %	16,67 %	1,19
Ind./Cont. M.	3,55	X	3,95	X	5,80	X	4,43 X	1,20

TABLE V. ^{99m}Tc UBI BIODISTRIBUTION IN INDUCED MICE

	No. 1		No. 2		No. 3			
	I.D.% / 1g tissue	I.D.% / w. organ	I.D.% / 1g tissue	I.D.% / w. organ	I.D.% / 1g tissue	I.D.% / w. organ	Average	SD±
15 min								
Tail	13,99	7,83	7,99	3,95	5,96	2,67	9,31	4,18
Blood	4,56	4,12	3,89	2,98	3,45	4,01	3,97	0,56
Control muscle	0,39	1,66	0,66	3,18	0,44	1,98	0,50	0,14
Induced muscle	1,58	0,44	1,20	0,33	0,79	0,13	1,19	0,40
Bone	2	3,98	2,13	4,00	2,86	5,23	2,33	0,46
Heart	1,23	1,23	1,45	0,24	1,34	0,16	1,34	0,11
Thyroid	0,12	0,01	0,92	0,02	0,24	0,03	0,43	0,43
Lungs	7,51	0,65	6,54	1,34	5,92	1,20	6,66	0,80
Liver	59,76	48,23	59,25	52,13	59,23	50,12	59,41	0,30
Spleen	23,12	2,56	40,12	2,87	40,12	3,01	34,45	9,81
Kidneys	12,23	3,94	19,12	3,29	20,26	5,12	17,20	4,34
Stomach	1,2	0,34	1,56	0,90	1,65	0,45	1,47	0,24
Small intestine	0,9	1,11	1,84	1,41	1,02	0,65	1,25	0,51
Large intestine	0,87	0,55	0,93	0,55	2,02	0,97	1,27	0,65
Activity %		76,65		77,19 %		75,73 %	76,52 %	0,74
Ind./Cont. M.	4,05	X	1,82	X	1,80	X	2,55 X	1,30
2 h								
Tail	15,02	2,01	2,02	0,2	3,02	1,06	6,69	7,23
Blood	0,87	0,86	0,92	0,98	0,87	0,99	0,89	0,03
Control muscle	0,22	0,65	0,23	0,78	0,26	0,99	0,24	0,02
Induced muscle	0,33	0,19	0,7	0,13	0,82	0,12	0,62	0,26
Bone	0,62	1,24	1,67	2,2	1,88	2,11	1,39	0,68
Heart	0,56	0,09	0,88	0,06	0,89	0,07	0,78	0,19
Thyroid	0,59	0,01	0,86	0,01	0,56	0,02	0,67	0,17
Lungs	6,34	0,3	6,23	0,32	5,92	1,34	6,16	0,22
Liver	48,97	46,78	52,54	50,12	49,78	46,43	50,43	1,87
Spleen	21,45	3,23	27,65	2,11	29,78	2,79	26,29	4,33
Kidneys	19,86	4,58	15,78	3,96	16,23	3,67	17,29	2,24
Stomach	0,44	0,07	0,66	0,34	0,98	0,08	0,69	0,27
Small intestine	1,45	1,9	1,66	1,87	1,45	0,92	1,52	0,12
Large intestine	0,45	0,25	0,44	0,19	1,23	0,98	0,71	0,45
Activity %		62,16		63,27 %		61,57 %	62,33 %	0,86
Ind./Cont. M.	1,50	X	3,04	X	3,15	X	2,57 X	0,92
24 h								
Tail	2.78	1.98	4.33	1.28	3.87	1.21	3.66	0.80
Blood	0,45	0,65	0,43	0,33	0,34	0,42	0,41	0,06
Control muscle	0,13	0,55	0,13	0,23	0,14	0,66	0,13	0,01
Induced muscle	0,28	0,03	0,44	0,14	0,22	0,04	0,31	0,11
Bone	1,56	2,98	1,45	1,94	1,23	1,92	1,41	0,17
Heart	0,34	0,04	0,32	0,03	0,32	0,02	0,33	0,01
Thyroid	0,31	0,01	0,44	0,02	0,32	0,01	0,36	0,07
Lungs	1,93	0,11	0,72	0,44	1,82	0,09	1,49	0,67
Liver	6,58	4,12	5,92	5,24	4,23	3,98	5,58	1,21
Spleen	34,23	2,65	31,23	1,99	29,34	2,12	31,60	2,47
Kidneys	12,45	1,87	11,67	2,14	16,78	2,34	13,63	2,75
Stomach	1,45	1,56	1,12	1,97	1,45	0,97	1,34	0,19
Small intestine	0,45	0,12	0,44	0,67	0,65	0,56	0,51	0,12
Large intestine	2,04	0,98	2,91	1,24	2,56	1,91	2,50	0,44
Activity %		17,65 %		17,66 %		16,25 %	17,19 %	0,81
Ind. / Cont. M.	2,15	X	3,38	X	1,57	X	2,37 X	0,93

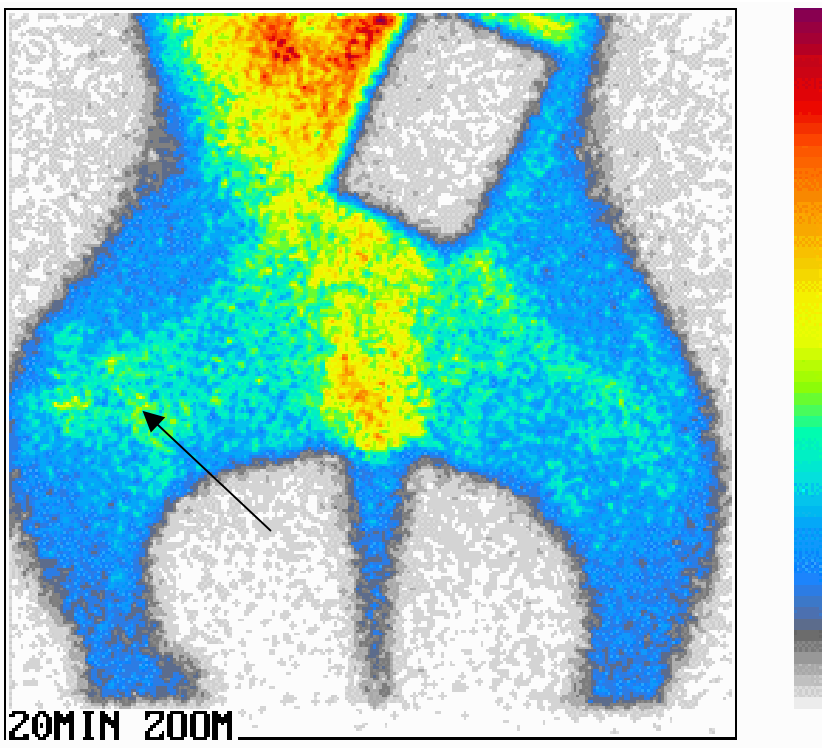

FIG.1. ^{99m}Tc *EB1 imaging in inflammation induced beagle dog.*

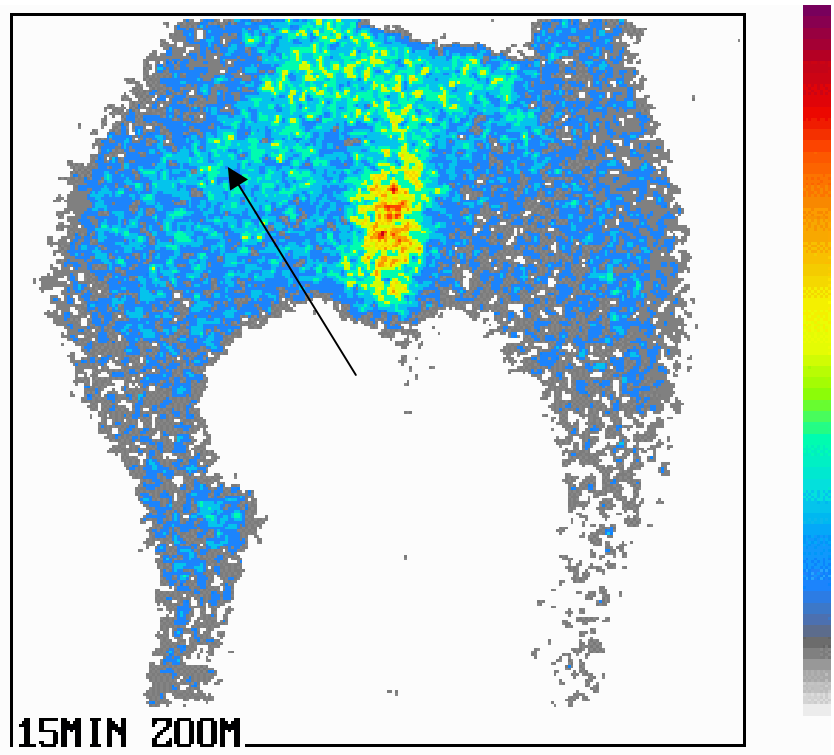

FIG.2. ^{99m}Tc *UBI imaging in infection induced beagle dog.*

4. CONCLUSIONS

On the basis of IAEA supported CRP we can conclude that ^{99m}Tc EB1 and ^{99m}Tc UBI have the potential for a diagnostic kit in the field of inflammation and infection imaging. Further studies are needed to evaluate available agents in term of differential diagnosis of bacterial and non-bacterial (sterile) inflammation.

ACKNOWLEDGEMENTS

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PREPARATION AND EVALUATION OF ^{99m}Tc LABELLED RADIOPHARMACEUTICALS AND FORMULATION OF KITS FOR IMAGING INFECTION/INFLAMMATION

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Abstract

Three agents were studied for infection/inflammation imaging under the CRP. EDTA-biotin monomer (EB1) offers advantages of ease of in-house synthesis, being inexpensive and showing better pharmacokinetics, as compared to currently used radiopharmaceuticals for inflammation imaging. Hence we had placed additional emphasis on this product starting from the synthesis step. The synthesized product was also provided under CRP. Lyophilized kit for ^{99m}Tc-EB1 was standardized and evaluated to satisfaction in terms, of >95% yield, >95% RC purity and ratio of uptake in inflamed tissue to normal being around 1.5–2 in mice. Among the other products, Ubiquicidine fragment, UBI 29-41, an antimicrobial peptide (made available under CRP) was radiolabelled by both direct method as well as by indirect method using DTPA as bifunctional chelating agent. Direct labelling of UBI resulted in product of high purity and stability (>90% labelling yield and >95% RC purity was obtained), but radiolabelling of UBI using DTPA as bifunctional chelating agent was not satisfactory. The biological efficacy of ^{99m}Tc-UBI was shown by both bacterial (S.aureus) binding studies (>30%) and higher uptake in muscle with induced infection, as compared to the results with ^{99m}Tc-scrambled peptide of UBI analog. The radiolabelling of UBI using HYNIC as bifunctional chelating agent was not fully optimized for obtaining high labelling efficiency. Human neutrophil elastase inhibitor (HNE2) (made available under CRP) was radiolabelled with ^{99m}Tc using MAG3 as bifunctional chelating agent. The procedures of conjugation with NHS-MAG3 and radiolabelling of the conjugate with ^{99m}Tc were first authenticated using the suggested reference compound, BPTI. The studies on radiolabelling of BPTI were also performed with DTPA as bifunctional chelating agent. However, the studies with HNE2 could not be completed due to non-availability of HNE2.

1. INTRODUCTION

The ability to image infection specifically is a very useful tool in the management of patients, especially suffering from fever (pyrexia) of unknown origin (FUO/PUO) and in such other suspected cases of occult infection. As inflammatory process is always associated with any active infection, many procedures depend on imaging inflammation more often. Since differential diagnosis from sterile inflammation would be more informative, the search for products for infection imaging based on sound rationale has been continued by many groups world over. IAEA accordingly supported the present CRP in an attempt to channel efforts towards the accomplishment of a specific infection imaging agent. The features of the existing products such as ^{67}Ga-citrate, leucocytes labelled with ^{111}In /^{99m}Tc, ^{111}In/^{99m}Tc-human immunoglobulin (HIG), ^{99m}Tc-ciprofloxacin, etc. are well known, as are their limitations. Based on detailed project formulation discussions held in the First RCM of the present CRP, three compounds were chosen for detailed investigation by the participant groups who were to follow a harmonized protocol in preparation and evaluation (Report of First RCM of the CRP). The three agents studied for infection/inflammation imaging under the CRP are: EDTA-biotin monomer (EB1), Ubiquicidine fragment, UBI 29-41, an antimicrobial peptide and human neutrophil elastase inhibitor (HNE2). The salient details of the studies carried out and the major findings are reported here.

2. METHODS

2.1. HNE-2 - Conjugation of BPTI and HNE2 with NHS-MAG3

Conjugation of BPTI and HNE2 with NHS-MAG_3 was carried out at the molar ratio of 4:1 of NHS-MAG3: BPTI and the reaction mixture purified over P4 column (20×0.7cm). 100 µl aliquots of suitable fractions were dispensed and stored at -20^{0}C until use.

2.2. UBI - Conjugation of UBI with cDTPA

Conjugation of UBI with cDTPA was carried out at the molar ratio of cDTPA : UBI:: 5:1. The conjugation of scrambled peptide for UBI was also carried out using the same procedure.

2.3. EB1 - Synthesis of biotinyl hydrazine-EDTA (EDTA biotin monomer, EB1)

To a stirred solution of biotin hydrazide (50 mg, 0.194 mM) in 20 mL of mixture of dry DMF: $CHCl_3$, 1:1 (v/v) at 65^{0}C, was added 20 mL of mixture of dry DMF: $CHCl_3$, 1:1 (v/v) containing EDTA bicyclic anhydride (65.0 mg, 0.254 mM). The mixture was stirred for 30 min, hydrolyzed by the addition of 0.5 mL of water and stirred for additional 10 min. The reaction mixture was poured into 300 mL of dry dichloromethane and extracted twice with 15 mL of distilled water. Water was removed by rotary evaporation. To the small amount of liquid that remained in the round bottom flask, dry dichloromethane was added. The precipitated EB1 was filtered, washed with dry acetonitrile and dried by vacuum desiccation. Crude EB1 was redissolved in 1 mL of 0.2 M sodium bicarbonate pH 8.5 and purified over P2 (120×1.4 cm) column using water as eluent. 1 mL fractions were collected and absorbance was measured at 220 nm. Suitable fractions were pooled and pure EB1 was recovered by rotary evaporation. White shiny crystals obtained were characterized for MP (186–192^{0}C dec), IR (IR cm^{-1} (KBr pellets): 1660 (-CO- of amide); 3300 (-NH- of amide)) and NMR. A sample of this EB1 was distributed to CRP participants in the 2nd RCM held in Mexico.

Kit formulation for EB1: Kit formulations with 200µg of EB1 and different stannous tin contents of 20, 50, 75 and 100 µg were prepared, lyophilized and evaluated. Kit containing 200µg EB1, 20µg $SnCl_2.2H_2O$, 2mg NaCl was finally chosen and lyophilized kits were made. Kit samples were distributed to all the CRP participants at the 2nd RCM held in Mexico.

2.4. Radiolabelling with ^{99m}Tc

Labelling of conjugates of BPTI-MAG3, HNE2-MAG3, UBI-DTPA and scrambled (UBI) peptide-DTPA was carried out and the labelling efficiency (LE) determined. In case of UBI, radiolabelling was also carried out by direct method.

Radiolabelling of EB1 with ^{99m}Tc: The kit vial was allowed to attain the ambient temperature. 2mL of ^{99m}Tc-pertechnetate was added to the kit vial. After mixing, the reaction vial was allowed to stand for 20 min at room temperature and the labelling efficiency determined.

2.5. Quality assurance and quality control testing

2.5.1. ^{99m}Tc HNE2

The radiolabelled conjugates of BPTI and HNE2-MAG3 were tested for kinetics and stability, stability in human serum and cysteine challenge test. The test for trypsin binding described below was carried out with BPTI-MAG3 conjugate labelled with ^{99m}Tc in order to ensure the retention of the biological activity of the molecule

Trypsin binding Studies: 10 µL aliquot of labelled BPTI solution was allowed to react with 30 µL solution of trypsin (2.5% in phosphate buffered saline). The reaction mixture was allowed to stand for 45 min at room temperature and passed over Sephadex G-100 column (20×0.7cm) with 0.1M phosphate buffer as the eluent. The labelled BPTI solution was also passed through another column for comparison.

Animal studies: Biodistribution studies with ^{99m}Tc-HNE2 and ^{99m}Tc-BPTI were performed with normal mice. 100 µL aliquots of ^{99m}Tc-HNE2 (11.7µg) and ^{99m}Tc-BPTI (25.9µg) were injected into the tail vein of the animals and the animals were sacrificed at 15min, 2h and 24h p.i.

2.5.2. ^{99m}Tc *UBI*

Radiolabelled UBI and scrambled peptide were tested for kinetics and stability, stability in human serum, cysteine challenge test and bacterial binding studies.

Bacterial binding studies: These studies were carried out as recommended with labelled UBI with 10^8 cells S.aureus ATCC 25923.

Animal studies: Infection was induced using S.aureus ATCC 25923 10^8 cells as recommended. 100µL of ^{99m}Tc-UBI was diluted to 1mL with normal saline. 100µL of this diluted ^{99m}Tc-UBI was injected per mouse and 200µL per rat, 24h after inducing infection with S. aureus. Animals were sacrificed at 15min, 2 h, & 24 h p.i. The biodistribution studies with ^{99m}Tc-UBI were performed in normal mice and in mice and rats with induced infection in the case of direct method and in normal mice and infection bearing mice in the case of indirect method.

2.5.3. ^{99m}*Tc EB1*

The labelled EB1 was tested for stability, stability in human serum and cysteine challenge test. Stability of the kit was assessed for the period of 3 months.

Test for avidin binding- Shift Assay: An aliquot of labelled EB1 (50µg) was allowed to react with 10mg avidin. Both these solutions were passed through Sephadex G-75 (20×0.7cm) columns and eluted with PBS (pH 6). 1 mL fractions were collected and radioactivity measured

Animal Studies: The biodistribution studies were carried out with ^{99m}Tc-EB1 prepared using 20 µg stannous tin content in inflammation bearing mice at 10min, 1h and 3h p.i.

2.6. Procedures commonly employed in the studies

2.6.1. Determination of labelling efficiency

Labelling efficiency was determined using ITLC-SG support (10×0.8 cm) with two solvents namely, acetone and normal saline.

2.6.2. Kinetics and stability

The labelling efficiency was studied as a function of time using TLC-SG as described above. The labelled preparation was evaluated for RCP for a period of 24 h.

2.6.3. Stability in human serum

A: An aliquot of labelled peptide containing 1–100 µg of peptide was added to 0.5 mL of fresh human serum. The reaction mixture was vortexed and incubated at 37 ^{0}C. The reaction mixture was analysed for RCP as described.

B: Suitable aliquot of labelled peptide containing 1-100 µg of peptide was added to 1mL of fresh human serum. The reaction mixture was vortexed and incubated at 37^0C. 0.5mL aliquots were loaded at the time intervals of 1h & 24h on the column of Sephadex G-75 (16×0.7 cm) and eluted with 0.1 M phosphate buffer. 1mL fractions were collected and measured for radioactivity.

2.6.4. Cysteine challenge test

Suitable aliquots of labelled preparation were allowed to react with suitable aliquots of solutions of cysteine with concentrations ranging between 0.01 mg/ mL to 10 mg/mL prepared in 0.2M phosphate buffer such that the molar ratios are ranging between 1:1 to 500:1 of cysteine: compound. The reaction mixtures were allowed to stand for 1 h at 37 ^{0}C and the RCP determined.

3. RESULTS AND DISCUSSION

3.1. ^{99m}Tc HNE2

Typically 93% labelling efficiency was achieved on labelling of BPTI-MAG3 and HNE2-MAG3. The preparations of ^{99m}Tc-BPTI and ^{99m}Tc-HNE2 were used without purification. In the case of BPTI, greater than 90% LE was observed after 20min, which remained more or less constant upto 24h, whereas in the case of HNE-2, greater than 90% LE observed at 1h reduced to 84% at 24h. Trypsin binding studies of ^{99m}Tc-BPTI clearly indicated shift in the radioactivity profile thereby confirming the binding of BPTI with trypsin (Fig. 1) and thus authenticating the procedures of conjugation with NHS-MAG3 and labelling of the conjugate with ^{99m}Tc. Studies with fresh human serum, indicated no significant fall in RCP in the case of ^{99m}Tc-BPTI, while ^{99m}Tc-HNE2 showed a maximum of 11% dissociation on incubation with human serum at 37^0C upto 24h (Fig. 2). About 21% of ^{99m}Tc-BPTI was bound to serum proteins after 1h at 37^0C whereas 4.4% and 18.7% of ^{99m}Tc-HNE2 was bound to serum proteins after 1h and 24h, respectively, at 37^0C. On challenge with cysteine maximum of 14% loss in RCP was observed in case of ^{99m}Tc-BPTI while fall of about 13–22% was observed in the RCP of ^{99m}Tc-HNE2 (Fig. 3). The results of biodistribution studies of ^{99m}Tc-HNE2 and ^{99m}Tc-BPTI in normal mice are given in Table I.

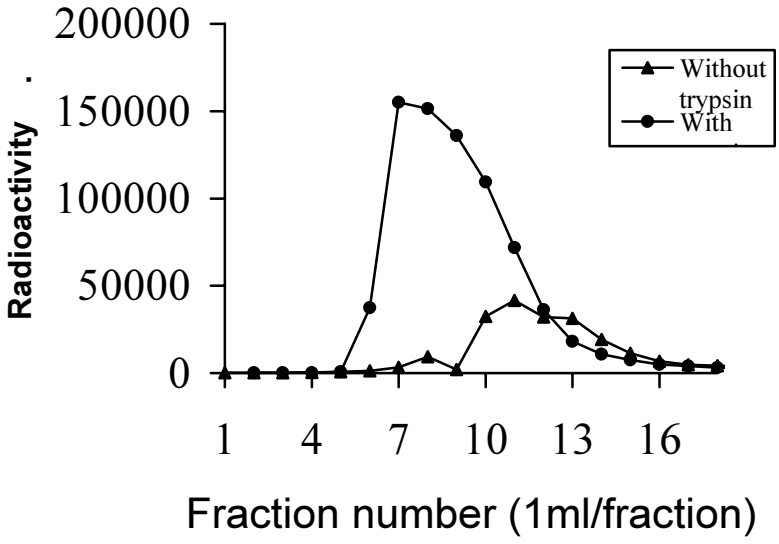

FIG. 1. Shift in radioactivity profile of ^{99m}Tc-BPTI on binding with trypsin over Sephadex G-100.

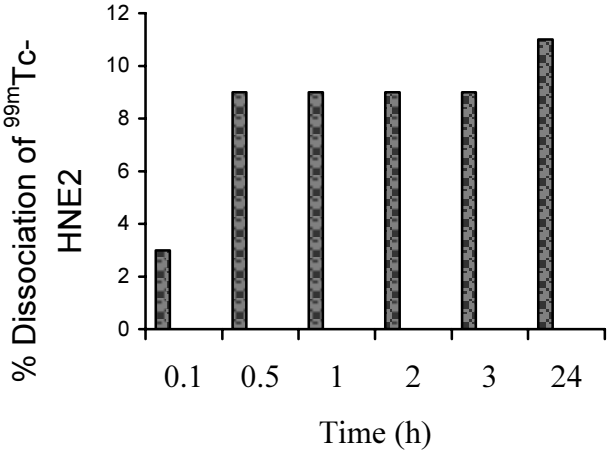

FIG. 2. Stability in human serum of ^{99m}Tc-*HNE2.*

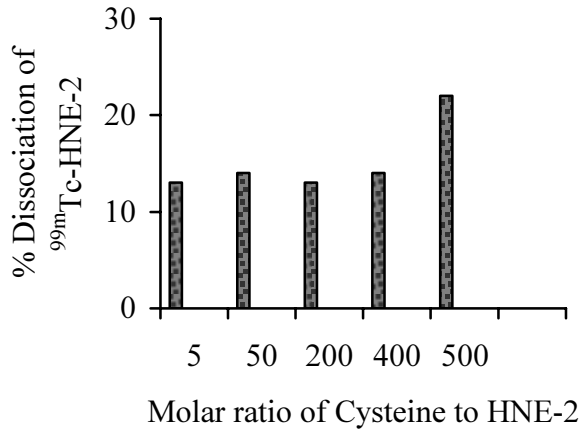

FIG. 3. Cysteine challenge test.

TABLE I. BIODISTRIBUTION RESULTS OF ^{99m}Tc-MAG3-HNE2 AND ^{99m}Tc-MAG3-BPTI IN NORMAL MICE (MEAN % I.D.)

	^{99m}Tc-BPTI		^{99m}Tc-HNE2	
Tissue	15 min	2 h	15 min	2 h
Blood/g	2.7	0.5	2.4	1.33
Lungs	0.95	0.14	0.63	0.23
Stomach	0.61	0.28	0.46	0.57
NM/g	1.52	0.4	2.26	2.3
Liver	5.41	4.8	2.5	4.8
Spleen	0.16	0.03	0.14	0.07
Kidneys	33.35	48.2	12.6	14.9
Bladder + Urine	4.63	9.47	5.9	9.96

3.2. ^{99m}Tc UBI

3.2.1. Direct method

The LE of greater than 90% of ^{99m}Tc-UBI was observed in different solvent systems (Table II). Acetone (LE: 98%) was selected as a solvent for further studies. ^{99m}Tc-UBI was found to be stable at RT upto 2h. Significant fall in RCP was observed at 4h (81%) and further at 24h (70%). However it was observed that on incubation with serum at 37^{0}C the RCP of ^{99m}Tc-UBI remained constant at about 94% upto 24h. Serum binding and bacterial binding studies indicated that about 8.9% of ^{99m}Tc-UBI was bound to the serum proteins and 30% was bound to bacteria, S.aureus at 37 ^{0}C after 1h. The observed ratio of uptake of ^{99m}Tc-UBI in infected muscle to normal muscle (IM/NM) at 15 min, 2 h and 24 h p.i. were 1.7, 2.8 and 6.7 respectively in mice and 1.3, 2.0 and 2.0 respectively in rats (Table III).

3.2.2. Indirect method

Relatively higher labelling efficiencies were obtained at pH 5.2 (>95%) as compared to pH 7.6 (~90%). For further studies, labelling was carried out at pH 5.2. There was no significant change in the RCP upto 24h. On incubation with serum at 37^{0}C, the RCP of ^{99m}Tc-UBI gradually reduced to 71% at 24h (Table IV). About 68% of ^{99m}Tc-UBI was bound to the serum proteins after 1 h, at 37^{0}C; 67% and 7% of ^{99m}Tc-UBI and ^{99m}Tc-scrambled peptide, respectively, were bound to bacteria, S.aureus at 37^{0}C after 1h. On challenge with cysteine, a fall of about 4-26% was observed in the RCP of ^{99m}Tc-UBI (Table V). The ratios of uptake in infected muscle to normal muscle (IM/NM) at 15 min, 2h and 24h p.i. for ^{99m}Tc-UBI were slightly higher as compared to that for ^{99m}Tc-labelled scrambled peptide

TABLE II. LABELLING EFFICIENCY (LE) OF ^{99m}Tc -UBI ASSESSED BY ITLC OVER DIFFERENT SOLVENT SYSTEMS

Solvent for chromatography	LE (%)
Saline	94
Acetone	98
MEK	91
MeOH	90
Acetonitrile	92

TABLE III. BIODISTRIBUTION STUDIES OF ^{99m}Tc-UBI (DIRECT METHOD)

	Injected dDose (% ID)					
	Infected mice			Infected rats		
Tissue	15 min	2 h	24h	15 min	2 h	24 h
*Blood	4.8 ± 0.6	2.5 ± 0.3	0.6 ± 0.3	1.5 ± 0.5	0.1± 0.03	0.02 ± 7
Lungs	0.7 ±0.1	0.3 ± 0.02	0.1 ± 0.1	2.2 ± 0.8	0.2± 0.08	0.05± .03
Stomach	3.4±0.4	0.8 ± 2.6	1.4 ± 1	1.6 ± 0.7	0.2± 0.05	0.4±0.32
*Muscle (normal)	0.3 ±0.2	0.5 ± 0.4	0.03± .01	0. ± 0.07	0.02	0.01
*Muscle (infected)	0.5 ± 0.3	1.4 ± 0.6	0.2 ± 0.1	0.5± 0.1	0.04	0.02±0.01
Liver	8.9 ± 0.5	5.7 ± 0.2	1.4 ± 0.9	4.1± 0.07	1.3± 0.3	0.3±0.06
Spleen	0.4 ± 0.02	0.2 ± 0.1	0.2 ± 0.1	0.5± 0.1	0.03± 0.01	0.04
Kidneys	6.6 ± 1.2	3.2 ± 0.4	0.2 ± 0.6	16.3± 6.3	6.5± 0.7	3.6±0.1
Small Intestine	4.1 ± 1.2	3.2 ± 1.0	1.1 ± 0.2	2.0± 0.6	0.9± 0.5	4.5± 0.3
Large Intestine	1.7 ± 0.1	6.1 ± 1.2	10.3 ± 5.6	5.2± 1.01	1.6± 0.9	7.4±2.2
Bladder + Urine	9.4 ± 3.0	17.8 ± 13.7	0.1 ± 0.2	1.2± 1.26	0.6± 0.6	5.7
Ratio (IM/NM)	*1.7*	*2.8*	*6.7*	*1.3*	*2.0*	*2.0*

*Uptake is given on the basis of %ID/g

TABLE IV. STABILITY IN HUMAN SERUM OF ^{99m}Tc-DTPA-UBI

Time interval	RCP (%) TLC/Saline
0	94
10 min	89
30 min	89
1 h	81
2 h	78
4 h	74
24 h	71

TABLE V. CYSTEINE CHALLENGE TEST OF ^{99m}Tc-DTPA-UBI

Cysteine concentration (mg/ml)	RCP (%) TLC/Saline
Control	99
0.01	95
0.1	91
1	87
10	73

3.3. ^{99m}Tc-EB1

Labelling efficiency of >95% was achieved. No significant difference in the LE was observed with ^{99m}Tc-EB1 prepared with different stannous tin content. The labelled preparation was found to be stable up to 24 h at RT. The kit was found to be stable for not less than about 3 months when stored at 4^0C. The studies with human serum indicated stability of the labelled preparation with no significant fall in the RCP up to 2 h and 67% was bound to human serum. On challenge with cysteine the fall of about 6–29% was observed in the RCP of ^{99m}Tc-EB1 (Fig. 4). The studies on avidin binding indicated shift in the radioactivity profile thereby confirming the binding of ^{99}Tc ^{m} -EB1 with avidin (Fig. 5) The ratios of uptake of ^{99m}Tc-EB1 (prepared with 20 µg stannous tin content) in inflamed muscle to normal muscle at 10min, 1h and 3h were 2.0, 1.5 and 1.7, respectively, with EB1 obtained under CRP and 1.9, 1.2 and 2.0, respectively, with in-house synthesized EB1 in mice (Table VI). The ^{99m}Tc-EB1 prepared from formulations with higher stannous tin (II) content resulted in higher liver uptake as expected.

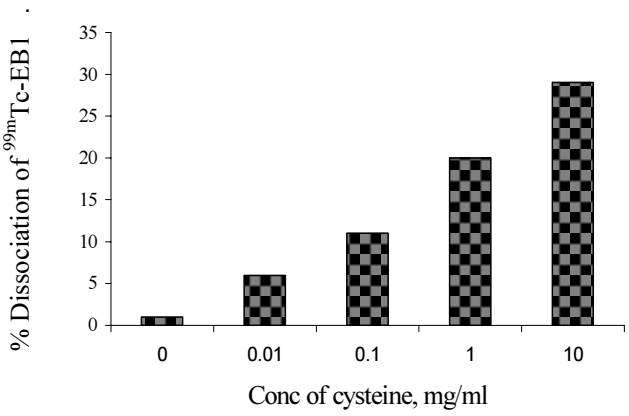

FIG. 4. Cysteine challenge test.

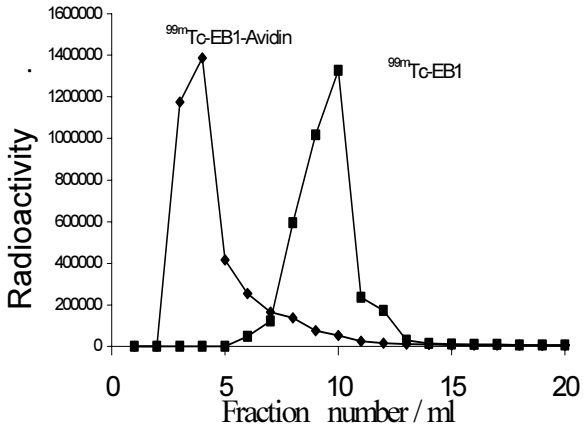

FIG. 5. Radiochromatogram of ^{99m}Tc-EB1-avidin and ^{99m}Tc-EB1.

TABLE VI. BIODISTRIBUTION RESULTS WITH ^{99m}Tc-EB1

Tissue / Organ	% I.D. (Mean±SD) In-house synthesized EB1			EB1 obtained under CRP		
	10 min	1 h	3 h	10 min	1 h	3 h
Blood / g*	4.0±0.6	1.8±0.4	0.8±0.01	2.5±0.3	1.2±0.4	0.7±0.4
S. intestine	1.4 ± 0.5	2.7± 0.4	2.3 ± 0.6	1.3± 0.3	1.1± 0.3	0.5±0.1
L. intestine	1.5 ± 0.4	0.7 ± 0.1	2.4±0.4	0.5±0.01	2.0±0.2	2.7±0.7
Stomach	1.6±0.2	1.1±0.2	0.5± 0.2	0.8±0.2	1.4±0.1	0.3±0.03
Liver	10.6 ±0.7	12.3 ±1.3	10.1±2.2	7.8±0.2	9.8±2.2	6.6±0.6
Kidneys	2.1±0.8	1.1±0.2	0.8±0.03	0.9±0.1	0.6±0.1	0.5±0.03
NM/g*	0.7±0.1	0.5±0.2	0.2±0.1	0.7±0.1	0.2 ±0.04	0.7± 0.03
IM/g*	1.3±0.3	0.6±0.2	0.4±0.1	1.4±0.1	0.3±0.2	1.2 (-)
IM/NM	1.9	1.2	2.0	2.0	1.5	1.7

*Uptake is given on the basis of %ID/g.

4. SUMMARY AND HIGHLIGHTS

Radiolabelling of HNE2 through MAG3 as BCA has demonstrated good labelling efficiency, stability and integrity of the molecule as indirectly determined by trypsin binding studies with BPTI.

Radiolabelling of UBI by direct method has demonstrated good labelling efficiency, stability, adequate bacterial binding and significant uptake in the infection bearing tissue.

Radiolabelling of UBI through DTPA as BCA has demonstrated good labelling efficiency, stability and bacterial binding as compared to the control scrambled peptide. However no significant advantage over direct labelling was noted.

Synthesis of EB1 and the formulation of the kit for labelling with ^{99m}Tc were successfully accomplished. The labelled product showed high labelling efficiency, stability and significant uptake in the inflamed muscle as compared with the normal muscle.

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^{99m}Tc LABELLING OF UBIQUICIDINE (UBI 29-41) AND EDTA-BIOTIN MONOMER (EB1)

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Abstract

Ubiquicidine (UBI 29-41) and EDTA-Biotin (EB1) labelled with ^{99m}Tc have been reported to have capability as infection and inflammation imaging agent, respectively. UBI 29-41 was labelled with ^{99m}Tc following direct and indirect methods using cyclic DTPA anhydride and NHS-HYNIC as bifunctional chelators with tricine and EDDA as coligands, and analysed by thin layer chromatography (TLC) and reversed phase HPLC (RP-HPLC). The stability of ^{99m}Tc-UBI and ^{99m}Tc-EB1 was assessed in human serum and by cysteine challenge. The efficacy of ^{99m}Tc-UBI for infection imaging was evaluated using in vitro bacterial binding assay and in vivo studies in infected mice. ^{99m}Tc-EB1 was subjected to shift assay after reacting with avidin, while its efficacy for inflammation imaging was assessed by in vivo study in inflammation induced mice and rats. The labelling efficiency (LE) of UBI and scrambled UBI using both direct and indirect methods did not show any significant difference (>85%), and EB1 also giae high labelling yield (>90%). Stability of ^{99m}Tc-HYNIC-UBI in cysteine seemed to be higher than that of ^{99m}Tc-UBI both in human serum and in cysteine. Bacterial binding of ^{99m}Tc UBI and ^{99m}Tc scrambled UBI prepared using direct and indirect methods was 40-75% and did not show any significant difference. Bioassay in infected mice showed higher radioactivity in infected thigh than in the normal thigh for both direct and indirect labelled UBI, and T/NT ratio 1.5 and 3 for ^{99m}Tc-HYNIC-UBI and ^{99m}Tc-UBI, respectively, at two h post injection. The ^{99m}Tc-scrambled UBI unexpectedly showed higher T/NT ratio than ^{99m}Tc-UBI, and it needs further evaluation. ^{99m}Tc EB1 showed binding for avidin (Gel permeation assay), and higher uptake in inflammation site in mice (T/NT ratio 2.3).

1. INTRODUCTION

Infectious diseases still pose a serious problem in developing countries, especially in tropical countries such as Indonesia, that need accurate and proper diagnosis for treatment and to minimize the risk of resistence to antibiotics by microorganisms. Nuclear medicine imaging has been used to localize infection sites and attempts have been continued to develop improved infection specific radiopharmaceuticals [1,2]. Synthesized ubiquicidine 29-41 (UBI) derived from human antimicrobial peptide has been reported to be able to bind with gram positive bacteria (Staphylococcus Aureus and Klebsiella pneumonia) and fungi either in vitro or in vivo after labelling with ^{99m}Tc, and furthermore it is reported to distinguish between infection and sterile inflammation [3,4,5].

^{99m}Tc labelling methods for small peptides should be carefully optimized to retain biological activity and achieve high labelling efficiency. High pharmacological activity often exhibited by peptides in vivo requires that the amount of peptides administered should be as small as possible. Peptide-based radiopharmaceuticals for imaging purpose should have high specific activity and stability. In addition to the originally reported direct labelling method for ^{99m}Tc UBI, bifunctional chelating agent (BFC) approach was considered interesting for investigation under the CRP programme. One of the agents commonly used now for ^{99m}Tc labelling of peptides is hydrazinonicotinamide (HYNIC) with tricine and EDDA as coligands [6]. In this project, ^{99m}Tc labelling of UBI 29-41 and a control peptide having scrambled sequence of same amino acids but poorer bacterial binding, was investigated by both direct and BFC methods and compared.

In addition, ^{99m}Tc complex of EDTA-biotin (EB1) reported to have good prospect as inflammation imaging agent, was also investigated.

2. MATERIALS

Ubiquicidine 29-41 (UBI) and Scrambled Ubiquicidine 29-41 (Scr.UBI) were supplied from Leiden University Medical Center (LUMC), Netherlands. EDTA-Biotin (EB1) was supplied from PINSTECH, Pakistan. NHS-hydrazinonicotinamide (NHS-HYNIC) was supplied from POLATOM, Poland. P-4 column (Biorad) and cyclic-DTPA anhydride (Sigma) were donated by Prof.D.Hnatowich (Massachussets Univ., USA). ^{99m}Tc generator was supplied by BATAN TECH, Indonesia. Basic chemicals such as potassium borohydride, stannous chloride dihydrate, sodium pyrophosphate etc. were purchased from Sigma, Aldrich and Merck. Staphylococcus Aureus was provided by P3TIR-BATAN, Indonesia.

3. METHODS

3.1. Synthesis of HYNIC-UBI and DTPA-UBI

100 µL of UBI or Scrambled UBI solution (3 mg/mL in bicarbonate buffer pH 8.5) was reacted with 10 µL of NHS-HYNIC solution (in dry DMF) at room temperature for 1 h, the ratio of NHS-HYNIC to peptide was 3. The HYNIC-peptide was purified through P-4 column using 0.05 M ammonium acetate buffer pH 5.2 as eluant. The amount of pure conjugate was measured using UV spectrometry. HYNIC-peptides solution was stored at -40°C. DTPA-UBI was synthesized in the same way.

3.2. Labelling UBI and Scrambled UBI with ^{99m}Tc using direct method

10 µL UBI or Scr.UBI solution (1 mM in 0.01 M acetic acid) was added with 4 µL stannous pyrophosphate solution (containing 0.95 mg/mL $SnCl_2.2\ H_2O$ and 2 mg/mL sodium pyrophosphate), 2 µL KBH_4 solution (10 mg/mL in 0.1 M NaOH) and 0.1 mL pertechnetate ^{99m}Tc solution (~10 mCi/mL), mixed and allowed to stand at room temperature for 10 min.

3.3. Labelling UBI and Scrambled UBI with ^{99m}Tc using indirect method

NHS-HYNIC and cDTPA were used as BFC. Tricine and EDDA were selected as coligands for labelling HYNIC-UBI.

To a solution of HYNIC-UBI or HYNIC-Scr.UBI (containing 10 µg of HYNIC-UBI or HYNIC-Scr.UBI) were added 20 µL tricine solution (100 mg/mL in water), 0.1 mL pertechnetate ^{99m}Tc solution (~10 mCi/mL), and 10 µL stannous chloride solution (1 mg/mL in 0.01 M HCl previously purged with nitrogen), mixed and allowed to stand at room temperature for 30 min.

Another method using mixture of tricine and EDDA (2:1) as coligand was also carried out. 500 µL of a solution containing 10 mg tricine and 5 mg EDDA was added to the the reaction vial containing 10 µg of HYNIC-UBI prior to adding 0.5 mL pertechnetate ^{99m}Tc solution (~10 mCi/mL) and 10 µL stannous chloride solution, followed by heating at 70°C for 1 h.

Solution of DTPA-UBI or DTPA-Scr.UBI (containing 10 µg of DTPA-UBI or DTPA-Scr.UBI) was mixed with 0.1 mL pertechnetate ^{99m}Tc solution (~10 mCi/mL), and 10µL stannous chloride solution (1 mg/mL in 0.01 M HCl previously purged with nitrogen), mixed and allowed to stand at room temperature for 30 min.

3.4. Labelling EB1 with ^{99m}Tc

3 µL of EB1 solution (10 mg/mL in sodium acetate) was mixed with 0.1 mL pertechnetate ^{99m}Tc solution (~10 mCi/mL), and 2 µL stannous chloride solution (1 mg/mL in 0.01 M HCl previously purged with nitrogen), and allowed to react at room temperature for 30 min.

3.5. Analysis of radiolabelled UBI, Scr.UBI and EB1

Radiolabelled UBI, Scr.UBI and EB1 were analysed using RP-HPLC (Shimadzu) and TLC. RP-HPLC was run using C-18 column (Microbondapack C-18, Waters), and acetonitrile and water containing 0.1 % TFA as eluants with gradient, i.e 0–3 min TFA/water, 3–13 min 0–50% ACN, 13-23 min 50% ACN, 23–26 min 50–70% ACN, 27 min 70–0% CAN and UV detection at 280 nm as well as radioactivity detection.

TLC was carried out in three separated chambers, i.e. the 1st chamber with acetone and Whatman-1 paper to determine % free pertechnetate, 2nd chamber with mixture of 0.05 M ammonium acetate pH 5.2 and methanol (1:1) and ITLC-SG strips to determine % Tc colloid and 3rd chamber with PBS pH 7.4 and TLC silica strips to determine % Tc-coligand and free pertechnetate. The radioactivity was measured using a TLC scanner (Veenstra Instrument), and % radiochemical purity of ^{99m}Tc peptides was calculated by substracting from 100 the sum of percentage of measured impurities.

3.6. Bacterial binding assay

To 0.1 mL of phosphate buffer containing 10 µL of ^{99m}Tc-UBI or ^{99m}Tc-Scr.UBI, were added 0.8 mL of phosphate buffer containing 50% acetic acid 0.01M and 0.01% Tween-80 and 0.1 mL of phosphate buffer containing about 2×10^7 CFU Staphylococcus Aureus. The mixture was incubated at 37ºC for 1 h followed by centrifugation for 5 min at 1000 rpm. The precipitate was separated and washed with 2×1 mL of phosphate buffer, and resuspended with 1 mL of phosphate buffer. Bacterial precipitate was measured for radioactivity, and % bacterial binding was calculated.

3.7. In vivo assay

In vivo assay was carried out using healthy mice and mice infected with bacterial culture. Assay on healthy mice was done to see the normal pharmacokinetics of radiolabelled UBI, while assay on Staphylococcus Aureus infected mice was done to see its effectiveness for marking infection. Mice were infected by injecting 0.1 mL of saline containing 10^7 CFU bacteria into right thigh. 18 h later, mice were injected with 0.2 ml saline containing 10% of ^{99m}Tc-UBI or ^{99m}Tc-Scr.UBI solution through tail vein. Injection of ^{99m}Tc-UBI or ^{99m}Tc-Scr.UBI was also done to normal mice as control. Mice were sacrificed after 30 min, 1 h and 2 h, and the organs including blood and the thigh were taken and measured for radioactivity.

3.8. Stability test

Stability test was carried out using fresh human serum and cysteine solution. Stability in serum was tested by incubating solution of 1-10 µg ^{99m}Tc-UBI or ^{99m}Tc-EB1 in 1 mL serum at 37ºC for 1 h, and the radiochemical purity was analysed with TLC. Stability against cysteine (cysteine challenge study) was tested by adding 20 µL of cysteine solution (1 mg/mL in 0.1 M PBS pH 7) into 90 µL of ^{99m}Tc-UBI solution (2.2 µM in 0.2 M PBS pH 7), incubating at 37ºC for 1 h, and analysing with TLC for radiochemical purity.

4. RESULTS AND DISCUSSION

Direct labelling of UBI and scrambled UBI with ^{99m}Tc showed similar labelling efficiency, of 87.8% and 85%, respectively (Table I). Labelling of UBI and scrambled UBI with ^{99m}Tc using NHS-HYNIC as BFC showed similar labelling efficiency, 85.2% and 83.1%, respectively. UBI, scrambled UBI and NHS-HYNIC were also separately labelled following the same protocol of that for HYNIC-UBI conjugate, and the results were 13.1%, 8.2% and 82.7%, respectively (Table II) confirming the specificity of BCA labelling.

TABLE I. LABELLING EFFICIENCY OF DIRECT LABELLED UBI

Labelled compounds	% free pertechnetate	% Tc colloid	% hydrophilic impurities	% labelled compound
Pyrophosphate	3.0	9.0		88.0
UBI		8.9	3.3	87.8
Scr.UBI		12.3	2.7	85.0

TABLE II. LABELLING EFFICIENCY OF INDIRECT LABELLED UBI (HYNIC-UBI)

Labelled compounds	% free pertechnetate	% Tc colloid	% hydrophilic impurities	% labelled compound
UBI	61.8	2.7	84.2	13.1
Scr.UBI	50.5	7.3	84.5	8.2
NHS-HYNIC	7.8	2.0	90.5	82.7
HYNIC-UBI		10.7	4.1	85.2
HYNIC-Scr.UBI		10.6	6.3	83.1

Experiments in labelling UBI using various coligands showed that tricine is better for higher yield than combination of tricine and EDDA (2:1). EDDA alone caused relatively poor labelling (Table III).

TABLE III. LABELLING EFFICIENCY OF HYNIC-UBI USING DIFFERENT COLIGANDS AND REACTION TEMPERATURES

Co ligand	Temperature/ Reaction time	% free pertechnetate	% Tc colloid	% hydrophilic impurities	% labelling efficiency
Tricine	RT/30 min	4.2	10.9	4.9	84.2
EDDA	RT/30 min	6.3	7.9	55	37.1
Tricine + EDDA	100°C/10 min	12	5.4	44.6	50
Tricine + EDDA	70°C/60 min	6.8	5.9	16.3	77.8

The low labelling yield of DTPA-UBI was attributable to the poor labelling of cDTPA with ^{99m}Tc (Table IV). The labelling protocol for DTPA-UBI needs to be optimized.

TABLE IV. LABELLING EFFICIENCY OF INDIRECT LABELLED UBI (DTPA-UBI)

Labelled compounds	% free pertechnetate	% Tc colloid	% hydrophilic impurities	% labelled compound
UBI	61.8	2.7	84.2	13.1
cDTPA	3.6	20.9		75.5
DTPA-UBI		21.9	19.5	58.6

EB1 was labelled with high yield (92.5%), even when it was no longer fresh (one month old kept in freezer) (Table V). Its reactivity to avidin was confirmed using Sephadex G-50 open column and by the shift notable in elution profile (Fig. 4). The stability of labelled UBI and EB1 in human serum was similar, 10% and 20% decrease after 1 h and 2 h incubation, respectively. ^{99m}Tc-HYNIC-UBI seemed to be more stable, with only 10% decrease after 2 h incubation (Table VI), also in cysteine solution.

EB1 can also be labelled with ^{99m}Tc in high labelling efficiency. ^{99m}Tc-EB1 was relatively stable in human serum and cysteine, and showed higher uptake in inflamed muscle compared to normal muscle.

TABLE V. LABELLING EFFICIENCY OF EB1

Labelled compounds	% free pertechnetate	% Tc colloid	% hydrophilic impurities	% labelled compound
Fresh EB1	2.8	4.7		92.5
EB1 (1 month)	4.4	9.8		85.8

TABLE VI. STABILITY TEST IN CYSTEINE SOLUTION AND FRESH HUMAN SERUM

Labelled compound/ Time observed (h)		Decrease in % radiochemical purity		
		Cystein solution	Human serum	PBS pH 7.4 (negative control)
^{99m}Tc-UBI	1	35.3	13.6	26.3
	2	49.3	22.4	44.3
^{99m}Tc-HYNIC-UBI	1	0.8	11.2	0
	2	17.6	11.7	2.9
^{99m}Tc-EBI	1		16.5	
	2		21.9	

Bacterial binding assay showed 40-80% binding for ^{99m}Tc-labelled scrambled UBI, UBI and HYNIC-UBI. The radiochemical purity value correlated with the percentage of bacterial binding. (Table VII and Fig. 1)

TABLE VII. BACTERIAL BINDING ASSAY

Labelled compound	% binding
^{99m}Tc UBI	58.7
^{99m}Tc Scrambled UBI	43.1
^{99m}Tc HYNIC-UBI	76.9

^{99m}Tc labelled UBI and HYNIC-UBI were identified using RP-HPLC, the retention time corresponding to the retention time of native UBI (Figs 2–3).

Biodistribution pattern in normal mice was similar for all labelled compounds, which showed high accumulation in kidney and bladder (Fig. 5). In infected mice, T/NT ratio was 1.5 for all labelled UBI after 1 h. It was 1.5 and 3, for labelled HYNIC-UBI and labelled UBI, respectively, after 2 h. In inflamed mice with induced inflammation, the T/NT ratio was 2.3 for ^{99m}Tc-labelled EB1 5 min post injection. (Figs 6–8 and 10). For labelling HYNIC-UBI with ^{99m}Tc use of tricine + EDDA and tricine alone as coligand did not show any significant difference (Figs 8 and 9). Gamma camera images of all labelled compounds are shown in Figs 11–14.

5. CONCLUSIONS

UBI and scrambled UBI can be labelled with high labelling efficiency either using direct or indirect method. The affinity to bind bacteria, both in vitro or in vivo, was also similar.

They showed higher uptake in infected site, but the stability in human serum and cysteine showed a slight difference in which labelled HYNIC-UBI seemed to be higher than direct labelled UBI.

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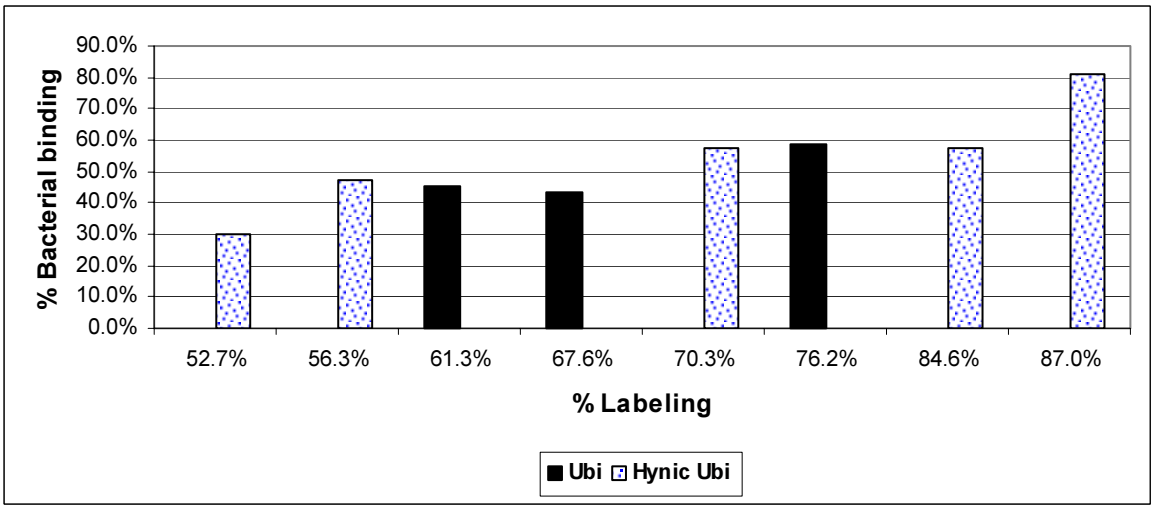

FIG. 1. Correlation between % labelling of ^{99m}Tc-UBI and % bacterial binding.

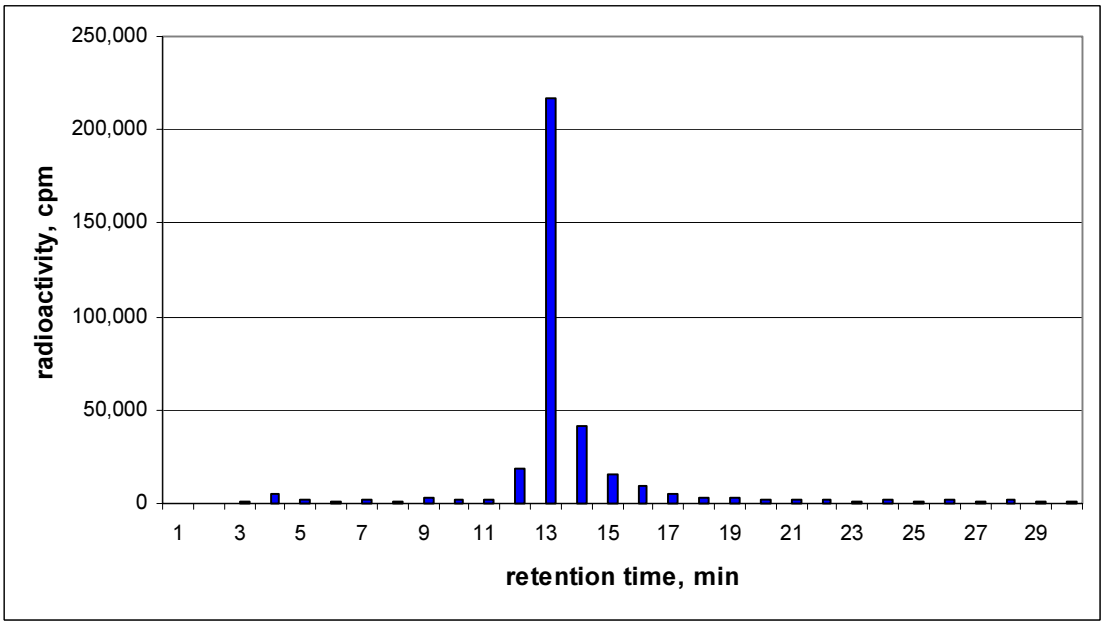

FIG. 2. Reversed phase HPLC of radiolabelled UBI.

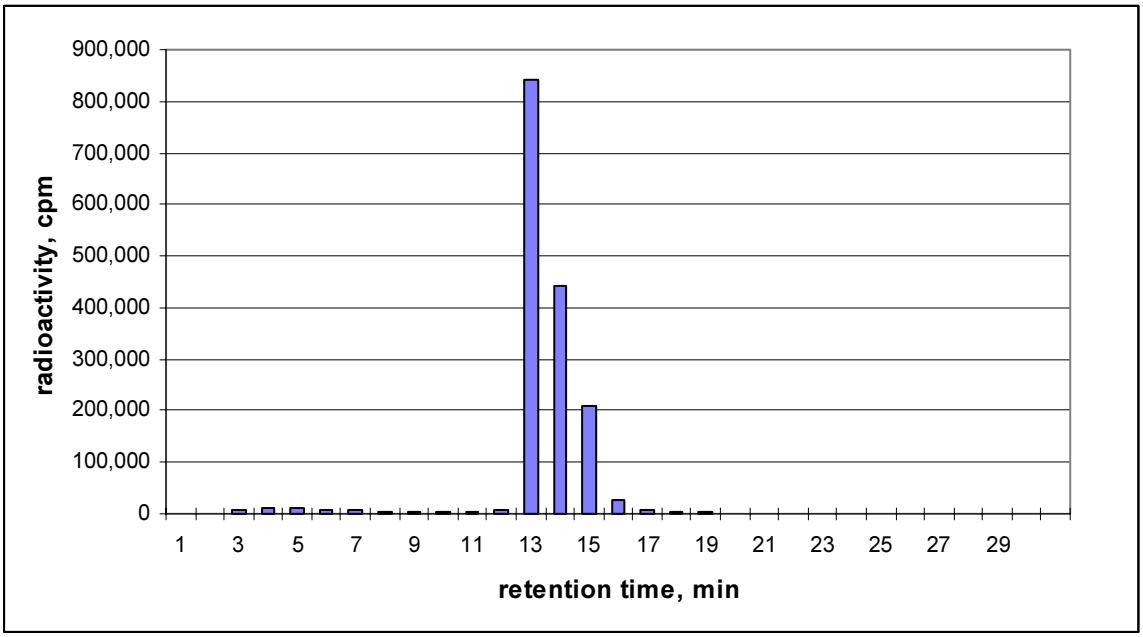

FIG. 3. Reversed phase HPLC of radiolabelled HYNIC-UBI.

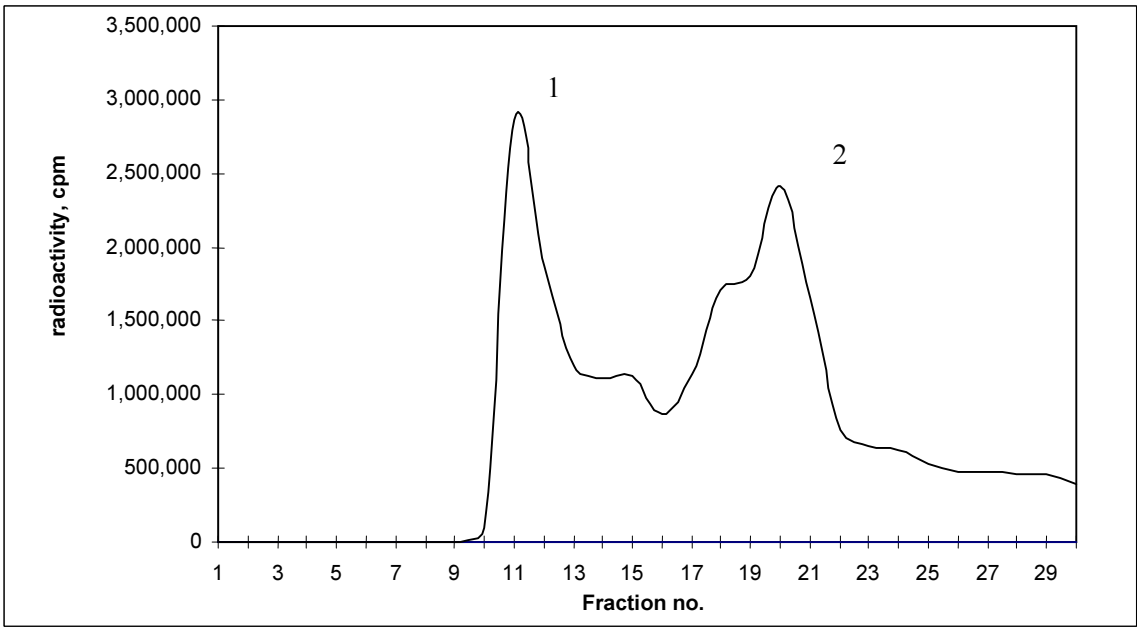

FIG. 4. Shift assay of radiolabelled EB1 — (1) ^{99m}Tc-EB1-avidin, (2) ^{99m}Tc-EB1.

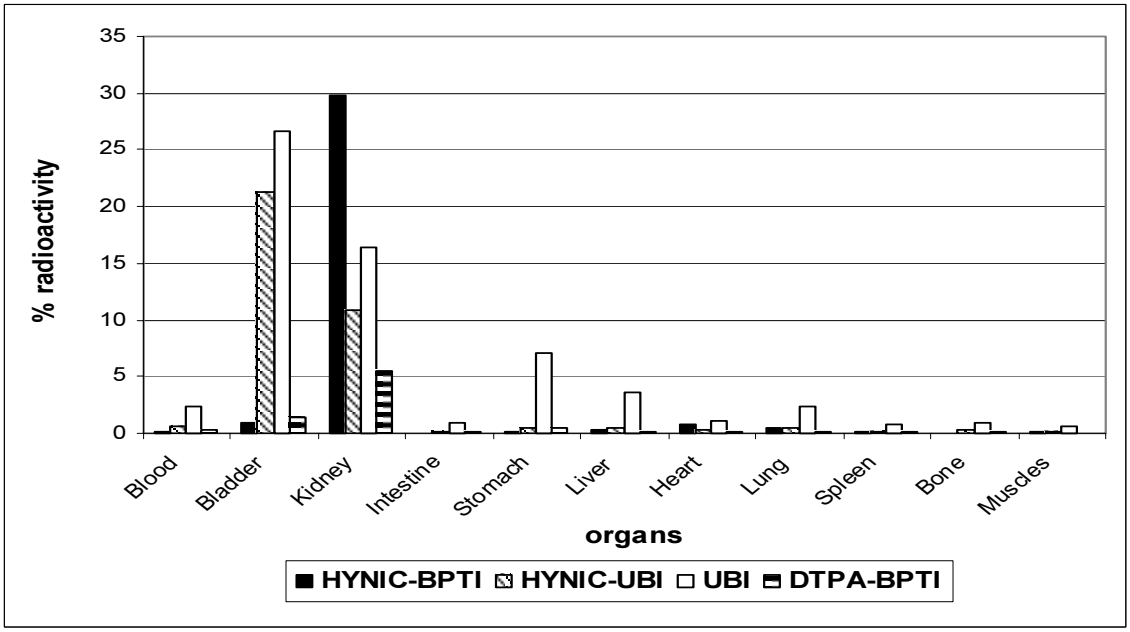

FIG. 5. Biodistribution of various ^{99m}Tc*-peptides in normal mice.*

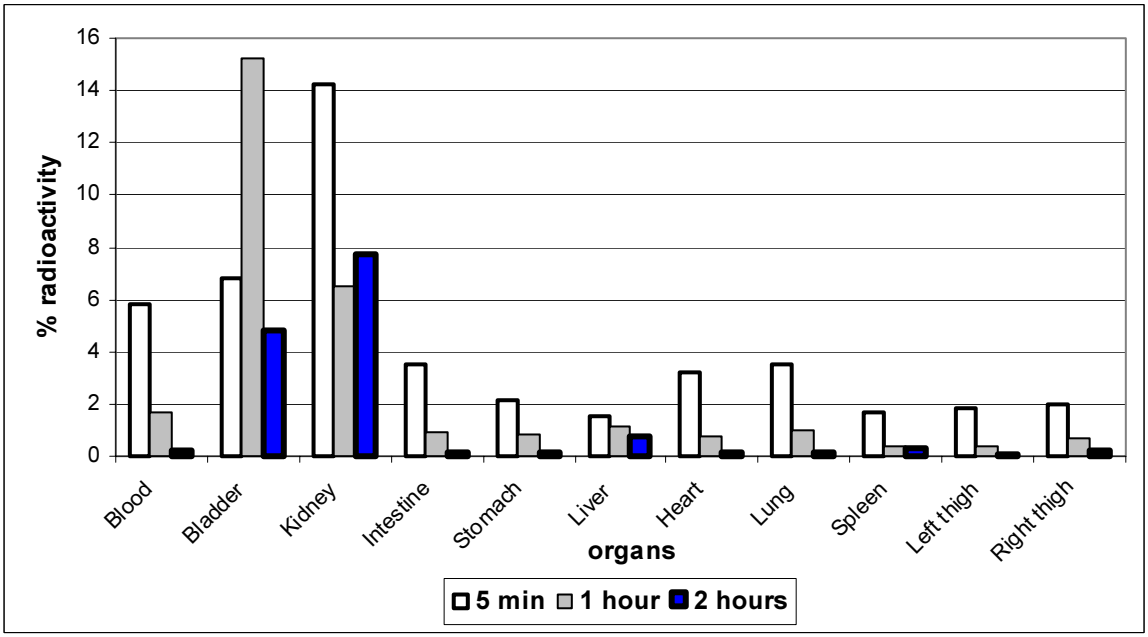

FIG. 6. Biodistribution of ^{99m}Tc*-UBI in mice with induced infection.*

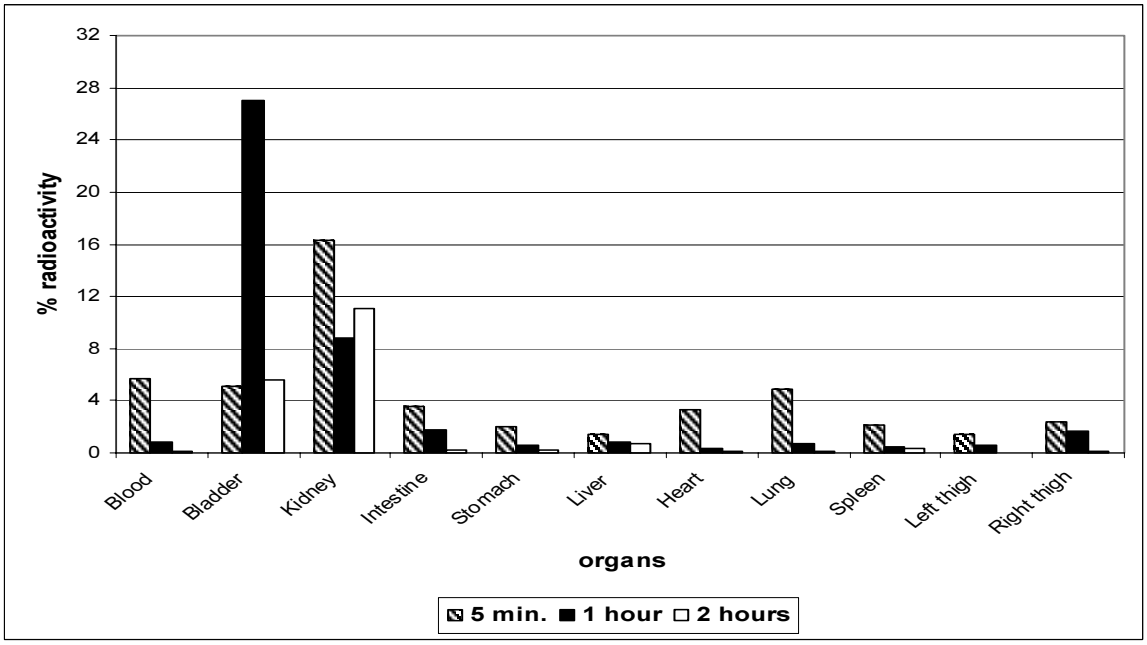

FIG. 7. Biodistribution of ^{99m}Tc-*scrambled UBI in mice with induced infection.*

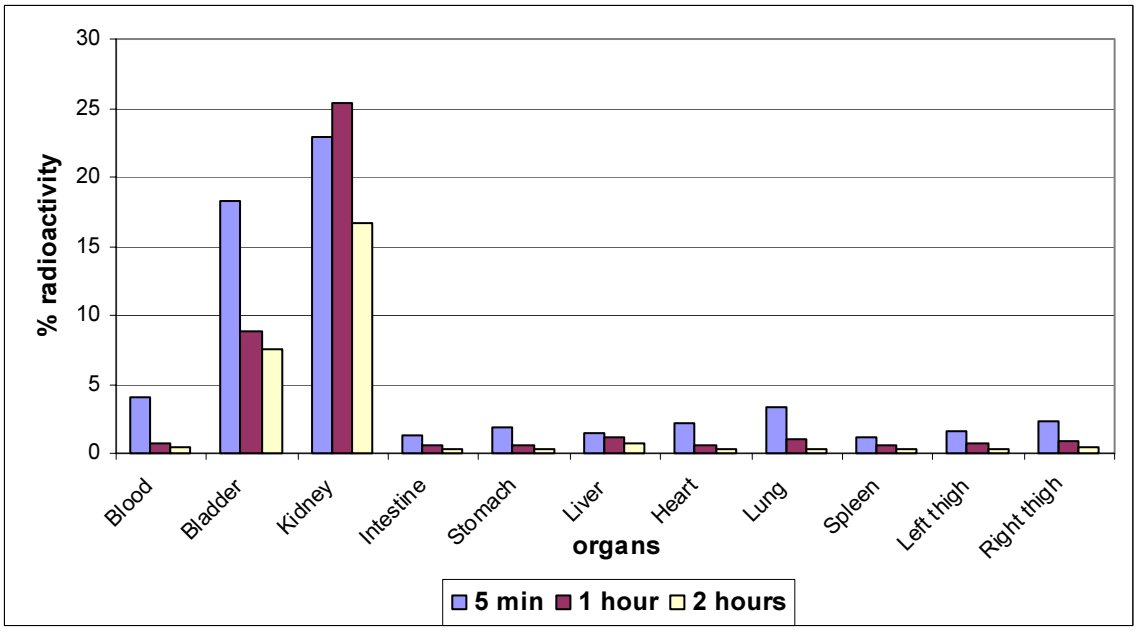

FIG. 8. Biodistribution of ^{99m}Tc-*HYNIC-UBI with tricine in mice with induced infection.*

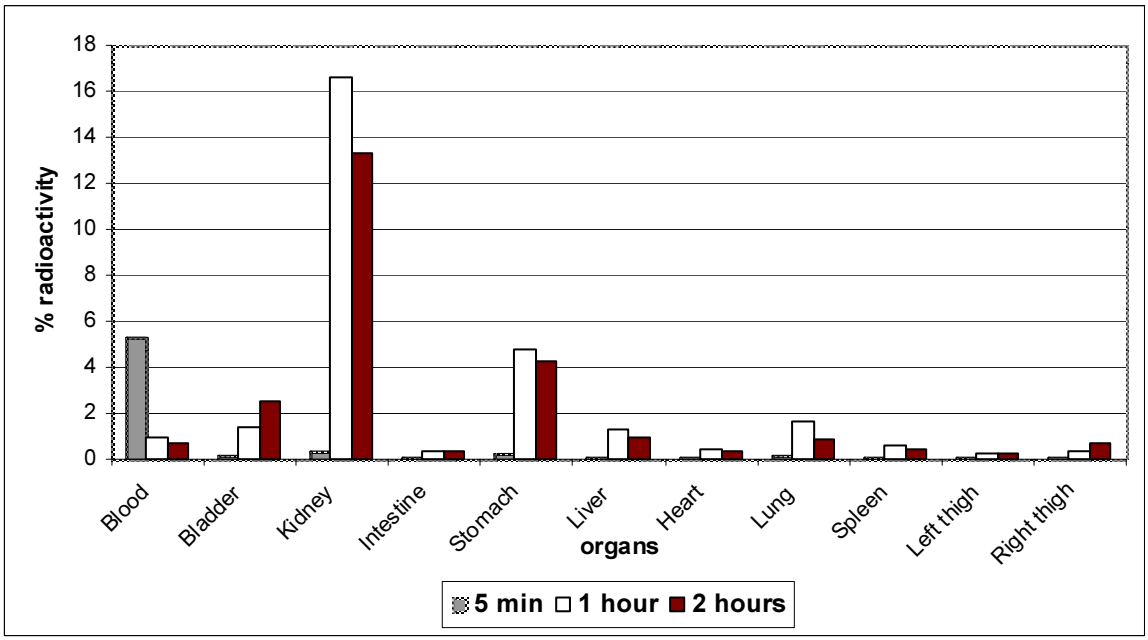

FIG. 9. Biodistribution of ^{99m}Tc-*HYNIC-UBI with tricine/EDDA in mice with induced infection.*

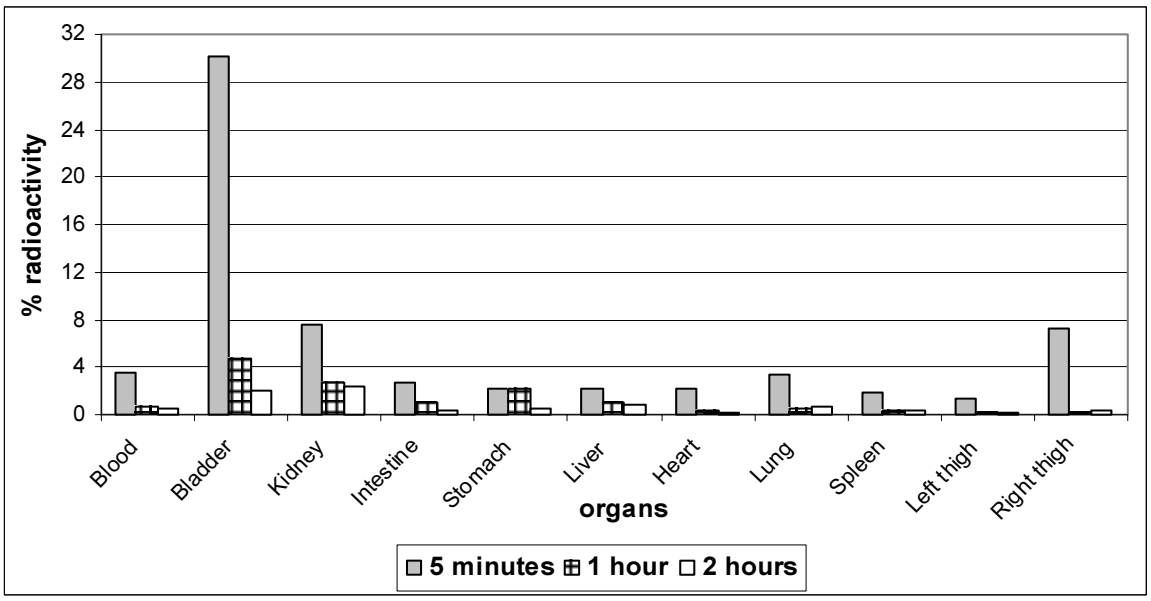

FIG. 10. Biodistribution of ^{99m}Tc-*EB1 in mice with induced inflammation.*

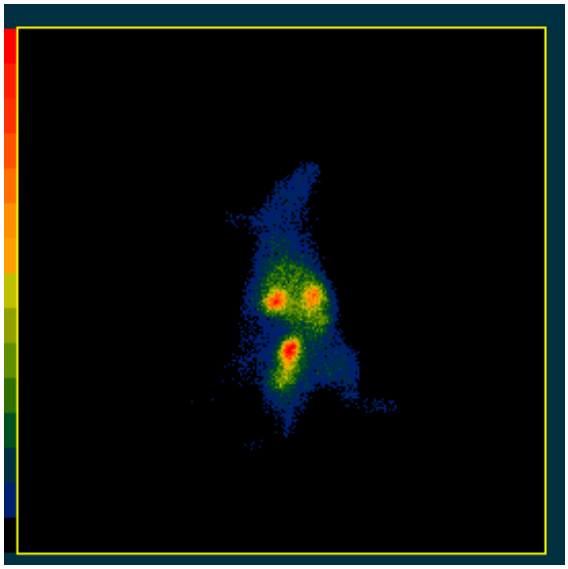

FIG. 11. Gamma camera imaging of ^{99m}Tc-*EB1 in Wistar rat with induced inflammation.*

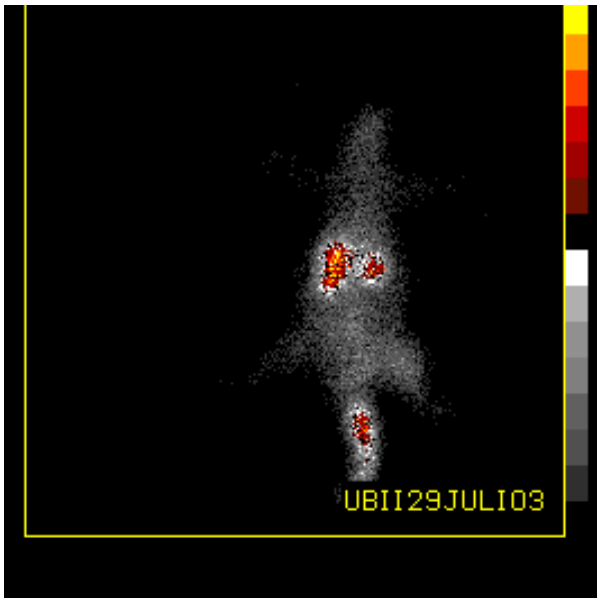

FIG. 12. Gamma camera imaging of ^{99m}Tc-*HYNIC-UBI/tricine in mice with induced infection 1 h p.i.*

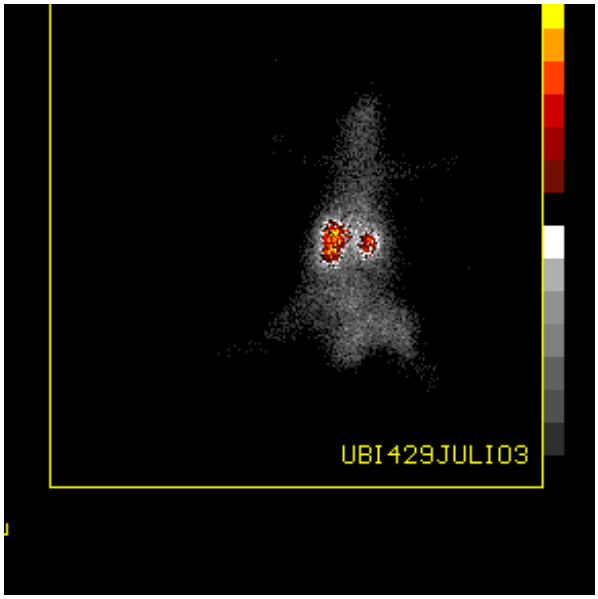

FIG. 13. Gamma camera imaging of ^{99m}Tc-*HYNIC-UBI/EDDA in mice with induced infection 1 h p.i.*

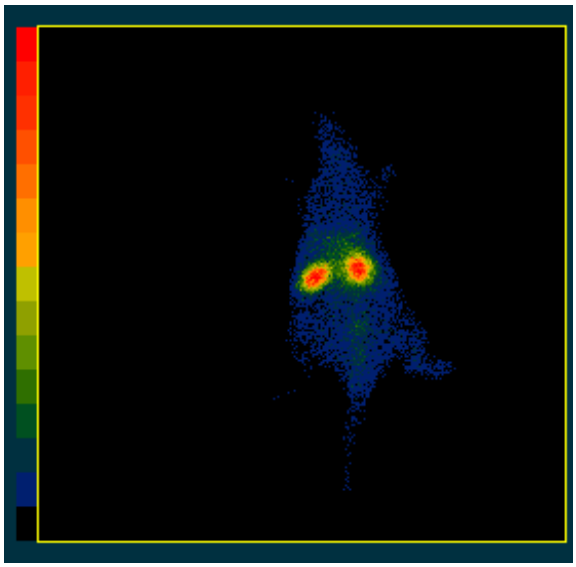

FIG. 14. Gamma camera imaging of ^{99m}Tc-*UBI in mice with induced infection 1 h p.i.*

PREPARATION OF ^{99m}Tc KITS FOR INFECTION IMAGING

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Abstract

The aim of this study was to label ubiquicidin fragment 29-41 (UBI) and biotin with ^{99m}Tc and evaluate their feasibility as infection imaging agents for in vivo use. ^{99m}Tc-UBI, labelled by a direct method, showed high in vitro and in vivo stability, specific uptake at the site of infection, rapid background clearance, minimal accumulation in non-target tissues and rapid detection of infection sites. ^{99m}Tc labelled biotin showed in vitro and in vivo stability, fast renal clearance and ability to detect infection and sterile inflammation processes in mice. Because of their stability and biological properties, both agents could be used in clinical applications.

1. INTRODUCTION

Recently technetium-99m labelled ubiquicidin peptide 29-41 fragment (^{99m}Tc-UBI) has been proposed as a new radiopharmaceutical for infection imaging [1,2]. UBI is a cationic human antimicrobial peptide fragment (MW 1.69 kDa) with the amino acid sequence Thr-Gly-Arg-Ala-Lys-Arg-Arg-Met-Gln-Tyr-Asn-Arg-Arg, therefore, with 6 positively charged residues (5 Arg + 1 Lys). Studies of the biodistribution of ^{99m}Tc-UBI in mice have shown a fast renal clearance with minimal hepatobiliary excretion. ^{99m}Tc-UBI is bound to bacteria in vitro and is accumulated at sites of infection in experimental animals [1,2].

Considering that five of the thirteen amino acids in UBI 29-41 fragment are arginine (with free $-NH_2$ groups), and 3 of these are close to a lysine with its own $-NH_2$ group, it is possible that some of these groups could form a stable complex with reduced technetium, such as the mono-oxo Tc(V). Therefore, the first aim of this study was to help establish if ubiquicidin peptide 29-41 fragment (UBI) contains a specific site for ^{99m}Tc labelling by a direct method under alkaline conditions. Since this peptide does not have cysteine residues, it is possible that neighbouring arginine and lysine in the peptide amino acid sequence (Thr-Gly-Arg-Ala-Lys-Arg-Arg-Met-Gln-Tyr-Asn-Arg-Arg) could be a coordination site to form a stable ^{99m}Tc-UBI complex. Following direct labelling, the in vitro stability of ^{99m}Tc-UBI was compared to UBI radiolabelled by one indirect method using HYNIC/tricine [3].

A second aim was to help determine if ^{99m}Tc-UBI is bound to the bacterial cell envelope by a simple nonspecific electrostatic interaction. A comparative study of the in vitro binding of ^{99m}Tc-UBI and two different ^{99m}Tc labelled cationic peptides (^{99m}Tc-Tat-1-Scr and ^{99m}Tc-Tat-2-Scr) to bacteria and to two tumor cell lines (LS174T and ACHN) was accomplished [4]. The in vivo specificity of ^{99m}Tc-UBI for infection in mice was also evaluated using dual labels in the same animal and comparing the target/non-target ratio for ^{67}Ga-citrate and ^{99m}Tc-UBI at sites of induced infection and sterile inflammation [4]. In addition, sequential gammagraphic images of ^{99m}Tc-UBI biodistribution in patients were obtained to calculate biokinetic parameters as residence time useful in absorbed dose calculations [5].

As a third aim, EDTA-biotin (EB1) and DTPA-bis-biotin were labelled with ^{99m}Tc and evaluated as non-specific infection imaging agents in mice [6].

2. MATERIALS

Ubiquicidin 29-41 fragment peptide (UBI) was synthesized and characterized at the Leiden University Medical Center, The Netherlands. Cationic control peptides with amino acid sequences Cys-Arg-Lys-Arg-Gly-Lys-Arg-Arg-Gln-Arg-Gly-Arg (Tat-1-Scr) and Arg-Lys-Arg-Lys-Arg-Gly-

Arg-Arg-Gln-Arg (Tat-2-Scr) were solid-phase synthesized and characterized at the University of Massachusetts Medical School (UMASS), USA. EDTA-biotin was supplied by the Pakistan Institute of Nuclear Science and Technology and NHS-HYNIC by POLATOM Radioisotope Centre and UMASS. Staphylococcus aureus microorganism, human renal adenocarcinoma cell line (ACHN) and the human colon cancer cell line (LS174T) were obtained from American Type Culture Collection. DTPA-bis-Biotin, tricine and other chemicals were purchased from Sigma Chemical Co.

3. METHODS

3.1. Direct techenetium-99m UBI labelling

For ^{99m}Tc labelling, 10 µL of a UBI aqueous solution (1 mg/0.2 mL) was added to a vial containing 5 µL of a fresh 1 mg/mL solution of stannous chloride in 10 mM HCl. The pH was adjusted to 9 by addition of 10 µL of 0.1 M NaOH, followed by 100 µL of ^{99m}Tc-pertechnetate (185-370 MBq). The resulting solution (pH 9-10) was left to react for 5 min at room temperature and finally, 1 mL of 0.9% NaCl solution was added to obtain a final pH 6.5-7.0. The KBH_4 and pyrophosphate used in an earlier direct labelling method were found to be unnecessary.

3.2. Indirect ^{99m}Tc UBI labelling using hydrazinonicotinamide (HYNIC)

Conjugation: UBI peptide was prepared at a concentration of 5 mg/mL in 0.1 M HEPES (N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)) buffer, pH 8.0, to which a fresh 10 mg/mL solution of NHS-HYNIC in dry dimethylformamide (DMF) was added dropwise with agitation. The final HYNIC-to-peptide molar ratio was 3:1, and the volume of DMF added was always <10% of the total volume. The mixture was reacted at room temperature for 30-60 min before purification on a 0.7×20 cm P4 size-exclusion open column (BioRad, Hercules, CA) with 0.25 M ammonium acetate, pH 5.2 or 0.05 M phosphate buffer pH 6.0 as eluants. Fractions were collected and analysed by ultraviolet (UV) absorbance at 280 nm (U-20000; Hitachi Instruments, Inc., Danbury, CT) using an extinction coefficient of 0.756 for UBI. The conjugated peptide was diluted to approximately 0.4-0.5 mg/mL with 0.25 M ammonium acetate pH 5.2 or 0.1 M phosphate buffer pH 6.0 (conjugated peptide solution).

^{99m}Tc-HYNIC-UBI/tricine: Approximately 20 µL of a 100 mg/mL tricine solution in water was added to 0.05 mg of HYNIC-UBI conjugate dissolved in 0.1 mL of 0.25 M ammonium acetate, pH 5.2. Then 111 MBq ^{99m}Tc-pertechnetate generator eluant was added followed by 6 µL of a fresh $SnCl_2 \cdot 2H_2O$ (1 mg/mL in 10 mM HCl) solution. After reacting for 30-60 min at room temperature, the labelled peptide was purified over the P4 column as above.

3.3. Quality control

Radiochemical purity analyses were performed by Sep-Pak C-18 cartridge open column chromatography, instant thin layer chromatography (ITLC) and reverse phase and size exclusion high-performance liquid chromatography (HPLC). The Sep-Pak cartridges were preconditioned with 5 mL of ethanol followed by 5 mL of 1 mM HCl. An aliquot of 0.1 mL of the labelled peptide was loaded on the cartridge followed by 5 mL of 1 mM HCl to elute ^{99m}Tc-pertechnetate. The labelled peptide was eluted with 3 mL of ethanol:saline (1:1) followed by 3 mL of 0.1 M HCl: methanol (15:85). The hydrolyzed-reduced ^{99m}Tc remainded on the column.

ITLC silica gel glass fiber 10 cm strips (Gelman Sciences) were used as stationary phase. Saline solution was the mobile phase to determine the amount of free $^{99m}TcO_4^-$ (R_f = 1). In this system the radiopeptide migrates when it is diluted in human serum.

Reverse Phase HPLC Method: HPLC analyses were carried out with a Waters instrument (Waters, Milford, MA, USA) running Millenium software with both radioactivity and UV-photodiode

array in-line detectors. YMC ODS-AQ S5 column (5µm, 4.6×250 mm) at a flow rate of 1 mL/min using 0.1% trifluoroacetic acid (TFA)/water (solvent A) and 0.1% TFA/acetonitrile (solvent B). The gradient started at 100% solvent A for 3 min, changed to 50% solvent A over 10 min, held constant for 10 min, changed to 30% solvent A over 3 min and finally back to 100% solvent A over 4 min.

Size-exclusion HPLC Method: Superdex-peptide column (1×30 cm, Pharmacia, Piscataway, NJ), at a flow rate of 1.0 mL/min with 0.1 M sodium phosphate, pH 7.0, as mobile phase.

3.4. Serum stability

Size exclusion HPLC analysis was used to estimate the serum stability of UBI labelled with ^{99m}Tc by the direct method and the indirect method using HYNIC. A volume of 100 µL of the labelled peptide solution (5µg/100µL) was incubated at 37^{o}C with 1 mL of fresh human serum. Radiochemical stability was determined taking samples of 10 µL at different times from 5 min to 24 h for analysis. A shift of the radioactivity profile to higher molecular weight indicates protein binding, while lower-molecular weight peaks indicate labelled catabolites or serum cysteine binding. Recovery of radioactivity was routinely determined.

3.5. Dilution stability

To determine the stability of ^{99m}Tc-UBI after dilution, 50 µL of radiolabelled peptide (25 µg/50 µL) was diluted to a ratio 1:20 with saline solution (0.9% NaCl), and incubated for 24 h at room temperature at pH 6.5 and pH 9. The reaction mixtures were analysed by C-18 Sep-Pak and reverse phase HPLC at various time intervals between 5 min and 24 h.

3.6. Cysteine challenge

The ^{99m}Tc-UBI, ^{99m}Tc-HYNIC-UBI/tricine and ^{99m}Tc-HYNIC-UBI/tricine/EDDA were tested for instability toward cysteine. A fresh cysteine solution was prepared (10 mg/mL in 0.1M PBS, pH 7) and diluted to different concentrations. Then 12 µL of each cysteine solution was mixed with 90 µL of the 2.2 µM of the labelled peptide solutions. The molar ratios of cysteine to peptide were between 5:1 and 500:1. Each test tube was incubated at 37°C and radiochemical purity analysed 1 h later by ITLC.

3.7. Semi-empirical and theoretical methodology

Molecular mechanics (MM) and quantum-mechanical calculations were performed on a PC using BioMedCAChe and CAChe Work System Pro 5.02 or 5.04 (Fujitsu America Inc., USA). UBI has thirteen amino acids including five arginines. Arginine contains a strongly basic guanidino group (pKa >12.0) that is protonated at neutral pH and therefore carries a positive charge.

The molecules of UBI peptide and protonated-UBI peptide were built considering different hybridization geometries, technetium oxidation state (V, IV, and III), bond type and coordination bond from the most probable coordination sites of the peptide to the mono-oxo-Tc(n+) cation. Also, geometries were optimized after adding water molecules from the reaction media into the coordination core, with MM calculations using Augmented MM3 force field. The energy minimization was performed until the energy change was <0.001 kcal/mol, or until the molecule had been updated 300 times. For Tc-UBI complex, several structures were calculated to identify donor sites able to coordinate technetium as $(Tc(O))^{n+}$ and the minimum energies found fluctuated between -100 and 1000 kcal/mol.

The optimum geometrical structures of UBI peptide and protonated-UBI peptide found by MM were confirmed by quantum-mechanical methods using the Schrödinger equation with MOPAC

(Molecular Orbital Package) which calculates heat of formation. Tc-UBI complexes were not calculated by MOPAC (technetium parameters are not available in MOPAC).

3.8. In vitro specificity test

Bacteria: Staphylococcus aureus ATCC 25923 microorganism, was used for in vitro and in vivo studies. The bacteria, previously preserved frozen (-80^{0}C) and after overnight culture incubation at 37^{0}C in a shaking water bath, were washed (0.1 M phosphate buffer, pH 7.5), and aliquoted at a concentration of about 4×10^7 cells per mL.

Tumor cells: The human renal adenocarcinoma cell line (ACHN) and the human colon cancer cell line (LS174T) were obtained from American Type Culture Collection (Manassas, VA). Cells were grown in Minimum Essential Medium (MEM, Gibco BRL Products, Gaithersburg, MD) with 2 mM L-glutamine, 1.5 mg/L sodium bicarbonate, 0.1 mM non-essential amino acids and 1.0 mM sodium pyruvate supplemented with 10% fetal bovine serum (FBS) and 100 mg/mL of penicillin-streptomycin (Gibco). Cells were maintained as monolayers in a humidified 5% CO_2 atmosphere, normally in T75 flasks (Falcon, Becton Dickinson, Lincoln Park, NJ)

The cells were trypsinated in the T75 flasks at 80-90% confluence using 0.05% trypsin/ 0.02% EDTA and were then suspended in MEM with 10% FBS to the desired density, normally 2×10^5 cells/mL. The cells were seeded in wells of 24-well flat-bottomed plates at 1 mL/well and incubated at 37°C in 5% CO_2 for 24 h for adherence and growth. The wells of the plates were pre-wetted with 1 mL of Dulbecco's phosphate buffered saline (DPBS, Gibco) to ensure homogeneity of cell adherence to the well bottom. The medium was changed to MEM with 1% FBS to slow cell growth and the cells were cultured further for another 10 h or until 80% confluence was reached. Cell growth status and viability was monitored by inverted phase contrast microscopy. Trypan blue exclusion was also used to assess viability. About 30 min before in vitro studies, tumor cells were washed using 0.1 M phosphate buffer, pH 7.5, and aliquoted at a concentration of about 4×10^7 cells per mL (tumor cell suspension).

In vitro competition assay for the binding of ^{99m}Tc-labelled cationic peptides: To 0.8 mL of the incubation buffer (0.05 M PBS containing 0.1% Tween 80 and 0.2% acetic acid, pH 5), was added 0.1 mL of the preparation containing the labelled cationic peptide and 0.1 mL of the cell suspension. The active suspensions were gently mixed in a vortex and incubated at 37^{0}C. In competition experiments, bacteria or tumor cells were pre-incubated for 1 h at 37^{0}C with 100 fold excess of unlabelled cationic peptides before the addition of a radiolabelled peptide. After incubation of the bacteria or tumor cells with radiolabelled peptides for 1 h, the tubes were centrifuged at 1000 g for 5 min. The supernatant was removed and the pellet resuspended in 1 mL of incubation buffer and re-centrifuged as described above. The supernatant was removed and the radioactivity in the pellet was counted in a NaI(Tl) detector. The radioactivity associated with cells was expressed as percentage of the total ^{99m}Tc-activity added.

3.9. In vivo specificity test

Biodistribution studies in balb/c mice were performed according to the Official Mexican Norm (NOM): rules and regulations for safe and adequate animal handling. ^{67}Ga-citrate was purchased at a radiochemical concentration of 37 MBq/mL.

Inflammation and infection induction in mice: Heat killed bacteria [endotoxin, lipopolysaccharide (LPS)] was used for creating inflammation. Gram negative bacteria (Klebsiella pneumoniae, sp) in saline solution were heated at 100^{0}C for 2 h and aseptically dispensed in 1 mL aliquots into sterile vials. These were subjected to several freeze-thaw cycles. Samples were checked for absence of bacterial growth after culturing. Finally 0.1 mL of the heat killed bacterial suspension was injected (i.m) at the same concentration in the left thigh of 5 mice. To create infection, 0.1 mL of

the bacterial suspension (*S. aureus*) was immediately injected i.m. in the right thigh of the same 5 mice.

Uptake of ^{67}Ga-citrate and ^{99m}Tc-UBI at sites of infection and inflammation in the same animal: After 24 h of inflammation and infection induction in mice, 1.5-2.3 MBq (0.05 mL) of ^{99m}Tc-UBI and ^{67}Ga-citrate were together administered intravenously in a tail vein. After 2 h post-injection the animals were sacrificed in a CO_2 chamber. Infected, inflamed and normal muscles (taken from the left anterior limb) were extracted. Selected tissues (blood, heart, liver, kidney, spleen, stomach, intestine, muscle and bone) were also removed. The organs were weighed in tarred test tubes. The radioactivity in the tissues was analysed by γ-spectrometry using a multichannel HPGe detector (Canberra). The 140 keV γ-rays of ^{99m}Tc and 300 keV γ-rays of ^{67}Ga were used for detection. The ^{67}Ga and ^{99m}Tc counts per gram of tissue were used to calculate the target/non target (T/NT) ratios for ^{99m}Tc-UBI and ^{67}Ga-citrate with respect to normal tissues at sites of infection and inflammation. For all tissues radioactivity was expressed as percentage of the injected dosage per gram of tissue (% I.D./g).

Infection and Inflammation Imaging: Imaging was performed 2 h after the radiopharmaceutical injection on an E-CAM, Siemens scintillation camera with a pinhole and the window adjusted for ^{67}Ga and ^{99m}Tc to avoid cross talk between radiolabels. The anesthetized live mouse was placed in a prone position with limbs spread out and secured with surgical tape. The image was taken for 35 min and stored in a 256×256 matrix. Two regions of interest (ROIs) were drawn over the infected muscle and inflamed muscle. Accumulation at damaged muscles was expressed as infection/inflammation ratio for ^{99m}Tc-UBI and ^{67}Ga-citrate. Differences between the in vivo data were evaluated with the Student pair *t* test.

3.10. ^{99m}Tc-UBI biokinetics

Whole-body images from 6 children with suspected bone infection were acquired at 1, 30, 120, 240 min and 24 h after ^{99m}Tc-UBI administration. To evaluate biokinetics, total-body images were analysed with the geometric mean of the anterior and posterior views (256×1024 pixel matrix). ROIs were drawn around source organs (heart, liver, kidneys and bladder) on each time frame. The same set of ROIs was used for all 6 scans and the counts per minute of each ROI were converted to activity using the system calibration factor. Counts were corrected by physical decay and by the best background correction factor derived from preclinical phantom studies. The image sequence was used to extrapolate ^{99m}Tc time activity curves in each organ and calculate the cumulated activity ($\tilde{A}$ **).** Urine samples were used to obtain the cumulative percent of injected activity (% I.A.) versus time eliminated via kidney. To establish the biokinetic model the % I.A. for each organ and the remainder of the body was fitted to a bi-exponential function using the Biexp 2.0 software. Blood activity curve was derived from the heart activity.

$\tilde{A}$ is the cumulated activity in the source organ and is given to the area under the time-activity curve for a source organ or region. As activity is the number of disintegrations per unit time, integrating this over time gives the total number of disintegrations:

$$\tilde{A}_h(0,\infty) = \int_0^\infty A_h(t)dt$$

residence time (τ) was calculated as:

$$\tau_h = \frac{\tilde{A}_h}{A_o}$$

The absorbed dose to organs was evaluated according to the general equation described in the MIRD formalism. In this study, absorbed dose estimates were obtained by entering the experimental τ values for all source organs in the MIRDOSE 3.1 software using a reference 10 years old child phantom.

3.11. ^{99m}Tc labelled EB1 and DTPA-bisBiotin

Diethylenetriaminepentaacetic-α,ω-bis(biocytinamide) (DTPA-bis-Biotin) or EDTA-Biotin, 1 mg, was dissolved in 100 µL of 1 M sodium acetate buffer pH 6. For labelling, 50 µL was added to a vial containing 37-185 MBq of ^{99m}Tc-pertechnetate eluant in a volume of 10-20 µL. To this was added 2 µL of a fresh 1 mg/mL solution of stannous chloride in 10 mM HCl. The reaction mixture was incubated at room temperature for 10 min. Radiochemical purity of the ^{99m}Tc-biotin complexes was assessed using SepPak C-18 cartridges (Waters), ITLC-SA/ 20% sodium chloride and ITLC-SG /85% methanol systems.

Size exclusion HPLC radiochromatograms (ProteinPak 125, Waters, phosphate buffer 0.1 M, 1.0 mL/min) were obtained of the radiolabelled EDTA-Biotin and DTPA-Biotin with and without the addition of avidin in a five molar excess. For measurement of the in vitro stability in human serum of the ^{99m}Tc-biotin conjugates, 10 µL of a solution containing 10 nmol of the labelled conjugate in saline was added to 1 mL of fresh human serum. At various intervals samples were taken and diluted with avidin solution (5 mg/mL). The stability was expressed as the percentage of Tc-Biotin bound to an excess of avidin as determined by HPLC and ITLC. ^{99m}Tc-biotin uptake at site of infection and inflammation in mice was accomplished as described above for ^{99m}Tc-UBI.

4. RESULTS AND DISCUSSION

Radiochemical purity of ^{99m}Tc-UBI averaged 97% compared to 88% for ^{99m}Tc-HYNIC BI/tricine. Both ^{99m}Tc-HYNIC-UBI and ^{99m}Tc-UBI showed stability in human serum and solutions of cysteine (Figs 1 and 2).

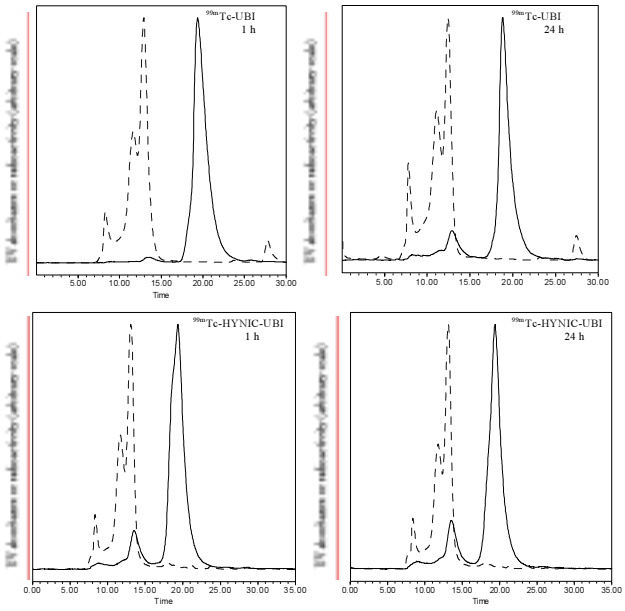

FIG. 1. Size-exclusion HPLC: Dash line represents the UV-chromatogram (280 nm) of human serum proteins and solid line the radiochromatogram of ^{99m}Tc-HYNIC-UBI (t_R= 19 min) and ^{99m}Tc-UBI (t_R= 19 min) after 4 and 24 h incubation in human serum at 37°C.

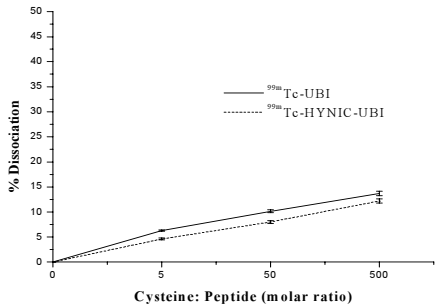

FIG. 2. Dissociation from ^{99m}Tc-HYNIC-UBI and ^{99m}Tc-UBI by exchange of the ligand with cysteine.

^{99m}Tc-UBI radiochemical purity 24 h after dilution in 0.9% NaCl was greater than 90% at pH 9 and greater than 95% at pH 6.5. Under one set of experimental conditions, in vitro binding to bacteria of ^{99m}Tc-UBI was 35% and similar to that of ^{99m}Tc-HYNIC-UBI at 32%. The biodistribution of ^{99m}Tc-UBI in mice showed a rapid renal clearance. Molecular mechanics and quantum-mechanical calculations showed that the amine groups of Arg^7 and Lys are the most probable sites of ^{99m}Tc binding following direct labelling. The calculations show that these groups can form a square pyramid with two water molecules for the Tc cation (*dxysp*^{3}) (Fig. 3). It will be necessary to isolate and characterize the ^{99}Tc(V)(*O*)-UBI $(H_2O)_n$ complex to confirm these results [6].

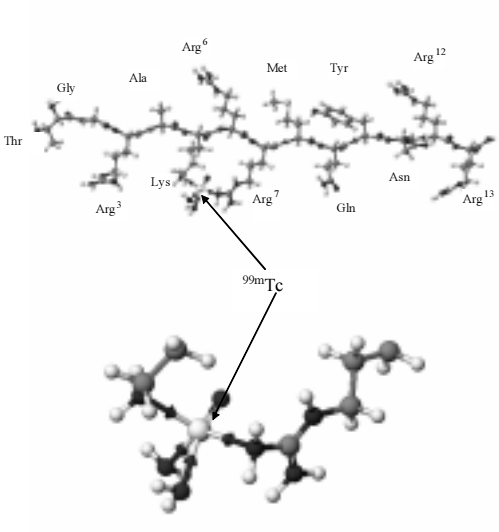

FIG. 3. Optimum molecular structure of Tc(V)(O)-(Arg^7-Lys-UBI)$^{5+}$ $(H_2O)_2$ complex in a square pyramidal coordination polyhedron calculated in Mechanics using Augmented MM3 parameters. Tc-UBI complex full view (up) and coordination polyhedron of Tc(V) only (down).

Under conditions of this study, the in vitro binding of ^{99m}Tc-UBI, ^{99m}Tc-Tat-1-Scr and ^{99m}Tc-Tat-2-Scr to *S. aureus* was 35%, 78% and 87%, respectively (Fig. 4). While the binding of ^{99m}Tc-Tat-1-Scr and ^{99m}Tc-Tat-2-Scr was 37 and 33% to colon tumor cells (LS174T) and 39 and 41% to renal tumor cells (ACHN) respectively, the binding of ^{99m}Tc-UBI to both cell types was much lower at less than 4% (Figs 5a and 5b). In vivo studies revealed that there is a significant difference ($p < 0.05$) in the radioactive accumulation of ^{99m}Tc-UBI between the sites of infection and inflammation compared to ^{67}Ga-citrate (Fig. 6). Thus, ^{99m}Tc-UBI showed an average infection/inflammation ratio of 2.08 ± 0.49 compared to 1.14 ± 0.45 for ^{67}Ga-citrate. The in vitro and in vivo results provide evidence that a specific mechanism is responsible of the ^{99m}Tc-UBI bacterial intracellular accumulation.

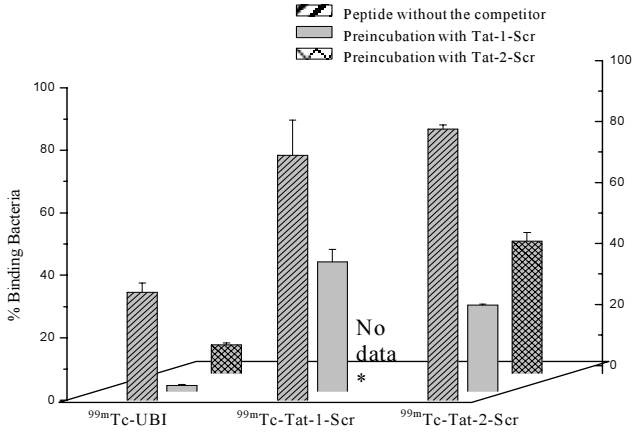

FIG. 4. In vitro binding of ^{99m}Tc-UBI, ^{99m}Tc-Tat-1-Scr and ^{99m}Tc-Tat-2-Scr to S. aureus. Competition binding of the peptides to bacteria showed that pre-incubation with cold UBI, Tat-1-Scr or Tat-2-Scr, decreased significantly the average binding of the labelled peptides.

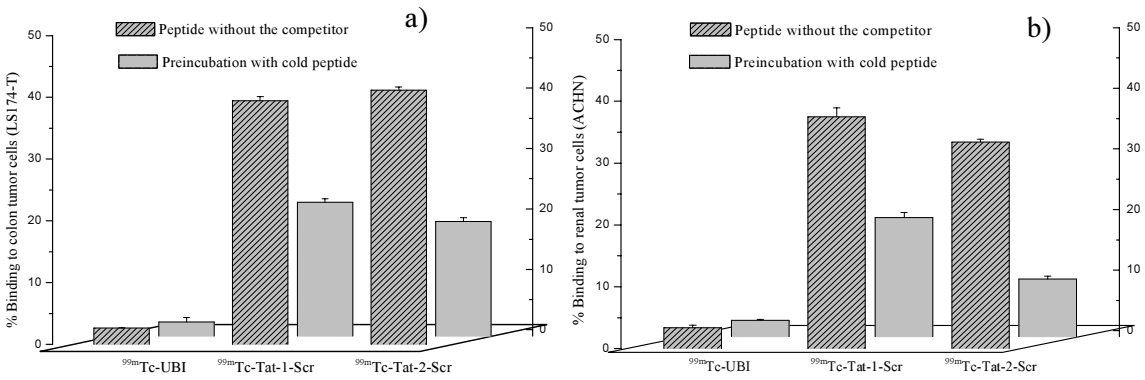

FIG. 5. a) In vitro binding of ^{99m}Tc-UBI, ^{99m}Tc-Tat-1-Scr and ^{99m}Tc-Tat-2-Scr to colon tumor cells (LS174T) b) In vitro binding of ^{99m}Tc-UBI, ^{99m}Tc-Tat-1-Scr and ^{99m}Tc-Tat-2-Scr to renal tumor cells (ACHN). In both cases there is a minimal accumulation of ^{99m}Tc-UBI compared to the Tat peptides.

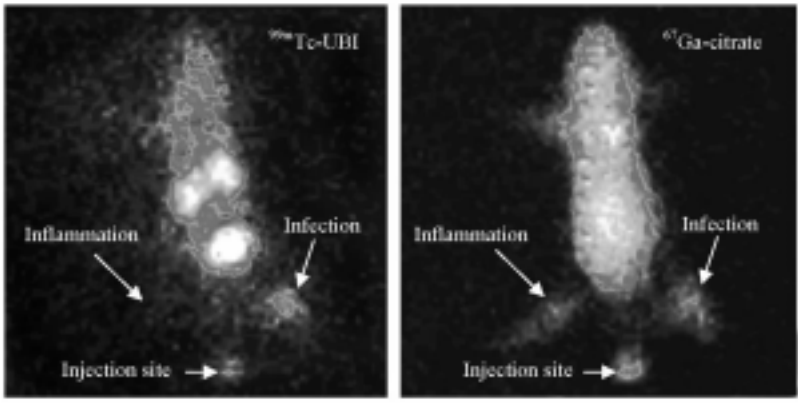

FIG. 6. Whole body images of the same mouse with induced infection (right thigh) and sterile inflammation (left thigh) 2 h after administration of both ^{99m}Tc-UBI and ^{67}Ga-citrate.

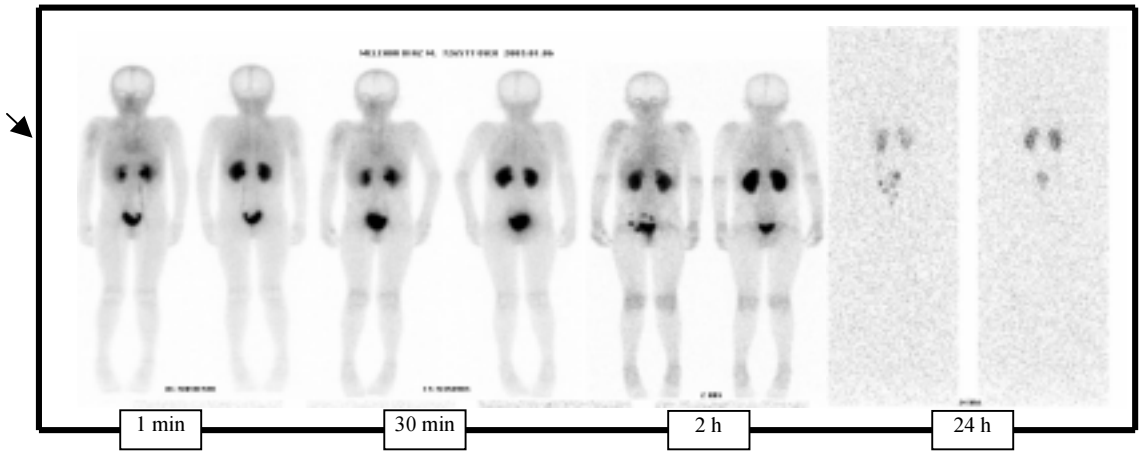

FIG. 7. Patient with chronic bone infection on right arm. Anterior and posterior views at 1 min, 30 min 2 h and 24 h after ^{99m}Tc-UBI administration.

^{99m}Tc-UBI showed a fast renal clearance in patients (Fig. 7). Two hours after administration a small percentage of the injected activity was removed by the liver in comparison with the kidney. Residence times and absorbed dose calculated from patients are shown in Table I and target non-target ratio at the infection sites are shown in Table II.

Radiochemical yield for ^{99m}Tc-EDTA-Biotin and ^{99m}Tc-DTPA-bis-Biotin was 93% and >98% respectively. A quantitative shift of the radioactivity profile to shorter retention times (avidin retention time) in the presence of the avidin demonstrated a good radiolabel and the avidity of the ^{99m}Tc -biotin complexes for avidin. Specifically ^{99m}Tc labelled biotin showed an average infection/inflammation ratio of 1.36 ± 0.27.

TABLE I. ABSORBED DOSE, RESIDENCE TIMES AND EFFECTIVE DOSE OF ^{99m}Tc-UBI CALCULATED FROM PATIENTS

Patient No.	Absorbed Dose, mGy/MBq [Residence time, h]		Effective Dose mSv/MBq
	Kidney	Liver	
1	3.94E-02 [0.74]	3.94E-03 [0.38]	1.01E-02
2	6.79E-02 [1.27]	5.43E-03 [0.52]	7.62E-03
3	3.97E-02 [0.74]	4.51E-03 [0.43]	4.21E-03
4	5.32E-02 [0.99]	3.06E-03 [0.29]	7.89E-03
5	7.56E-02 [1.41]	5.37E-03 [0.51]	6.68E-03
6	3.86E-02 [0.72]	7.92E-03 [0.76]	3.53E-03

TABLE II. TARGET/ NON-TARGET RATIO OF ^{99m}Tc-UBI AT INFECTION SITES IN PATIENTS

Patient No.	^{99m}Tc-UBI Target/non-target ratio
1	2.83
2	3.09
3	1.58
4	1.45
5	1.02 (negative)
6	1.97

5. CONCLUSIONS

^{99m}Tc-UBI, labelled by a direct method, showed high in vitro and in vivo stability, specific uptake at the site of infection, rapid background clearance, minimal accumulation in non-target tissues and rapid detection of infection sites. ^{99m}Tc labelled biotin showed to be stable in vitro and in vivo, fast renal clearance and ability to detect infection and sterile inflammation processes in mice. Because of their stability and biological properties, both agents could be used in clinical applications.

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DEVELOPMENT OF KITS FOR ^{99m}Tc RADIOPHARMACEUTICALS FOR INFECTION IMAGING

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Abstract

The aim of the present work was to evaluate three agents selected in this CRP for their possible role as ^{99m}Tc labelled infection imaging agents: human neutrophil elastase inhibitor (HNE2), Ubiquicidin (UBI 29-41) and Ethylene diamine tetraacetic acid biotin monomer (EB1). ^{99m}Tc labelling of EB-1 was carried out by direct labelling method with a yield of 90–95%. UBI was labelled by direct labelling as well as indirect labelling method using cyclic DTPA and HYNIC as conjugates with labelling efficiency of >95% in both cases, while HNE-2 was labelled only by indirect method and >96% labelling with ^{99m}Tc could be achieved. Chromatographic studies like ITLC, paper chromatography and HPLC were carried out for radiochemical separation and for determining the labelling yields. Kit formulations were also developed for these agents. Since availability of HNE-2 was doubtful detailed study of ^{99m}Tc-UBI (29-41) involving cysteine challenge, in vitro binding with bacteria as well as infection imaging in mice and rabbits was carried out. Experimental thigh muscle infection was induced by injecting 2×108 CFU of live S. aureus or E. coli bacteria into right thigh muscle of rabbits while turpentine oil and formalin killed S.aureus were utilized for inducing sterile thigh muscles inflammation. Significantly higher ($p<0.05$) accumulation of ^{99m}Tc-UBI (29-–41) was seen at sites of S.aureus infected subjects (T/NT ratio 2.2 ± 0.5) as compared to E. coli (T/NT ratio 1.7±0.4). Maximum tracer accumulation was seen at 60 min post injection followed by gradual decline. No accumulation of ^{99m}Tc-UBI was observed in thighs of rabbits injected with either turpentine oil or killed S.aureus with significant lower T/NT ratios ($p<0.05$) compared with S. aureus and E. coli infected thighs. Hence, it can be concluded that ^{99m}Tc-UBI 29-41 scintigraphy is a good technique for differentiating infection with S. aureus and E. coli. This study can be extended to clinical study after toxicity clearance of ^{99m}Tc UBI 29-41.

1. INTRODUCTION

One of the best studied antimicrobial peptides is UBI (29-41) [TGRAKRRMQYNRR, 1,693 Da) which was originally isolated from the cytosolic fraction of IFN-γ-activated cells of mouse macrophage cell line RAW264.7. Later on, an identical UBI was isolated from human, H 292 airway epithelial cells [1]. Similarly, another microbial peptide which has been studied for antibacterial therapy in experimental infections is human neutrophil elastase inhibitor (HNE2) [2]. The antibacterial effect was found to be associated with an increased influx of neutrophils in the infected area [3]. Use of this agent in experimental thigh muscle infection in mice allowed rapid visualization of bacterial infection, but abscess to background ratios were low and decreased with time [4,5].

The various methods of labelling peptides with ^{99m}Tc including indirect labelling using the preformed chelate approach or bifunctional chelating agents and the direct labelling method have been discussed extensively [6]. The direct labelling method is a simple procedure in which the peptide is labelled in absence of exogenous chelators. EB1 and UBI can be labelled by direct labelling with ^{99m}Tc in presence of reducing agent. The labelling of UBI is rapid and labelling yield quite good (>95%) [7]. UBI can also be labelled through indirect labelling while HNE2 can only be labelled through bifunctional chelating agent procedures [8]. Welling et al [9] have shown significant accumulation of ^{99m}Tc-UBI (29-41) in rabbits with experimental thigh muscle infection induced by two different types of S.aureus (ATCC25923) and MRSA. They found mean target to non-target ratio of 3.3±0.2 in both cases and found similar results in mice [10–11].

The purpose of the present investigation was to study in detail ^{99m}Tc labelling conditions for three agents HNE2, EB1 and UBI and formulate them into easily available freeze-dried kit form. The work on HNE2 and EB1 was limited to determining the labelling conditions only. However, UBI was found quite promising. So a detailed study of this antimicrobial peptide was carried out which included: (i) Direct labelling of UBI with ^{99m}Tc in in-situ form, (ii) Indirect labelling, (iii) Formulation

of freeze-dried kit, (iv) Labelling of freeze-dried kit, (v) Formulation of quality control procedures to find out labelling efficiency, (vi) In vitro binding assay of ^{99m}Tc UBI to bacteria, and scintigraphic visualization of infection in animal species. Results found have been very encouraging and comparable to literature results [11].

2. MATERIAL

UBI (29-41) and Scrambled UBI (S.UBI) were obtained from Lieden University, Netherlands [7]. EB1 was synthesized locally from the chemicals obtained through IAEA according to the literature method [12]. A sample of EB1 was also received from India. HNE2 was obtained through Dr. Hnatowich. All other chemicals used were analytical grade and obtained commercially. S. aureus and E. coli strains were obtained from National Institute of Health, (NIH) Islamabad. Rabbits and mice were also obtained either from NIH or University of Agriculture, Faisalabad (PINUM studies).

3. METHODS

3.1. Preparation of conjugated compounds

3.1.1. Conjugation of UBI with HYNIC

UBI was conjugated to HYNIC following the 1st RCM protocol. The mixture was purified by passing through Sephadex G-25 column, 0.75×20 cm. Elution was then carried out with 0.25 M ammonium acetate buffer pH 5.2 at slow rate (about 2.5 mL/h) to effectively separate the unconjugated HYNIC. The elution was monitored at 280 nm UV. The peptide was eluted in 8 mL. The peptide dispensed in ten vials, each containing 0.15mg peptide, and lyophilized for further studies. The HPLC analysis confirmed the absence of unreacted HYNIC.

3.1.2. Conjugation of UBI with DTPA

UBI (1.5 mg) was dissolved in 0.15 mL water in a sterile vial and equal volume of sodium bicarbonate buffer pH 8.5 added. A suspension of DTPA anhydride (3.5 mg) was prepared in 0.1 mL DMF (dried over molecular sieve for two days) and added in portions to the solution of UBI with agitation. The mixture was kept at room temperature for 1 h and then purified either through Sephadex G-25 or Sep-pak (Waters USA) C-18 cartridge as described in conjugation of UBI with HYNIC.

3.1.3. Conjugation of HNE2 with DTPA

HNE2 solution (50µg) in 0.1 mL was added to 0.1 mL 0.5 M $NaHCO_3$ solution. A suspension of 0.1 mg DTPA anhydride in 0.1 mL DMF (dried over molecular sieve for two days before use) was prepared and added in portions to the solution of HNE2 with agitation. The mixture was kept at room temperature for 1 h and then purified either through Sephadex G-25 or Sep-pak (Waters USA) C-18 cartridge as described above.

3.1.4. Conjugation of BPTI with NHS-MAG_3

1.5 mg peptide (BPTI) and 1.5 mg NHS-MAG_3 were weighed in sterile vials. The peptide was dissolved in 0.5 ml HEPES buffer. The NHS MAG_3 dissolved in 0.1mL DMF (dried over molecular sieve) was immediately added to the peptide solution in small portions with manual mixing. The mixture was kept at room temperature (25^{0}C) for 2 h and purified with Sephadex G-25 column 0.75×20 cm. by elution with ammonium acetate buffer (2.5 mL/h.). The peptide was eluted in 8 mL volume (UV 280 nm), dispensed in ten vials and lyophilized for further use. HPLC analysis confirmed the absence of unconjugated NHS-MAG_3.

3.2. Procedure for kit formulation

3.2.1. Direct labelling kit for ^{99m}Tc UBI

The labelling procedure recommended by Leiden University was slightly amended as lyophilized pyrophosphate kit was used as source of stannous ions and sodium borohydride as reducing agent. Various formulations have been tried as shown in Table I.

The following composition has given more than 90% labelling efficiency in repeated tests: UBI 100µg dissolved in 10 µL 0.01 M acetic acid; 5 µl from pyrophosphate lyophilized kit consisting of 10 mg sodium pyrophosphate, 2.5 mg $SnCl_2.2H_2O$ and reconstituted with 5.7 mL distilled water; 8µL from a solution of 7 mg/mL sodium borohydride in 0.1N NaOH; 0.15 mL activity (^{99m}Tc 2~3 mCi) freshly eluted from Generator; pH 6~7

Lyophilized kits with 4 times above amount were also prepared in order to use the higher amounts of activity for studies in higher animals.

3.2.2. Direct labelling kit for ^{99m}Tc S-UBI

The procedure and formulation developed for ^{99m}Tc UBI was tested for ^{99m}Tc S-UBI and found that the same can be used without any change. Therefore the S-UBI kits used in our experimental work were prepared by the same procedure as for UBI kits.

3.2.3. ^{99m}Tc HYNIC-UBI kit

Following procedures were investigated:

Ligand exchange procedure: The lyophilized kit of heptagluconate was used containing 100 mg calcium heptagluconate and 0.12 mg stannous chloride. The kit was labelled by addition of 4 mL activity (^{99m}Tc, 5~7 mCi) at room temperature. The kit was labelled within 20 min and 0.1mL peptide conjugate solution was then added. After further half an hour the kit was analysed for quality control

Co-ligand procedure: Tricine was used as co-ligand for labelling of UBI-HYNIC. In order to determine the optimum quantity of the reagents to get labelling yield >90%, the amount of peptide, tricine and tin have been varied as shown in Table II. The first four experiments were carried out at room temperature while in 5 and 6, the reaction mixture vial was heated in boiling water for 15 min.

The following composition was considered the best for kit production: 50µg UBI-HYNIC in 50µL ammonium acetate buffer pH 5.2; 5µL from 1 mg/ml $SnCl_2.2H_2O$, solution in HCl; 50µl from 1 mg/50µL aqueous solution of tricine; 0.1 mL activity (^{99m}Tc 3~4 mCi) (with heating); pH 6~7

3.2.4. ^{99m}Tc DTPA -UBI kit

The following formulation provided labelling efficiency in the range of 80-90%: 150 µg UBI-DTPA; 18µL from 0.5 M sodium bicarbonate buffer pH 9.2; 5 µL from $SnCl_2.2H_2O$ 1 mg/mL solution in HCl; 0.1mL activity (^{99m}Tc 3~4 mCi) from generator; pH 7.5.

However the formulation was not much investigated and the labelled solution used after removal of the hydrolyzed activity by elution through the Sephadex G-25 column (3×0.75 cm).

3.2.5. ^{99m}Tc DTPA -HNE2 kit

The following formulation provided above 90% labelling: 5µg DTPA-HNE2 (lyophilized in the vial); 18µL from buffer consisting of 0.5 M $NaHCO_3$, 0.25 M ammonium acetate and ammonium

hydroxide upto pH 9.2; 5 µL from 1 mg/mL $SnCl_2.2H_2O$ in HCl; 0.1 mL activity (^{99m}Tc, 2~3 mCi) from generator; pH 7.6. The solution was analysed for labelling after 30 min.

3.2.6. ^{99m}Tc MAG_3-BPTI kit

The following formulation has been used for labelling the conjugated peptide: 20µg MAG_3-BPTI in 0.1 mL ammonium acetate buffer; 50µL tartarate buffer pH 9.2; 10µL from 0.15 mg/mL $SnCl_2.2H_2O$ in HCl; 0.2 mL activity (^{99m}Tc 4~5 mCi); pH 7.0–7.5

However the above formulation gave excessive amount of hydrolyzed activity (about 30–40%) and purification through Sephadex on Sep-pak was necessary.

3.2.7. ^{99m}Tc EB1 kit

Various protocols were tried as given in Table III. Finally, following protocol was adopted. EB1 (1mg) was dissolved in acetate buffer 10mL of pH~6. Then to this solution freshly prepared Sn Cl_2. $2H_2O$ solution was added (10 µg/0.01mL). This solution was dispensed into 10 vials and lyophilized. Each vial contains 100 µg EB1 in 1mL acetate buffer of pH~6 and 1µg $SnCl_2$.

3.3. Labelling procedures

3.3.1. ^{99m}Tc EB1

Each time a single vial was taken and 1ml of ^{99m}Tc added and heated in boiling water for 10 min and 15 min. Labelling efficiency was determined by paper chromatography with two solvents, acetone and saline. The results show that 92–96% labelling is obtained with 15 min heating while 80% labelling was obtained with 10 min heating.

3.3.2. ^{99m}Tc UBI and ^{99m}Tc HNE2

The lyophilized kits of direct labelled UBI, scrambled UBI, UBI-DTPA conjugate and HNE2-DTPA conjugate were labelled by addition of 0.1~0.15 mL ^{99m}Tc activity freshly eluted from generator and was kept at room temperature for 30 min. When a kit was prepared by taking multiple amount of the formulation, the activity was increased in the same ratio. In case of UBI-HYNIC kit with tricine as co-ligand, heating in boiling water bath for 10 mts followed addition of ^{99m}Tc activity.

3.4. Quality control

For determination of free activity (TcO_4^-), labelled activity and reduced/hydrolyzed activity in direct labelled peptide kits as well as conjugated peptide kits, the following systems were used.

Whatman 3-acetone/ethanol: The chromatographic system of Whatman-3 paper with acetone solvent was very reliable to differentiate TcO^-_4 from other species. The free TcO^-_4 moves with the front while other species remain at the origin. In ethanol only hydrolysed Tc stayed at the origin while TcO^-_4 and labelled peptide moved.

ITLC-SG-85% Ethanol, pH 3: The system is very reliable to differentiate hydrolyzed activity from other species because all other species move while hydrolyzed activity remains at the origin. However some times the movement of labelled compound was so small that it was difficult to decide whether the activity was at the origin or moved.

HPLC: The gradient programme as given in Leiden University protocol was adopted by minor adjustment by using 0.01 M TFA (A) and acetonitrile (B) as mobile phase as follows: 0-5 min.(Constant) 100% A, 0% B; 5–15 min (Gradient) 20% A, 80% B; 15–20 min

(Constant) 20% A, 80% B; 20–25 min (Gradient)100% A,0% B; 25-30 min (Constant)100% A,0% B. In the above mentioned system Bonda PAK RP-columns 0.5×25 cm was used. The elution time for free TcO^{-}_{4} was 3.6 min. and for peptide 12–16 min. with peak splitting.

3.5. Room temperature stability of labelled kits

The quality control of all the labelled kits was performed after 30 min of labelling. The analysis repeated after the intervals of 2 h in order to determine the stability of the label. It was found that all the kits were stable up to 6.0 h. However the analysis of the next day showed that the labelled kit was not stable on the next day.

3.6. Cysteine challenge

The cysteine challenge test was performed only with direct abeled ^{99m}Tc UBI. The instability towards cysteine was checked according to the protocol recommended in first RCM report. The results are given in Table IV.

3.7. In vitro binding studies with bacteria

Two different strains of bacteria have been used in the subject studies. The standard strain of S.aureus ATCC 25923 as recommended has been obtained from National Institute of Health (NIH) Islamabad. The standard strain of E.coli ATCC No. 25922 was also obtained from NIH Islamabad. The primary culture provided was Mac'Conky agar culture petri dish. Microscopic slides were prepared and stained with grahm's strain to check the purity of the culture.

The binding of labelled compounds to bacteria (S.aureus ATCC 25923) was assessed at 4^{o}C following 1st RCM protocol. The supernatant was removed and the bacterial pellet resuspended in 1 mL PBS and re-centrifuged. The supernatant was recombined and the activity associated with bacterial pellet was determined in the activity meter.

3.8. Animal studies

The animal studies can be subdivided in three categories (I) mice studies (II) rabbit studies at PINSTECH (III) rabbit studies at PINUM, Faisalabad.

3.8.1. Mice studies

Induction of infection in mice: The standard culture containing 2×10^{7} viable bacteria per mL was thawed 2 h before injection. The mice were anaesthetized given anesthesia by exposure to vapors of anesthetic ether. 0.1 mL culture injected was into left or right thigh muscle. After 24 h the symptoms of infection were obvious.

Testing the labelled kit: The standard kit of UBI(29-41) was labelled with addition of 0.1 mL ^{99m}Tc elution from the generator as standard procedure. After 1 h the kit was diluted to 2 mL and analysed for radiochemical purity as standard procedure. The mice were anaesthesized with anaesthetic ether and 0.1 mL (5 μg UBI content) was injected through the tail vein. Three groups of three mice each were sacrificed after 1 h, 5 h and 24 h, respectively, for biodistribution. The uptake in infected compared with the normal thigh muscle are given in Table V.

3.8.2. Rabbit studies at PINSTECH

In order to confirm the findings in mice, New Zealand white rabbits (weighing from 1.5 kg to 2.0 kg and fed on fresh vegetables) have been used for imaging to assess the accumulation of radiotracer in infected lesions, induced by injecting 2×10^{9} CFU fresh cultured S.aureus ATCC 25923

or 2×10^8 CFU of ATCC 25922 E. coli. The bacteria cultured in BHI broth, were resuspended in normal saline before injection in order to avoid the effect of accumulated toxins. Right thigh muscle was infected using 0.4 mL suspension two to four days before the imaging studies. The development of infection was confirmed by physical examination and collection of blood/puss from the lesion. The imaging was performed using Siemens Spect Gamma Camera using parallel hole low energy collimator and 256×256 matrix. The rabbit was laid anaesthesized with surgical tapes on legs under the gamma camera and images taken at 15, 30, 45 min and 1 h post injection with time interval 2 min and 5 min. The sterile inflammation was induced by injecting 0.3 mL sterilized solution of E.coli in saline (containing the endotoxins). The rabbit was injected 2 mL diazepam in the muscles of fore leg. The anesthesia remained effective for about 1 h, sufficient for our imaging purpose.

Infection imaging: Following kits have been used for infection imaging after labelling with ^{99m}Tc: UBI 29-41 lyophilized kit, UBI Scrambled lyophilized kit, Infecton (labelled ciprofloxacin) lyophilized kit, UBI-DTPA lyophilized kit, UBI-HYNIC lyophilized kit

Summary of the protocols: Lyophilized kit of UBI or DTPA-UBI or HUNIC-UBI or S.UBI containing 0.4 mg peptide was reconstituted with 0.5 mL ^{99m}Tc (about 3 mCi). After about 30 min the quality control was performed and 0.4 mL injected in the ear vein of the rabbit. Separate kits were used for imaging gram positive, gram negative or sterile inflammation. The Infecton kit, consisting of 2 mg ciprofloxacin and 1 mg FSA adjusted to pH 7.5 was labelled by addition of 1 mL ^{99m}Tc (8 mCi). After Q.C. 0.1 mL was injected in the ear vein of the rabbits. The imaging was performed as described above after 45 min.

3.8.3. Rabbit studies at PINUM

Thirty three rabbits were used in the study. Twenty-five were females and eight males. Weight of each animal ranged from 1.2 – 1.6 kg, with an average of 1.4 kg.

Induction of inflammation in rabbits: sterile inflammation was induced by injecting 0.3 mL of saline containing 2×10^8 formalin killed S.aureus. into the right thigh muscle. After 48 h, when swelling of thigh muscles was present, ^{99m}Tc UBI 29-41 imaging was carried out. Group of the animals and agents used to induce infection and sterile inflammation are summarized below.

Group		No. of animals	Infection/Inflammation inducing agent
I (control)		3	Nil
II	IIa	10	S.aureus (2×10^8 CFU/animal)
	IIb	10	E.coli (2×10^8 CFU/animal)
III	IIIa	5	Killed S.aureus (2×10^8 CFU/animal)
	IIIb	5	Turpentine oil (0.3 ml/animal

Biodistribution of ^{99m}Tc UBI 29-41 in rabbits: Total body counts were determined by drawing ROI over the image of entire animal. To find out the uptake (counts) of tracer in different organs, ROI's were drawn over the liver, both kidneys and urinary bladder in the images taken at 5 min, 30 min, 60 min and 120 min.

4. RESULTS

4.1. Direct labelling of UBI with ^{99m}Tc

Various protocols were studied for formulation of UBI in kit form. For direct labelling the products are given in Table I. Radiochemical analysis showed >95% labelled fraction. The stability of the labelled kit was studied up to 6 h during which it remained intact.

TABLE I. DIRECT LABELLING OF UBI WITH ^{99m}TC

Peptide		Sn^{+2}		Borohydride		Results after 1.5 h		
Volume	From	Volume	From	Volume (Weight)	From	Free TcO_4	Hydro-lysed	Peptide labelled
10µL (10µg)	Acetate buffer pH 2.5,1µ g/µL	5µL	$SnCl_2.2H_2O$ 0.1 mg/mL in HCl	5µL (15µg)	3mg/mL in 0.1 NaOH	84.6%	7.0%	8.4%
10µL	-do-	5µL	-do-	5µL (25µg)	5mg/mL in 0.1N NaOH	5.4%	45.6%	49.0%
10µL	-do-	5µL	10mL water to pyrophosphate kit	5µL (25µg)	5mg/mL $NaBH_4$ in 0.1N NaOH	45%	0.0%	45.03%
20µL	0.01 N acetic acid 1µg/µL	5µL	5.7mL water to pyrophosphate kit	6µL (42µg)	7mg/mL $NaBH_4$ in 0.1N NaOH	3.1%	30.8%	66.1%
10µL	0.01 N acetic acid 10µg/µL	5µL	5.7mL water to pyrophosphate kit	8µL (56µg)	7mg/mL $NaBH_4$ in 0.1N NaOH	10.4%	0.0%	89.6%
Lyophilized kit sample 1						8.4%	0.0%	91.6%
Lyophilized kit sample 2						7.3%	0.0%	92.7%
10µl	0.01 N acetic acid 10µg/µl	5µL	$SnCl_2.2H_2O$ 0.44 mg/mL in HCl	8µL (56µg)	7mg/mL $NaBH_4$ in 0.1N NaOH	2.6%	0.0%	97.4%

4.2. Indirect labelling of UBI with ^{99m}Tc

For indirect labelling two techniques ligand exchange procedure with Hepta gluconate kit as well as co-ligand procedure using tricine as co-ligand with UBI-HYNIC has been studied as given in Table II.

TABLE II. ^{99m}TC LABELLING OF HYNIC-UBI USING TRICINE CO-LIGAND

Peptide		Sn^{+2} ($SnCl_2.2H_2O$)		Tricine		Result after 1.5 h		
Vol.	From	Vol.	From	Vol.	From	Free TcO^-_4	Hydro-lysed	Peptide labelled
0.1mL	Acetate buffer 100µg/0.1mL	5µL	1mg/mL in HCl	50µL	4mg/50µL	0%	0%	56.%
0.1mL	Acetate buffer 100µg/0.1mL	5µL	1mg/mL in HCl	50µL	2mg/50µL	0%	0%	60%
0.1mL	Acetate buffer 100µg/0.1mL	5µL	1mg/mL in HCl	50µL	1mg/50µL	67%	0%	33%
0.1mL	Acetate buffer 100µg/0.1mL	5µL	1mg/mL in HCl	50µL	0.5mg/50µL	91%	1%	8%
50µL	Acetate buffer 200µg/0.1mL	5µL	1mg/mL in HCl	50µL	1mg/50µL (heating for 15 min)	0%	0%	96%
50µL	Acetate buffer 100µg/0.1mL	5µL	1mg/mL in HCl	50µL	1mg/50µL (heating for 15 min)	0%	0%	93%

UBI was labelled with ^{99m}Tc efficiently (>95%) by both direct and indirect labelling techniques. Since the direct labelling method is convenient this was followed for the production of kits and further studies.

4.3. Cysteine challenge

The results of cysteine challenge studies given in Table III show that labelled peptide is sufficiently stable toward cysteine.

TABLE III. CYSTEINE CHALLENGE OF DIRECT LABELLED ^{99m}TC UBI(29-41)

$^{99m}TcO_4^-$ in standard kit	$^{99m}TcO_4^-$+^{99m}Tc Cysteine after 1.5 h at Cysteine ratio to peptide		
	5:1	50:1	500:1
1.3%	14.5%	26.7%	34.5%
4.7%	21.4%	30.2%	44.1%

4.4. In vitro binding assay of ^{99m}Tc UBI to bacteria

The in vitro bacterial binding study at 4^{o}C is found to be a good indicator for the development of infection imaging agents. The kits having better binding properties in vitro, have shown also the good in vivo binding. The results revealed the highest binding of ^{99m}Tc direct labelled UBI peptides to bacteria. The results are presented in Table IV.

TABLE IV. BACTERIAL BINDING PROPERTY OF DIFFERENT ^{99m}TC UBI KITS

Description	% Activity bound n=5
^{99m}Tc UBI Direct labelled	31 ± 5%
^{99m}Tc UBI Scrambled	9 ± 3%
^{99m}Tc DTPA- UBI	22 ± 5%
^{99m}Tc HYNIC -UBI	25 ± 4%

4.5. Biodistribution and accumulation of ^{99m}Tc UBI in infection in mice

The results as given in Table V show that the activity is accumulated in infected site within 1 h, which is useful for rapid scanning. The IM/NM activity ratio is 4.1, 4.9 and 5.8 after 1, 5 and 24 h, respectively. The activity is rapidly excreted mainly through the urine. The IM/NM ratio is improved with the passage of time but the total activity in the IM is rapidly decreased.

TABLE V. BIODISTRIBUTION STUDIES OF ^{99m}TC UBI IN MICE (%ID/ORGAN, SD N=3)

Tissue	1 h	5 h	24 h
Liver	3.6 ±3.0	2.7 ± 2.0	1.4 ± 0.4
Spleen	0.12 ± 0.1	0.06 ± 0.02	0.02 ± 0.01
Stomach	1.0 ± 0.5	0.1 ± 0.05	0.01 ± 0.04
Intestine	13.9 ± 5.0	7.3 ± 4.0	6.1 ± 2.0
Lungs	0.1 ± .02	0.003 ± 0.01	0.003 ± 0.02
Kidneys	17.5 ± 7	6.1 ± 2.0	2.1 ± 1.2
Urine//Bladder	42.8 ± 10	78.1 ± 12	94.3 ± 5.0
Heart	0.6 ± 0.3	0.003 ± 0.01	0.002 ± 0.01
Head	2.1 ± 1.5	0.68 ± 0.2	0.12 ± 0.05
Normal Muscle(NM)	0.3 ± 0.1	0.04 ± .02	0.01 ± 0.05
Infected Muscle (IM)	1.3 ± 0.3	0.19 ± 0.05	0.058 ± 0.01
IM/NM	4.1	4.9	5.8

4.6. Infection imaging studies in rabbits (PINSTECH)

A summary of the results is, as follows (Table VI):

^{99m}Tc UBI from lyophilized kit for direct labelling showed highest uptake (T/NT ratio >2) in gram positive, S. aureus infection and comparative uptake in gram negative E.coli. The uptake in sterile inflammation was negligible. 45 min is the appropriate time for imaging.

^{99m}Tc scrambled UBI from lyophilized kit for direct labelling showed negligible uptake of activity in the lesion.

The imaging properties ^{99m}Tc DTPA -UBI and ^{99m}Tc HYNIC- UBI were inferior to the direct labelled ^{99m}Tc UBI.

TABLE VI. INFECTION IMAGING STUDIES IN RABBITS

Kit for ^{99m}Tc labelling	Target	T/NT ratio (Average) n=3			
		15 min.	30 min.	45 min.	60 min.
UBI-29-41	S.aureus Inf	1.20±0.20	2.00±0.12	2.10±0.22	1.25±0.07
UBI-29-41	E.coli Inf	1.15±0.04	1.90±0.31	1.95±0.24	1.30±0.11
Scrambled UBI	S.aureus Inf	1.01±0.03	1.15±0.12	1.15±0.11	1.07±0.05
UBI-29-41	Sterile Infl.	1.03±0.01	1.10±0.05	1.12±0.04	1.10±0.03
Infecton	S.aureus Inf	1.10±0.04	1.51±0.21	2.21±0.20	2.20±0.15
UBI-DTPA	S.aureus Inf	1.07±0.01	1.23±0.12	1.87±0.16	1.31±0.10

Infl= Inflammation
Inf= Infection

4.7. Rabbits studies at PINUM

The biodistribution of ^{99m}Tc UBI in various organs of rabbit determined scintigraphically are presented in Table VII. Kidney displayed gradual excretion of radioactivity with mean percent uptake values of 10.6 ± 2.1 and 4.2 ± 0.3 at 5 and 120 min p.i., respectively. Liver also showed gradual decline in uptake values with the passage of time having mean percent uptake values of 6.6 ± 1.6 and 2.5 ± 0.8 at 5 and 120 min p.i., respectively. Rapid accumulation of radioactivity was seen in urinary bladder with almost 80% of injected tracer present in bladder at 120 min p.i.

TABLE VII. BIODISTRIBUTION OF ^{99m}TC UBI 29-41 IN RABBITS (N=3)

% of total body activity per organ												
Organ	Liver				Kidneys				Urinary bladder			
Time	5 min	30 min	60 min	120 min	5 min	30 min	60 min	120 min	5 min	30 min	60 min	120 min
Mean (± SD)	6.6 (1.6)	5.2 (0.8)	4.1 (0.5)	2.5 (0.8)	10.6 (2.1)	8.5 (1.4)	5.9 (0.8)	4.2 (0.3)	16.7 (6.0)	46.6 (5.6)	66.6 (7.2)	81.0 (6.7)

On the ^{99m}Tc UBI scintigram both S.aureus and E.coli infection were visualized within 5-30 min p.i. Target to non-target (T/NT) ratios increased with the passage of time. Maximum mean T/NT for both S. aureus and E. coli was observed at 60 min p.i. (Table VIII). The highest (T/NT) ratio that was observed in S.aureus infected rabbits was 3.5 at 60 min p.i. E. Coli infected rabbit showed lesser accumulation of tracer as compared to S.aureus group, with maximum T/NT ratio of 2.45 at 30min p.i. Examples of E. coli infected lesions in rabbits are shown in Fig. 1. Cultures of aspirate taken from site of infection were positive in all cases of S.aureus and E-coli groups (growth of S.aureus & E.coli on blood agar and Mac' Conkey's agar).

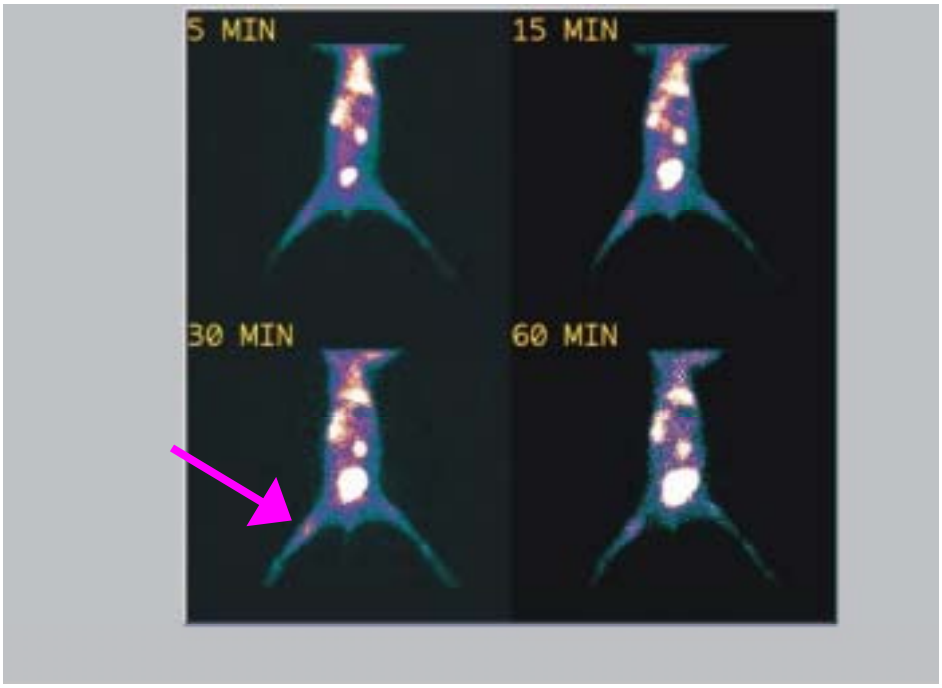

FIG. 1. Tc-99m-UBI 29-41 scintigram of a rabbit with E. coli thigh muscle infection (indicated by an arrow).

TABLE VIII. TARGET TO NON-TARGET RATIOS FOR ^{99m}Tc UBI IN INFECTED THIGH MUSCLES IN RABBITS

	Time	5 min	30 min	60 min	2 h	3 h	4 h	Culture
S. aureus	Mean (± SD)	1.7 (0.6)	2.0 (0.6)	2.2 (0.5)	1.8 (0.5)	1.7 (0.3)	1.5 (0.4)	+
E. coli	Mean (± SD)	1.2 (0.2)	1.5 (0.4)	1.7 (0.4)	1.4 (0.3)	1.5 (0.4)	1.4 (0.4)	+

In rabbits with sterile inflammation induced by turpentine oil and formalin killed S.aureus ^{99m}Tc UBI did not show significant accumulation in the lesions. Target to non-target ratios calculated in case of inflammation induced by turpentine oil were (1.2 $\pm$ 0.14), (1.25 $\pm$ 0.08), (1.16 $\pm$ 0.15) and (1.03 $\pm$ 0.17) at 5, 30, 60 and 120 min p.i. respectively. These ratios are lower than T/NT ratios in cases of both S.aureus and E.coli infected rabbits. In the same way, lower T/NT ratios were seen in rabbit with formalin killed S.aureus induced inflammation. Results are shown in Table IX. ^{99m}Tc-UBI scintigram of a rabbit with turpentine oil induced thigh muscle inflammation is depicted in Fig. 2.

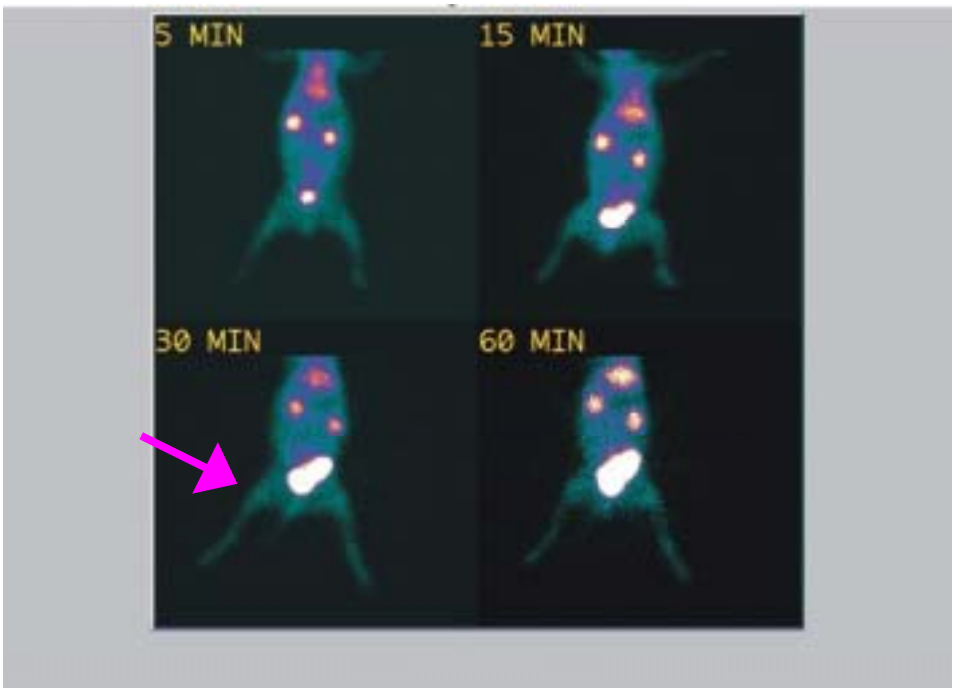

FIG. 2. ^{99m}Tc-UBI 29-41 scintigram of a rabbit with turpentine oil induced thigh muscle inflammation (arrow).

TABLE IX. TARGET-TO-NON-TARGET RATIOS FOR ^{99m}Tc UBI IN INFLAMED THIGH MUSCLES IN RABBITS

Time (min) p.i	5	30	60	120
Turpentine Ind, Mean (± SD)	1.20(0.14)	1.25 (0.08)	1.16 (0.15)	1.03 (0.17)
Formalin Ind, Mean (± SD)	1.05(0.09)	1.11 (0.35)	1.01 (0.27)	1.11 (0.39)

4.8. ^{99m}Tc HNE2

HNE2 was with >90% yield through indirect labelling after making conjugate with cyclic DTPA. Since, the availability of HNE2 was not certain further work could not be extended.

4.9. ^{99m}Tc EB1

In order to develop lyophilized kit with EB1, locally produced various parameters were studied as shown in Table X. Finally a protocol giving >95% yield was adopted. Radiochemical analysis with paper chromatography (10 cm) in acetone was carried out to assess the percentage of ^{99m}Tc present as pertechnetate ($R_f \sim 0.9$) while using saline as eluent the percentage of ^{99m}Tc present as colloid was assessed.

TABLE X. ^{99m}TC LABELLING OF EB1

EB1	$SnCl_2$	^{99m}Tc (~2mCi)	Free ^{99m}Tc %	Colloid %	Labelled %
30 µg	1 µg (shaking for 30 min)	0.5 mL	5–15	10–26	58–80
100 µg	1 µg (Heating for 30 min)	0.5 mL	5–15	1–5	80–94
30 µg	(1 µg–1.5 µg) Mannitol (5–15mg) (Shaking for 30 min)	0.5 mL	0–1	0–11	88–93
90 µg	1 µg Mannitol =15mg Heating for 15 min	0.5 mL	nil	nil	100
25 µg	1.5 µg Hepta gluconate (5–15 mg)	0.5 mL	nil	11–15	85–89
90 µg	1.5 µg Hepta gluconate (5–15 mg) Heating for 15 min	0.5 mL	nil	0–5	95–100
25 µg	1.5 µg ascorbic acid = 10mg	0.5 mL	48	16	36

5. CONCLUSIONS

Out of the three agents selected for possible use as infection imaging agent after labelling with ^{99m}Tc. UBI looked quite promising. UBI has been formulated into kit form, it can be labelled with ^{99m}Tc by direct labelling as well as through bifunctional chelates with a labelling yield of >95%. Radiochemical analysis technique is quite easy and rapid. It binds well to the bacteria during in vitro studies. ^{99m}Tc UBI scintigraphy can be used for differentiating infections with S.aureus and E.coli with significantly higher scintigraphic intensity as compared to sterile inflammatory sites. The optimum time for infection imaging using ^{99m}Tc UBI was found to be 60 min post injection in this study. The

encouraging results of ^{99m}Tc-UBI (29–41) warrant detailed study in other types of bacteria than S.aureus and E.coli). Once toxicology studies of UBI are complete, clinical trials will be quite challenging and interesting.

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DEVELOPMENT OF KITS FOR ^{99m}Tc RADIOPHARMACEUTICALS FOR INFECTION IMAGING

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Abstract

UBI (a derivative of antimicrobial peptide ubiquicidin) labelled with ^{99m}Tc is reported to discriminate between bacterial infections and sterile inflammatory processes. In this study we compared UBI labelled with ^{99m}Tc by a direct method and using hydrazinonicotinamide (HYNIC) as a bifunctional chelator. We tested four dry kits, two of them based on the direct labelling method, with or without KBH_4 and two based on HYNIC conjugation via BOC (one of them contained EDDA). After addition of $^{99m}TcO_4^-$ solution the kits were labelled under specific conditions and ITLC, HPLC analyses (reverse-phase and ion exchange) were performed. The binding of labelled UBI to bacteria, S. aureus, was estimated and stability experiments in human serum were performed. The accumulation in infected tissues was studied using ex vivo countings and scintigraphy. Data were compared to the UBI 29-41 labelled with ^{99m}Tc in solution (control). Conjugate of UBI 29-41 with HYNIC was prepared with high purity (>97%). Radiochemical analysis indicated rapid and high labelling yield (90-95%) for all four kits, the labelled compounds being stable (RCP>90%) for 24 h in vitro in human serum. The kit containing HYNIC-UBI 29-41 and the control labelling showed highest binding of 40% of the added activity to bacteria, being significantly higher than the direct labelling kits (30%). The range of pH was from 6 to 9 for direct labelling kits and 4 for the kits containing HYNIC. After injection into infected mice, all tracers were rapidly removed from the circulation by renal excretion (50–200%ID/g), but blood activity of kits containing HYNIC-UBI 29-41 remained higher than that of directly labelled UBI 29-41. Specific accumulation in infected thigh muscles as indicated by a T/NT ratios between 2.3 and 2.9 until 2 h after injection was observed for all the kits. For all preparations we calculated good correlations between the number of microorganisms and T/NT ratio. The UBI 29-41 directly labelled in presence of $SnCl_2$ and the HYNIC-UBI 29-41 kit without EDDA showed the most promising results for further in vivo evaluation.

The native human neutrophil elastase inhibitor (HNE-2) radiolabelled with ^{99m}Tc is reported to localized specifically in inflammations in experimental animals and provide early images of diagnostic quality. The aim of the present work was to study the radiochemical behaviour of the peptide HNE-2 and BPTI when radiolabelled with ^{99m}Tc by indirect method using HYNIC as a bifunctional chelator. The conjugation of HNE-2 and BPTI with HYNIC for labelling with ^{99m}Tc was previously described. Chromatographic studies were performed using size-exclusion HPLC.

1. INTRODUCTION

In several previous papers, the ^{99m}Tc-labelled cationic antimicrobial peptide derived from human ubiquicidine (UBI) was introduced for the detection of bacterial and fungal infections but not sterile inflammatory processes in mice and rabbits [1]. Originally, UBI was isolated from murine macrophages [2,3] and later from human airway epithelial cells. ^{99m}Tc-labelled synthetic UBI 29-41 was successful at imaging the infections in animal studies [4]. Furthermore this labelled peptide allowed monitoring the efficacy of antimicrobial treatment in mice having an experimental infection [5]. Moreover, it was reported that ^{99m}Tc-UBI peptide can be useful in infection detection in both immunocompetent and immunocompromised animals [6]. UBI-derived peptides can be good candidates for radiolabelling and studies on detection of bacterial infections in humans. Application in humans requires standardization of the radiolabelling. Diverse dry kits of this peptide were developed in our work for ^{99m}Tc labelling. The aim of the present study was to evaluate the radiochemical and biological characteristics of four kits for ^{99m}Tc UBI 29-41.

In recent years radiolabelled polyclonal IgG, has been shown to be useful in the diagnosis of infection and inflammation as a ‘non-specific’ agent [7]. In animal model streptavidin may be a more useful agent to detect infection [8]. Unlabelled streptavidin was administered and allowed sufficient time to accumulate non-specifically in the leison. Thereafter, radiolabelled biotin (EB1) derivative was administered. The labelled EB1 may be retained in the lesion because biotin has extremely high

affinity for streptavidin. Radioscintigraphy with ^{111}In-biotin in this approach was successfully used in 15 patients with chronic osteomyelitis [9]. In this study we report the radiochemical characteristics and stability of two kits for preparing ^{99m}Tc EB1.

The radiolabelled peptide HNE-2, is a potential radiopharmaceutical with properties permitting specific localization in inflammation/infection and rapid pharmacokinetics. The peptide HNE-2 has a molecular weight of 6.7 kDa and 2 lysines plus the terminal amine available for conjugation. The peptide was conjugated with the *N*-hydroxysususuccinimide ester of mercaptoacetyltriglycine (MAG3), mercaptoacetyltriserine (MAS3), HYNIC, and the cyclic anhydride of diethylenetriaminepentaacetic acid (DTPA). The ability of ^{99m}Tc labelled conjugates to image inflammation/infection was evaluated in studies in monkeys [10].

This report describes the small-scale preparation of conjugate of HNE-2 with HYNIC for labelling with Tc-99m. Bovine pancreatic trypsin inhibitor (BPTI), which differs from HNE-2 in only 8 amino acid changes at the 58 BPTI-homologous positions plus 4 additional residues at the amino terminus, was radiolabelled with ^{99m}Tc in identical manner.

2. MATERIALS AND METHODS

2.1. ^{99m}Tc- UBI

2.1.1. UBI peptide

Synthetic peptide UBI 29-41 in powder form was received from Prof. Ernest Pauwels (Department of Radiology, Division of Nuclear Medicine, LUMC, Leiden, The Netherlands). Peptide was dissolved to get a 1 mM solution in 0.01 M acetic acid, pH 4 and stored at –20°C.

2.1.2. Synthesis of peptide conjugate

The method for peptide conjugation with 6-BOC hydrazinopiridine-3-carboxylic acid (BOC-HYNIC) and synthesis of HYNIC has been previously described [11]. A total of 8.31 mg of BOC-HYNIC (Radioisotope Centre POLATOM, Otwock-Swierk, Poland), 12.49 mg of O-(7-azabenzotriazolyl)-1,1,3,3-tetra-methyluronium hexafluorophosphate (HATU) (Merck KGaA Darmstadt, Germany), in 5.60 µL of N-ethyldiisopropylamine (DIPEA) (Merck KGaA Darmstadt, Germany) and 150 µL of N-methyl-2-pyrrolidone (NPM) (Merck KGaA Darmstadt, Germany) were reacted for 30 min. This solution was added to 100 mg of UBI 29-41 in 5.60 µL of DIPEA and 150 µL of NPM and allowed to react for 2 h. To this solution 1mL of 5% aqueous $NaHCO_3$ solution and 1mL of ethyl acetate were added. The $NaHCO_3$ solution was extracted three times with 1 mL ethyl acetate. The combined organic phases were washed four times with water and evaporated to dryness. The deprotection was achieved by dissolving the white solid in 5% H_2O 89% TFA (Merck KGaA Darmstadt, Germany), 3% thioanisole, 3% TIS, solution and reacted for 4 h at room temperature. After incubation the conjugate was precipitated with ethyl ether and centrifuged. Reversed-phase HPLC with an on-line UV detector set at 220 nm was used for analysis and purification of crude conjugate HYNIC-UBI 29-41. A gradient with two different eluents, i.e. 0.1% TFA/H_2O (A) and 0.1% TFA/CAN (B), at a flow rate of 15 mL/min was used. The gradient was performed as follows: 0-9 min 40%B, 9-15 min 40%B, 15-16 min 0%B, 16-22 min 0%B. The purity of the conjugate amounted to approximately 97%. The molecular weight of the purified conjugate was confirmed by mass spectrometry.

2.1.3. Preparation of kits

The compositions of the 4 types of lyophilized kits per vial were as follows - kit A: 50 µg UBI, 5 µg $SnCl_2$ (Fluka Chemie GmbH, Busch); kit B: 50 µg UBI, 10 µg stannous pyrophosphate (Aldrich Chemical Company, Inc, Milwaukee WI, USA), 100 µg KBH_4 (Sigma Chemical Co. St. Louis, MO

USA); kit C: 20 µg HYNIC-UBI, 20 µg $SnCl_2$, 20 mg tricine, 50 mg mannitol (Calbiochem); kit D: 20 µg HYNIC-UBI, 20 µg $SnCl_2$, 20 mg tricine, 10 mg ethylenediamine-*N,N*'-diacetic acid (EDDA) (Fluka Chemie GmbH, Buchs, Siwtzerland), 50 mg mannitol. Parameters of lyophilization: -45°C: 21 h, -45°C to + 40°C: 10.3 h, + 40°C: 14.3 h, total time: 46 h, vacuum: 0.12 mbar, final temperature: 16 °C. Kits were sealed in N_2 atmosphere.

2.1.4. Labelling procedure

UBI 29-41 peptide in solution was directly labelled with ^{99m}Tc as control. Briefly, 10 µL of a peptide stock solution was added to 4 µL of an aseptic solution of 950 mg/L $SnCl_2 \cdot 2H_2O$ and 2g/L sodium pyrophosphate decahydrate (Department of Clinical Pharmacy and Toxicology, LUMC). Immediately thereafter, 2 µL of a solution of KBH_4 (crystalline, Sigma Chemical Company, St. Louis, Mo., USA) 10 mg/mL in 0.1 M NaOH was added. After addition of 0.1 mL of ^{99m}Tc-sodium pertechnetate solution (200–700 MBq/mL) obtained from a ^{99m}Tc generator (Ultratechnekow, Mallinckrodt Medical, Petten, The Netherlands), the mixture, having a final pH between 8 and 9, was gently stirred at room temperature for at least 10 min.

Kits were reconstituted and labelled with 200 µL (kit A and B) or 500 µL (kit C and kit D) of ^{99m}Tc-sodium pertechnetate solution (200-700 MBq/mL) obtained as above. The mixture (A,Band C) was gently stirred at room temperature for at least 1h. Kit D was incubated for 10 min at 100°C and then cooled at room temperature for 30 min. The range of pH was between 6 and 9 for direct labelling kits (kit A and kit B) and 4 for the kits containing HYNIC (kit C and kit D).

2.1.5. HPLC analysis

The reaction mixtures after labelling of UBI or HYNIC-UBI were analysed by anion exchange HPLC to evaluate charged species (method 1) and reverse-phase HPLC to evaluate lipophilic species (method 2). Method 1: samples (10–50 µL) were applied on a MONO-S column attached to a chromatography apparatus equipped with an on line UV detector set at 220 nm and NaI (Tl) crystal gamma detection system. A gradient with two different eluents, i.e. 0.01% (v/v) Tween in PBS (PBST) (A) and 2M NaCl in PBST (B), at a flow rate of 1mL/min was used. The gradient was performed as follows: 0–5 min 0%B, 5–15 min 0-100%B, 15-20 min 100%B, 20–25 min 100–0%B, 25–30 min 0%B. Method 2: samples (5–10 µL) were applied on a 4.6×250 mm C_{18} column (Vydac, The Separations Group, Hesperia, CA) attached to a chromatography apparatus equipped with an on line UV detector set at 220 nm and NaI (Tl) crystal gamma detection system. A gradient with two different eluents, i.e. 0.1% TFA in H_2O (A) and, 0.1% TFA in methanol/ H_2O [(80/20,v/v),(B)] at a flow rate of 1mL/min was used. The gradient was performed as in method 1.

2.1.6. ITLC analysis

Labelling yields of ^{99m}Tc-UBI 29-41 and ^{99m}Tc-HYNIC-UBI 29-41 were determined using instant thin-layer chromatography (ITLC). 1×6.5 cm ITLC/SG strips (Gelman Sciences, Ann Arbor, MI), were dried in a microwave at 500 W for 1 min directly before use. Samples of the preparations containing labelled peptides (2 µL) were applied at approximately 1 cm from the bottom (baseline) of the ITLC strips and were immediately placed in air tight containers with methyl ethyl ketone or saline solvents. With both methods ^{99m}Tc in the form of pertechnetate can be quantitated. After 2 min of development, strips were dried in a microwave oven, cut into 2 fragments and radioactivity in the front section or remaining on the baseline was determined in a dose-calibrator (VDC 101, Veenstra Instruments, Joure, the Netherlands).

2.1.7. Stability of ^{99m}Tc-labelled peptides and ^{99m}Tc-labelled conjugate in neat human serum

Stability ^{99m}Tc-labelled UBI 29-41 and HYNIC-UBI 29-41 was challenged by incubating one volume of the labelling solution with one volume of neat human serum for 1 h and 24 h at 37°C. Next,

the amounts of free pertechnetate and ^{99m}Tc-peptide in the samples were determined by ITLC using methyl ethyl ketone and saline as solvent as described before.

2.1.8. In vitro binding of ^{99m}Tc-UBI and ^{99m}Tc-HYNIC-UBI to microorganisms

The multi-drug-(including vancomicine) resistant S. aureus type 2141 (*MRSA*) was a clinical isolate (Department of Infectious Diseases, LUMC). Overnight culture of bacteria was prepared in brain heart infusion broth (BHI, Oxoid, Basingstoke, UK) in a shaking waterbath at 37°C. The next day, bacteria were washed, counted and aseptically aliquoted. Stocks of 0.5 mL containing 1.2×10^9 viable bacteria were stored at -20°C. Before use the bacteria were washed twice in saline.

Binding of ^{99m}Tc-labelled UBI 29-41 and HYNIC-UBI 29-41 to bacteria was assessed at 4°C. In short, 0.1 mL PBS containing 1/10 of the mixture containing ^{99m}Tc-labelled peptides was transferred to an Eppendorf vial. Next, 0.8 mL of 50% (v/v) of 0.1% (v/v) acetic acid in PBS containing 0.01% (v/v) Tween-80 (PBST/HAc) and 0.1 mL of PBS containing approximately 2×10^8 of viable bacteria were added. The mixture, with a final pH of 5, was incubated for 1 h at 4°C and then the vials were centrifuged in a pre-cooled centrifuge at 2000xg for 5 min. The supernatant was removed and the bacterial pellet was gently resuspended in 1 mL PBST/HAc and centrifuged as above. The supernatant was removed and the activity of bacterial pellet was determined in dose calibrator (VDC 101, Veenstra Instruments, Joure, The Netherlands). The radioactivity related with bacteria was expressed as % of added ^{99m}Tc activity bound to viable bacteria.

2.1.9. Studies of ^{99m}Tc-labelled UBI 29-41 in mice with induced infection

Specific pathogen-free, female, Swiss mice, weighing 23–30 g were used in this study. The animals were housed in the animal housing facilities of the LUMC for at least 1 week before the onset of the experiments. Food and water were given *ad libitum*. All animal studies were done in compliance with the National laws related to the conduct of animal experiments and approved by the local Committee for Animal Experiments.

Mice were anaesthetized with a single intraperitoneal injection of a freshly prepared 0.1 mL of saline containing 1 mg of fluanisone and 0.03 mg fentanyl citrate (Hypnorm, Janssen Pharmaceutics, Tilburg, The Netherlands). Immediately thereafter, 2×10^7 of viable MRSA in water were injected aseptically into the right thigh muscle. After 18-24 h the mice were anaesthetized as above and preparations containing 1/10 (v/v in saline) of the radiolabelled peptide injected into tail vein, plus a subcutaneous injection of 0,1 ml of 10-fold (v/v) diluted in saline of valium to induce muscle relaxation. After injection of ^{99m}Tc-UBI 29-41 (2-7 MBq), accumulation of radioactivity in various organs was determined using scintigraphy.

Whole body images for each duplicate of mice were made until 2 h post-injection. Radioactivity counts in infected thigh muscles were assessed by means of anatomically fitted ROI over the entire infected (target) and equivalent size non-infected (non-target) thigh muscle and data are expressed as target-to-non-target (T/NT) ratios. Thereafter, the mice were sacrificed with an intraperitoneal injection of 0,50 ml of sodium pentobarbiturate (60mg/ml, Nembutal, Sanofi BV, Division Algin, Maassluis, The Netherlands). Blood was immediately collected by cardiac puncture, followed by removal of urine by a syringe from the bladder. Infected and non-infected thigh muscles and various organs were dissected, weighed and counted for radioactivity. Data are expressed as the percentage of total ^{99m}Tc activity (%ID) and then calculated as %ID per gram of tissue (%ID/g). To confirm the bacterial infection, the number of viable bacteria in the thigh muscle was determined microbiologically and expressed as CFU per gram of tissue (CFU/g) or CFU/thigh muscle (CFU/thigh muscle).

2.1.10. ^{111}In-DTPA-UBI 29-41

The coupling of the cyclic anhydride of diethylenetriaminepentaacetic acid (DTPA) (Aldrich Chemical Company, Gillingham, Dorset, England) to the UBI 29-41 was prepared by the same method as described above in 2.2. The conjugate DTPA-UBI 29-41 was labelled with ^{111}In.

2.2. ^{99m}Tc EB1

2.2.1. Preparation of kits

Synthetic peptide EB1 in powder form was received from Dr Mustansar Jehangir (Pakistan Institute of Nuclear Science and Technology — PINSTECH, Islamabad, Pakistan)

The compositions of 2 types of lyophilized kits per vial was as follows - kit I: 25 µg EB1, 1 µg $SnCl_2$; kit II: 25 µg EB1, 2 µg $SnCl_2$ 10 mg mannitol. Mannitol was added to the kit as an agent for improving the quality of the freeze-dried pellet. Parameters of lyophilization were same as that given for UBI kits.

2.2.2. Labelling procedure

Kits were dissolved in 100µL water and labelled with 74-220 MBq (200 µL) ^{99m}Tc-sodium pertechnetate obtained from a ^{99m}Tc generator. The mixture was incubated for 15 min at 80°C and then cooled at room temperature for 15 min

2.2.3. Quality control

Samples of 10-50 µL were applied on size exclusion HPLC (gradient pump, Merk Hitachi) column (BioSep-SEC-S 4000, size: 300×7.80 mm, Phenomenex) and eluted with 0.1 M phosphate buffer at a flow-rate of 1 mL/min. UV and radioactivity profile recorded as before.

Labelling yield and stability of ^{99m}Tc-EB1 with time was assessed by instant thin layer chromatography (ITLC) and mini paper chromatography. Samples of the preparations containing labelled peptides (2 µL) were applied at approximately 1.5 cm from the baseline of the 1×10 cm ITLC-SG strips and were placed in airtight containers with saline. The Whatman1 paper strips (1×10 cm) were placed in airtight containers with acetone. With both systems, only pertechnetate would migrate to solvent front while other ^{99m}Tc species are retained at origin.

2.2.4. Stability in human serum

Stability experiments in neat human serum were performed with two methods:

Method 1: Incubating one volume of the labelling solution with one volume of human serum for 1 h and 24 h at 37°C challenged stability of kit containing EB1. Next, the amounts of ^{99m}Tc-EB1 in the samples were determined by ITLC using saline and acetone as eluent.

Method 2: After incubation 100 µl aliquots (prepared as in method 2) were taken and treated with 200 µl of ethanol. Samples were cooled (4°C) and centrifuged for 15 min at 3000 rpm to precipitate serum protein. 100 µl of supernatant was removed for activity measurement. The sediment was washed twice with 1 ml of ethanol and measured. The activity in the supernatant was compared with the activity in the pellet to give the percent peptide not bound to proteins. Next, the amounts of ^{99m}Tc-EB1 in the samples were determined by ITLC using saline as solvent.

2.2.5. Shift assay with avidin

Shift assay was carried out by reacting ^{99m}Tc-EB1 with an excess of avidin (molar ratio 1:20) and followed by size exclusion HPLC analysis (as described before). The shift in retention time was assessed by comparing with that of ^{99m}Tc-EB1 without avidin. ^{99m}Tc-EB1 was also checked by reacting with excess of avidin (molar ratio 1:1 and 1:2) followed by by open column P4 chromatography (0.7×20 cm) using ammonium acetate as eluent.

2.2.6. Biodistribution in mice with strile inflammation

Pathogen free, female mice (Balb/c) were used in the study. The animals were housed in the animal housing facilities of the Military Institute of Hygiene and Epidemiology.

Mice were anaesthetized with single intraperitoneal injection of 0.2 ml of vetbutal 20-fold (v/v) diluted in saline. Immediately thereafter, 0,3 of *Oleum Terebinthnae* was injected aseptically into the right thigh muscle to induce sterile inflammation. After 48 h, the mice with typical symptoms of inflammation and healthy mice (as control) were anaesthetized as above and 0.2 ml solution of ^{99m}Tc-EB1 (approximately 37MBq), injected into tail vein.

Accumulation of radioactivity in various organs was determined using scintigraphy. Whole body images for at least 2 mice were made 6 h post-injection. Radioactivity counts in inflamed thigh muscles were carried out by means of anatomically fitted ROI over the entire inflamed (target) and equivalent size non- inflamed (non-target) thigh muscle and data are expressed as target to non target (T/NT) ratios.

2.3. ^{99m}Tc-HNE2

2.3.1. Conjugation with HYNIC

The peptide HNE-2 was received from Professor D. Hnatowich (University of Massachusetts Medical School, USA). The HYNIC-conjugated peptide was prepared following a method previously described [11].

To 9 µL of a 1.1 mg/mL HNE-2 solution in water 86 µL of 0.1 M phosphate buffer, pH 7.3 was added. Next, a 3 mM solution of HYNIC (4,88 µg) in dimethylforamide (DMF) was added dropwise to final HYNIC-to-peptide molar ratio of 5:1. The reaction mixture was incubated at room temperature for 2.5 h before purification on a Sephadex G-25 column with 0.1 M phosphate buffer, pH 7.3 eluant. The conjugated peptide eluted from the column was collected in the fractions between 5 and 7 mL.

2.3.2. ^{99m}Tc labelling procedure

To 100 µL of conjugated peptide in 0.1 M phosphate buffer, pH 7.3 was added 5 µl $SnCl_2$ solution (1mg/mL in 0.1 M HCL) and approximately 7.4 MBq ^{99m}Tc-pertechnetate (50 µL). The solution was incubated at 37°C for 30 min.

2.3.3. Quality control

^{99m}Tc labelled peptide was analysed by size-exclusion HPLC using a BioSep SEC-S-2000 [300×7.80 mm (Phenomenex)], with 0.1 M phosphate buffer, pH 7.3, as eluant at a flow-rate of 1 ml/min and with on-line radioactivity and UV detection (absorbance 280 nm).

RESULTS

2.4. ^{99m}Tc-UBI

The results for kits were compared to the UBI 29-41 labelled with ^{99m}Tc in solution (control) [1]. Radiochemical analyses showed high labelling yield (90-95%) for all kits except D, as assessed by ITLC and HPLC. ITLC analysis with MEK as solvent showed, that the different preparations of ^{99m}Tc-UBI 29-41 revealed small amounts of relased/free ^{99m}Tc after 1 h incubation in human serum (~2% of the total activity) and after 24 h (~6% of the total activity). The kit prepared ^{99m}Tc-HYNIC-UBI 29-41 and the ^{99m}Tc- UBI 29-41 control showed highest binding of ~40% of the added activity to bacteria, being significantly higher than the direct labelling kits 30%. The range of pH was from 6 to 9 for direct labelling kits and 4 for the kits containing HYNIC. After injection into infected mice, all tracers were rapidly removed from circulation by renal excretion (50-200%ID/g) but blood activity of kit prepared ^{99m}Tc-HYNIC-UBI 29-41 remained high. Specific accumulation in infected thigh muscles as indicated by a T/NT ratios between 2.3 and 2.9 for the direct labelling kits until 2 h after injection was observed. For all preparations good correlations between the number of microorganisms and T/NT ratio were observed.

The results with ^{111}In-DTPA-UBI 29-41 indicated that the peptide preparation was >98% pure and stable in human serum.

2.5. ^{99m}Tc EB1

Radiochemical analyses showed high labelling yield (93-99%) for both kits, as assessed by ITLC and HPLC. The labelled peptide with avidin was eluted out earlier than the one without avidin from both the size exclusion HPLC column and open column P4. ITLC analysis showed that ^{99m}Tc-EB1, after 1 h incubation in human serum, showed very small amounts of released/free ^{99m}Tc. But ~13% of other ^{99m}Tc complexes for both kits II and I at 1 h and ~64% for kit I and ~44% for kit II after 24 h were observed,. The range of pH for kits was from 5.0 to 7.7. The accumulation of ^{99m}Tc-EB1 in inflammed thigh muscle resulted in the T/NT ratio of 3.4 for the labelling kit II after 6 h p.i.

2.6. ^{99m}Tc-HNE2

The size-exclusion HPLC analysis of ^{99m}Tc-HYNIC-HNE-2 revealed a single peak at retention time of approximately 9.5 min. The recovery from HPLC was higher than 99%.

3. CONCLUSIONS

Both peptides UBI and HYNIC-UBI can be prepared in the dry kit form for labelling with ^{99m}Tc. All four kits containing UBI 29-41 or HYNIC-UBI 29-41 can be easily and quickly labelled with ^{99m}Tc with high yields. All tracers can be used without further purification. All the labelled preparations were stable in human serum up to 24 h. On the basis of these results, the UBI 29-41 directly labelled in presence of $SnCl_2$ and the HYNIC-UBI 29-41 kit without EDDA seem to be the most promising candidates for infection imaging.

EB1 can be easily and rapidly labelled with ^{99m}Tc using dry kit form wothout need for post-labelling purification. The shift in elution proflie towards higher molecular weights of ^{99m}Tc EB1 with excess avidin was proved. ^{99m}Tc EB1 was slightly less stable than ^{99m}Tc UBI 29-41 on 24 h in vitro incubation in human serum

ACKNOWLEDGEMENTS

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DEVELOPMENT OF ^{99m}Tc LABELLED INFECTION IMAGING AGENTS BASED ON PEPTIDES

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Abstract

The aim of this research project was to develop a kit for ^{99m}Tc radiopharmaceuticals for infection imaging. Conditions for ^{99m}Tc labelling of EB1 and UBI were optimized. For UBI, direct as well as HYNIC and DTPA conjugates labelling were studied. ^{99m}Tc-EB1 shows shift of retention time to higher molecular weight when avidin is added. It is stable in human serum. Labelling of EB1 seems to be affected by pH. Quality control and in vitro evaluations of ^{99m}Tc-UBI were done. Kits for ^{99m}Tc HYNIC-UBI showing very high labelling efficiency were prepared and studied for in vivo and in vitro stability. ^{99m}Tc-UBI is decomposed to free technetium when incubated in human serum, while ^{99m}Tc- HYNIC -UBI binds to serum protein. Biodistribution in mice infected with10^8 CFU S.aureus, 2 h after injection showed infected/normal thigh ratio of 4.83 for directly labelled ^{99m}Tc-UBI and 2.46 for ^{99m}Tc HYNIC-UBI. ^{99m}Tc labelling yields of DTPA-UBI at pH 5.2 and 7.6 were very low. In place of HNE2, its less expensive analogue, BPTI, was studied to optimize conditions for conjgating to MAG3, ^{99m}Tc labelling and in vitro control. Purified ^{99m}Tc-BPT1-MAG_3 showed 'trypsin shift' on both Sephadex column chromatography and size exclusion HPLC.

1. INTRODUCTION

Most infections can be diagnosed by physical examination and other techniques. However in some cases the diagnosis is more difficult. Nuclear medicine is making an important contribution to these conditions due to its sensitivity. Recently, many ^{99m}Tc radiopharmaceuticals have been developed for the diagnosis of infection owing to physical properties and availability at reasonable cost of ^{99m}Tc. However, the ^{99m}Tc-radiopharmaceuticals for the imaging of infection at present have some disadvantages [1]. For example, the preparation of ^{99m}Tc-leukocyte requires laborious preparation and specialized personnel, while labelled immunoglobulin has slow blood clearance.

In an attempt to develop more desirable ^{99m}Tc radiopharmaceuticals for infection imaging, many small biomolecules have been investigated, such as chemotactic peptides [2,3], cytokines [4,5], antimicrobial peptides [6,7], which show promising results. The aim of this investigation was to develop ^{99m}Tc imaging agents for infection imaging from the most promising peptides. Three agents Ubiquicidin 29-41(UBI), EDTA-biotin monomer (EB1) and human neutrophil elastase inhibitor (HNE2) were selected for study in this Coordinated Research Project. For ^{99m}Tc labelling direct method as well as an indirect method by conjugation with various types of chelating agent including NHS-MAG3, NHS-HYNIC and cDTPA were studied. Moreover, in vitro stability, in vitro binding and in vivo assay were evaluated. Due to scace availability of HNE2, BPTI which has similar proprties to HNE2 was used to optimize ^{99m}Tc labelling.

2. MATERIALS AND EQUIPMENT

All peptides, EB1, BPTI, UBI, scrambled UBI, NHS-HYNIC and NHS-MAG3, are provided by IAEA. Cyclic DTPA anhydride is purchased from Sigma. $SnCl_2.2H_2O$ is a product of Merck. Mo/Tc generator, Elumatic III, is from CIS, France. ITLC-SG is from Gelman Sciences, Ann Arbor, MI, USA. Raytest radiochromatogram scanner eqiupped with NaI(Tl) detector(Germany) and Packard automatic gamma counter (USA) are employed. Two HPLC instruments are used. Waters 600E System Controller and Waters 490E Programmable Multiwavelength Detector and in-house made radiometric detector were connected in series. The other system is JASCO, PU980, equipped with UV-detector and γ-RAM.

3. METHODS

3.1. Labelling of EB1 with ^{99m}Tc

40 μg of EB1 in 4 μL of 1 M sodium acetate buffer pH 6 is added to a vial containing 37 to 150 MBq of ^{99m}Tc-pertechnetate, followed by 1 μL of stannous chloride dihydrate solution (1 mg/mL in 10 mM HCl). The radiolabelled of EB1 is purified by P4 column (0.7 mL P4 in 1 ml syringe) with 0.05 M phosphate buffer pH 7 as eluant. Methods for radiochemical purity analysis and optimum conditions for labelling are studied. Quality assurance of ^{99m}Tc-EB1 is done by *Avidin Shift* by addition of 120 μg of avidin into 17 μg of the labelled EB1. ^{99m}Tc-EB1 and ^{99m}Tc-EB1-avidin are analysed by both size exclusion HPLC column and P-4 column (0.7×15 cm.). After incubation for 30 min, the labelled product is transferred into P-4 column, and the HPLC analysis was done after 1 h incubation. Phosphate buffer (0.05M. pH 7.2) is used as mobile phase for both systems (flow rate 1 ml/min for HPLC). In vitro stability of the purified product was studied. To maximize labelling efficiency, EB1 kits with higher amount of EB1 (50-80 μg) at various pH values have been evaluated.

3.2. ^{99m}Tc labelling of UBI

3.2.1. Direct labelling

UBI 29-41 is labelled using ^{99m}Tc pyrophosphate kit (Office of Atomic Energy for Peace, Bangkok, Thailand). Each kit consisted of tetrasodium pyrophosphate 4.17 mg and stannous chloride 100 μg). Briefly, 10 μL of UBI solution (1 mM in 0.01M acetate buffer pH 4.0) is added 12 μL of a solution of pyrophosphate kit (dissolved in 550 μl sterile water for injection). Then 2 μL of a 10 mg/mL solution of KBH_4 (crystalline, Sigma Chemical Company, St. Louis, MO, USA) in 0.1M NaOH is added. After addition of 100 μL of ^{99m}Tc sodium pertechnetate solution (approximately 200 MBq/mL), the mixture is gently swirled and let stand at room temperature for 30 min. The labelling yield of ^{99m}Tc-UBI is determined by ITLC-SG strips. Saline solution and 35%ACN in 0.1%TFA/H_2O are used as mobile phases. The ^{99m}Tc activity in ITLC strips are quantified by the radiochromatogram scanner.

^{99m}Tc UBI is also analysed by reverse-phase HPLC on a C18 column (Ultremex 4.6×250 mm, Phenomenex,) and eluted with a 0.1%TFA/H_2O (A) and acetonitrile (B) gradient as follows : 0%B for 5 min, 0-100%B in 10 min, 100%B for 5 min, 100-0%B in 5 min and 0%B for 5 min at 1.0 mL/min flow rate. ^{99m}Tc UBI is monitored by NaI(Tl) detector and UV detector at 280 nm.

Effect of the quantity of KBH_4 and ^{99m}Tc on the labelling of UBI is also studied by varying amounts of KBH_4 from 1.25 to 20 μg and of ^{99m}Tc from about 20 MBq to 0.2 GBq in 100 μL.

3.2.2. ^{99m}Tc labelling of HYNIC-UBI

Conjugation of UBI to HYNIC: UBI is dissolved in 0.1M pH 8.0 HEPES buffer to a concentration of 3 mg/mL. To the UBI solution is added freshly made NHS-HYNIC in dry DMF (20 mg/mL) at a 5:1 HYNIC/UBI molar ratio. The amount of DMF is kept less than 10% of the total volume. The reaction mixture is purified on P4 column with 0.25M ammonium acetate pH 5.2 as eluant. The peak fractions were collected and lyophilized to dryness and then stored at -20 ºC.

^{99m}Tc labelling: 37-185 MBq ^{99m}Tc-pertechnetate is added into a tricine kit (5 mg tricine, 10 ug $SnCl_2.2H_2O$, pH 5.5). To solution of 50 μg of UBI-HYNIC is added various amounts of ^{99m}Tc-tricine. The labelled peptide is incubated at room temperature. Labelling efficiency is determined using instant thin-layer chromatography (ITLC) in 0.9% NaCl, Whatman#3 in acetone as well as Sep Pak and HPLC. Optimum amount of ^{99m}Tc-tricine for 50 μg. UBI-HYNIC is studied for kit formulation.

3.2.3. ^{99m}Tc labelling of DTPA -UBI

Conjugation of UBI to DTPA: The conjugate was prepared according to a procedure previously described [8]. A 10 µg/µl solution of UBI in water is mixed with an equal volume of 0.2M HEPES buffer pH 8 and a suspension of cyclic DTPA anhydride in DMF is added dropwise with agitation. The final DTPA/peptide molar ratio is 5:1 and the addition of DMF is kept less than 10% of total volume. After 30 minute incubation at room temperature, the conjugated peptide is purified by size exclusion HPLC (Superdex, Pharmacia biotech) and eluted with a 0.1%TFA/H_2O (A) and acetonitrile (B) gradient as follow : 0%B for 5 min, 0-50%B in 10 min, 50%B for 5 min, 50-0%B in 5 min and 0%B for 5 min at 1.0 mL/min flow rate. The peak at 10.6 min is collected and the concentration of the fractions is determined by UV absorption at 280 nm. The solution of the coupled peptide is dispensed to 20 µg/vial and lyophilized for further use.

^{99m}Tc labelling: The DTPA-conjugated peptide was radiolabelled with ^{99m}Tc at both pH 5.2 and 7.6. The concentration of conjugated peptide is 0.5-1.0 µg/µL in 0.25M ammonium acetate buffer at pH 5.2. When labelled at pH 7.6, to 100 µL of the DTPA-peptide solution is added 18 µL of a buffer consisting of 0.5M sodium bicarbonate, 0.25M ammonium acetate and 0.18M ammonium hydroxide at pH 9.2. Finally, about 10 µL (about 37-74 MBq) of the ^{99m}Tc-pertechnetate solution is added. Immediately thereafter, 4 µl of a fresh solution of $SnCl_2.2H_2O$ (1 mg/mL in 10 mM HCl) is added and the solution is agitated. Labelling efficiency is determined using instant thin-layer chromatography (ITLC) in 0.9% NaCl and 35% acetonitrile in 0.1%TFA water.

3.3. ^{99m}Tc labelling of BPTI with using NHS-MAG3

3.3.1. Conjugation of BPTI to NHS-MAG3

Freshly made 3 to 5 fold molar excessive NHS-MAG3 in dry DMF solution (12-40 mg/mL) is added to 0.2-1.0 mg BPTI solution (3-5 mg/mL in 0.1 M HEPES buffer, pH 8.0) The addition of DMF is kept less than 10% of the total volume. The reaction mixture is placed at room temperature for 30 to 60 min, and then purified by HPLC using BioSep-SEC S2000 Column (Phenomenex), 7.8×300 mm. Phosphate buffer 0.05 M, pH 7.2 is used as mobile phase with flow rate 1.0 mL/min. The peak fractions are pooled. The concentration of MAG3-BPTI conjugate is determined by UV absorpbance at 280 nm. The purified MAG3-BPTI is stored at –20^{o}C before use.

3.3.2. ^{99m}Tc labelling

To 100 µL of purified MAG3-BPTI (10-80 µg) are added 100 µL PBS, 25µL sodium tartrate buffer, pH 9.2 (0.5M $NaHCO_3$, 0.25 M NH_4OAc, 0.175 M NH_4OH, and 50 mg/mL sodium tartrate), 0.1-10 mCi of ^{99m}Tc-pertechnetate, and 3-4 µL of freshly made $SnCl_2$ solution (1 mg/mL $SnCl_2$ in 0.01 N HCl). After incubation for 30 min, the radiolabelled MAG3-BPTI is purified by either column chromatography (Sephadex G-50, 0.05M PBS, pH 7.2 as eluant) or HPLC (Waters 600E System Controller, Waters 490E Programmable Multiwavelength Detector, 215 nm, NaI detector, BioSep-SEC 2000 Column (Phenomenex), 7.8×300 mm, 0.05 M PB, pH 7.2, flow rate 1.0 mL/min). The radioactive fractions monitored by UV detector are pooled and tested by thin layer and paper chromatography (SG-ITLC/methanol (MeOH) and Whatman 1/acetone), then stored for further experiments. Trypsin shift is performed to assure quality of the labelled product: A ten fold molar excess of Trypsin (1 mg/mL in saline) is added to radiolabelled MAG3-BPTI in 0.2 M PBS before or after purification. The mixture is incubated at room temperature for 30 min before analysis by HPLC.

In vitro stability

3.3.3. Human serum stability

Approximately 8 µg of ^{99m}Tc-peptide was added to 1 mL of human serum. The solution was incubated at 37°C. Then the samples were removed and analysed by ITLC and HPLC at various time intervals.

3.3.4. Cysteine challenge assay

The concentration of radiolabelled peptide is adjusted to 2.2 µM in 0.2M, PBS pH 7.2. The freshly made solution of L-cysteine at 13 mg/mL (0.083 M) in 0.1M PBS, pH 7.0 is diluted successively with 0.2M PBS pH7.2 to obtain three solutions from 0.0083 M to 0.000083 M. To 12 µL of each of the cysteine solution is added 90 µl of 2.2 µM radiolabelled peptide. Thus the molar ratios of cysteine to peptide are 500:1, 50:1 and 5:1 respectively. Each solution is incubated in water bath at 37°C for 1 h and analysed by Whatman paper no.1/acetone and paper/saline or 0.1M phosphate buffer pH 7 [9].

3.4. In vitro binding study

To 0.8 mL of 50% (v/v) of 0.01M acetic acid in 15mM PBS containing 0.01%(v/v) Tween-80 is added 10 µL of ^{99m}Tc labelled peptide. Then 0.1 mL of 15mM PBS containing viable bacteria is added. After the mixture is incubated at 4°C for 1 h, it is centrifuged in a pre-cooled centrifuge at 2,000xg for 5 min. The supernatant is removed and the bacterial pellet is gently resuspended in 1 mL PBS and recentrifuged as mentioned above. The supernatant is collected. The radioactivity of the supernatant and the bacterial pellet is determined in a gamma counter. The radioactivity of ^{99m}Tc-peptide bound to bacteria is expressed as percentage of total activity added.

3.5. In vivo study

Staphylococcus aureus 6538p is used to induce infection. A suspension of 0.1 ml aliquot containing about 10^8 bacteria per mL is injected intramascularly in the thigh of the mice. The radiopharmaceuticals are administered 24 h later for the biodistribution study.

Sets of 3 animals are used for both normal and infected animal study. After confirmation of radiochemical purity of products, approximately 100 µCi per mouse is administered. After 2 h, the animals are sacrificed and the tissues of interest are isolated. Percent injected dose in all major tissues are calculated and presented as mean ± S.D.

4. RESULTS

4.1. ^{99m}Tc EB1

ITLC-SG in acetone is the best to assess free pertechnetate (Rf = 0.9-1), meanwhile, ITLC-SG in either 50% EtOH in saline or 35% of acetonitrile in 0.1%TFA in water is used to determine ^{99m}Tc-colloid (Rf=0). Either P-4 cloumn or HPLC can be used to separate the compound of interest from each other, with or without avidin under the condition of study. The presence of avidin binding to EB1 is confirmed by the shift in the elution profile on P-4 column and HPLC. On P-4 column, without addition of Avidin, the labelled sample is eluted out at fraction of 8 to 12, while the Avidin added product is eluted out earlier at fraction of 4 to 6. The HPLC (Fig. 1) shows the shift of radioactivity profile to shorter retention time in the presence of avidin. The retention time shift in the presence of avidin indicated that the labelled product is ^{99m}Tc-EB1.

For 40 µg EB1 in the presence of 1 µg $SnCl_2 \cdot 2H_2O$, 37 MBq of ^{99m}Tc is optimal to label and have better stability when purified after labelling. ^{99m}Tc-EB1 is stable in human serum up to more than 20 h. Dissociation rate is increased with increasing molar ratio of cysteine: EB1 (Fig. 2*)*. Though labelling efficiency of the above conditions is ≥90%, most contaminant in the labelled product is ^{99m}Tc colloid. To improve labelling yield, kits containing 50 up to 80 µg are prepared. Better yields observed in all of these kits, but without any significant difference between each other. Therefore 50 µg EB1 was selected for kit formulation.

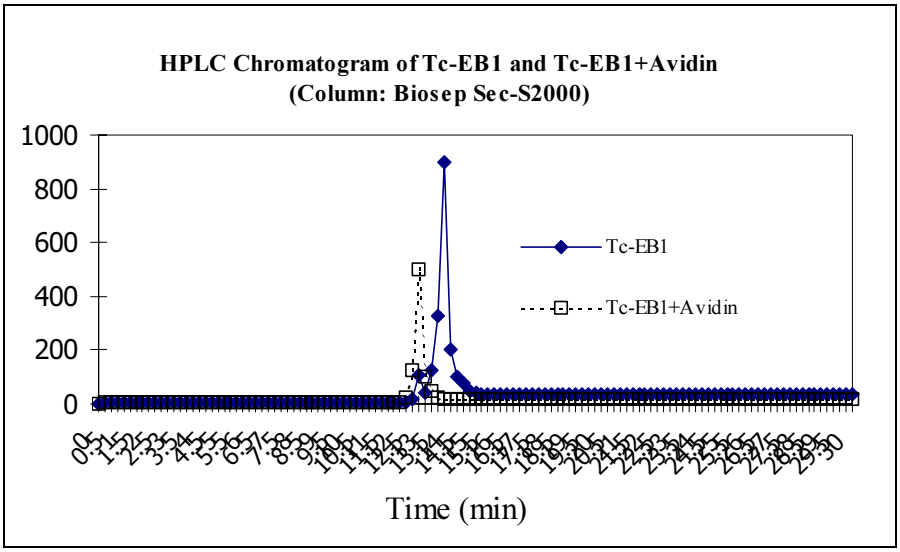


FIG. 1. HPLC radiochromatogram of avidin binding to ^{99m}Tc-EB1.

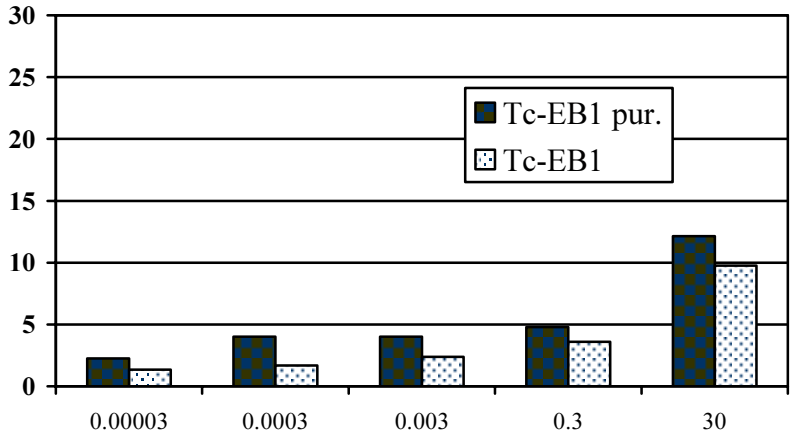


FIG. 2. Cysteine challenge of ^{99m}Tc-EB1.

4.2. ^{99m}Tc-UBI

4.2.1. Direct labelling

The radiochemical purity of ^{99m}Tc-UBI was determined as follows: ITLC-SG in saline is used to determine $^{99m}TcO_4^-$ (Rf ~1.0). ^{99m}Tc-colloid is determined by Sep-pak (remaining in cartridge after acid methanol elution.) Purity of the labelled compound is greater than 90% in the presence of ≥25 µg

of KBH_4 (in 100 µL reaction volume) and stable up to 5 h. Effect of KBH_4 and ^{99m}Tc activity on labelling are studied. The results showed that 40 µg of KBH_4 provided highest effective labelling of UBI with greater than 90% purity with the ^{99m}Tc concentration up to 1 GBq/ml. HPLC is successfully utilized to purify ^{99m}Tc-UBI with retention time of 15.6 min (Fig. 3). However, ^{99m}Tc-UBI is poorly stable in human serum. HPLC chromatogram (Fig. 4a) shows decomposition to free pertechnetate at about 3 h after incubation. Fig 5. shows dissociation of ^{99m}Tc in cysteine challenge . Furthermore, there is no binding of ^{99m}Tc-UBI to either *S. aureus* or *K. pneumonia* at 10^7 CFU, but the binding at

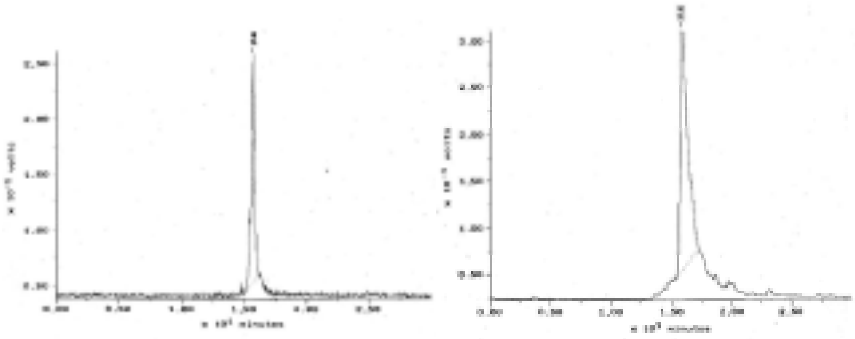


FIG. 3. HPLC profile of ^{99m}Tc-UBI, [left]: UV detector at 280 nm, [right] NaI(Tl) detector.

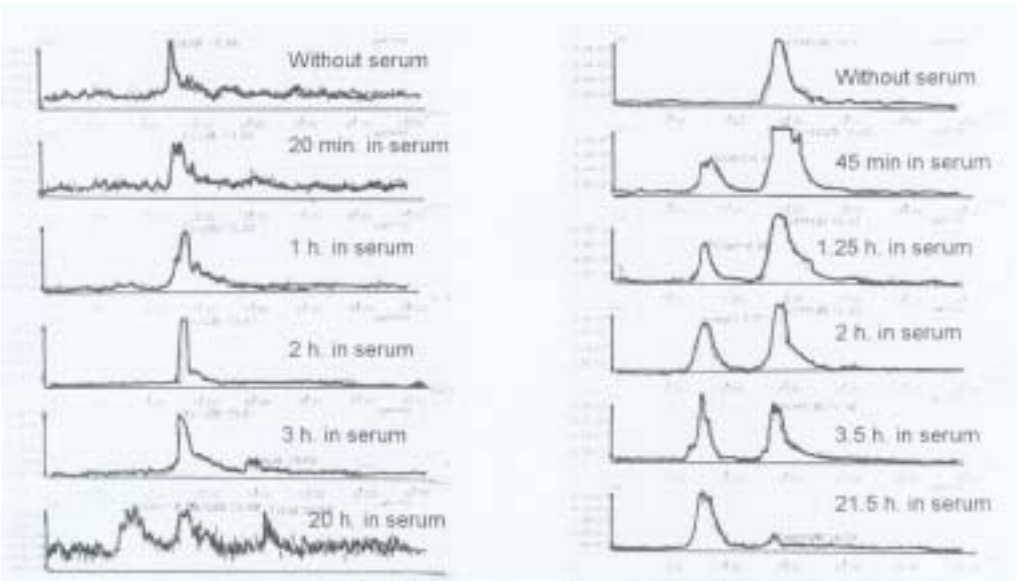


FIG. 4. Serum stability study on size exclusion column- [A]: ^{99m}Tc -UBI [B]: ^{99m}Tc -HYNIC-UBI.

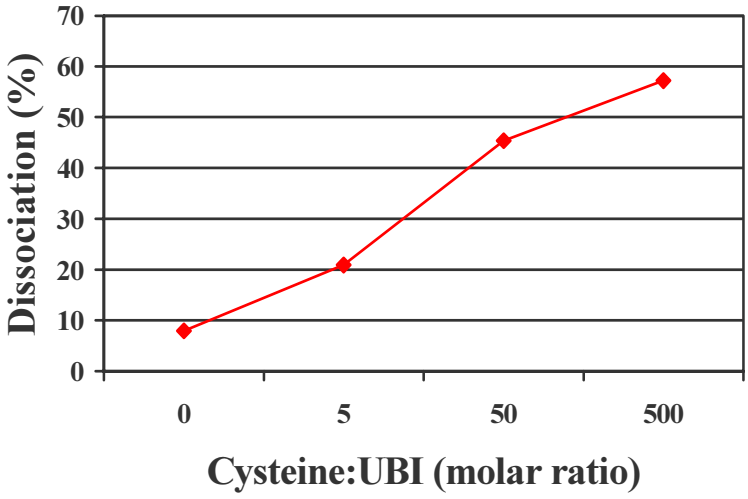
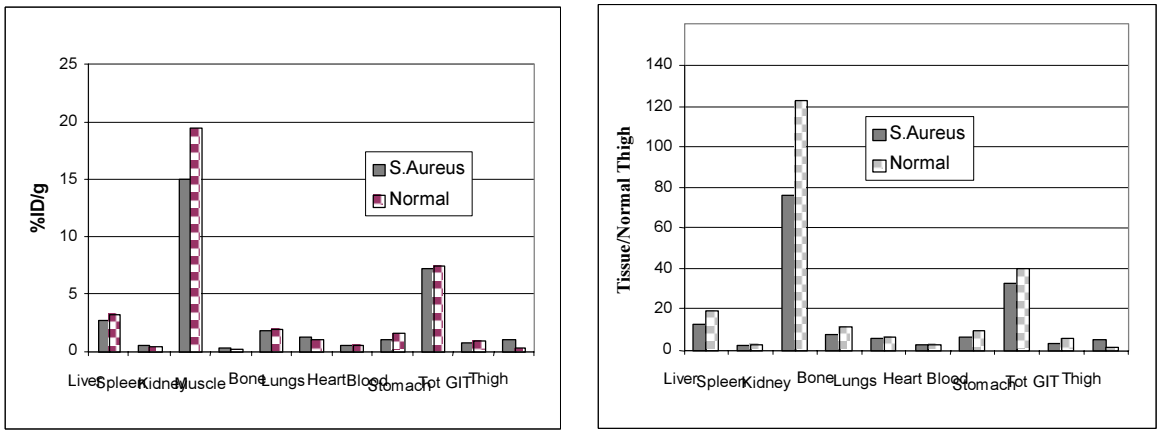


FIG. 5. Cysteine challenge of ^{99m}Tc-UBI.



[A] [B]

FIG. 6. Biodistribution of ^{99m}Tc-UBI in S.aureus infected and normal mice: (A)%ID/g(B) tissue/normal thigh.

2×10^9 CFU was about 30.4% and 23.1%, respectively. Biodistribution study of 10^8 CFU *S. aureus* infected mice at 2 h after injection shows 1.0%ID/g (± 0.05) at infection site, with T/NT of about 4.84 (±1.24) and in normal mice, 0.23 %ID/g (± 0.03) in the thigh (Fig. 6).

4.2.2. Labelling of HYNIC-UBI

This kit consists of 50 µg of HYNIC-UBI, 6.67 µg $SnCl_2.2H_2O$ and 3.33 mg tricine, at pH 5.5 . Labelling efficiency of the kit with 40-150 MBq of ^{99m}Tc is more than 95%. Radiochemical purity of ^{99m}Tc-HYNIC-UBI could be determined by ITLC/paper chromatography in acetone for free pertechnetate, in saline for hydrolyzed ^{99m}Tc and either in acetonitrile or in 2% methanol/ethylacetate for ^{99m}Tc-tricine (Table I.) ^{99m}Tc -HYNIC-UBI is eluted out of size exclusion column (BioSepSec-S2000) at about 13.8 min, while ^{99m}Tc-tricine and pertechnetate are eluted out at about 20 min. Incubation in serum shows increased binding of the labelled product as a function of time only very small amount of free ^{99m}Tc was observed (Fig. 4b). Biodistribution at 2 h after injection study in S.Aureus infected mice shows 0.50%ID/g (±0.07) at infection site, with T/NT about 2.5 (±0.47) and in normal mice show 0.14%ID/g (±0.02) in the thigh. (Fig. 7).

TABLE I. MIGRATION OF SAMPLES OF ^{99m}TC UBI-HYNIC IN ITLC WITH VARIOUS SOLVENTS

Eluant	Tc-colloid	Tc-HYNIC-UBI	Tc-tricine	$NaTcO_4$
Saline	Origin	Solvent front	Solvent front	Solvent front
Acetone	Origin	Origin	origin	Solvent front
Acetonitrile	Origin	Origin	Solvent front	Solvent front
MeOH	Origin	Solvent front	Solvent front	Solvent front
2%MeOH/EtOAc	Origin	Origin	Solvent front	Solvent front
0.5M PB pH 7.4	Origin	Solvent front	Solvent front	Solvent front

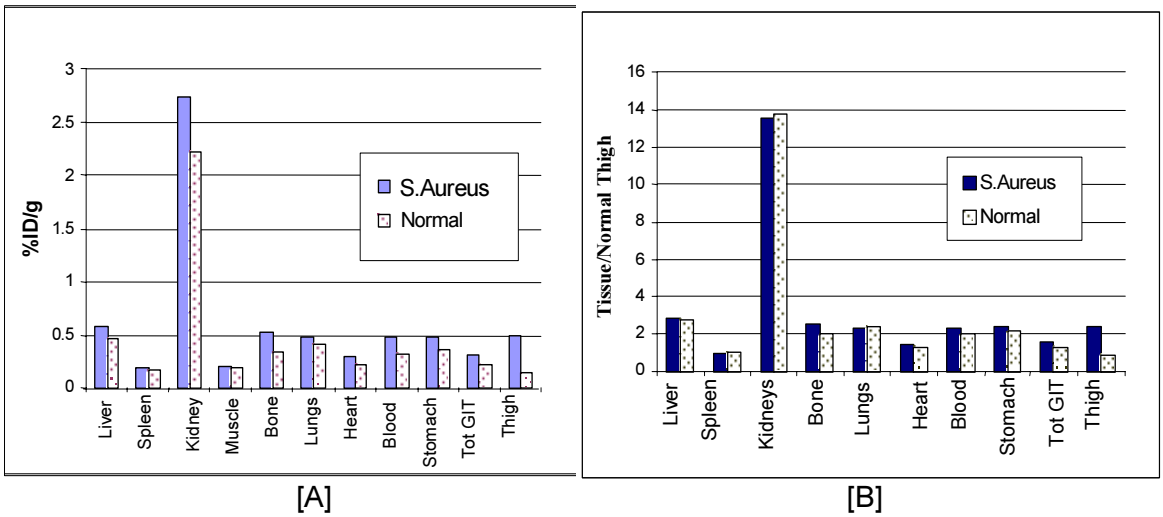


FIG. 7. Biodistribution of ^{99m}Tc-HYNIC-UBI in S.aureus infected and normal mice: (A) %ID/g (B) tissue/normal thigh.

4.3. ^{99m}Tc -MAG3-BPTI

Elution profile of samples on Sephadex G-50 column is better than on Sephadex G-100. Hence the former is chosen for purification or identification of the non-radioactive or radioactive compounds. The column can separate Trypsin, BPTI, NHS-MAG3, BPTI-MAG3, and pertechnetate from one another **(**Figs 8 and 9**).**

On a reverse phase column (Crest Pak), BPTI and NHS-MAG3, monitored at 235 nm, are separated with retention times of 11 and 6 mins respectively. Comparing the purified samples (peak A and peak B in Fig. 9) to the retention time of BPTI and NHS-MAG3 (Fig. 8), peak B has retention time corresponding to NHS-MAG3, but peak A showed different retention time than that of BPTI or NHS-MAG3. It could hence be assumed that peak A is that of conjugate of MAG3-BPTI and therefore, the pooled fraction of peak A is used for radiolabelling studies.

The MAG3-BPTI is labelled with ^{99m}Tc at room temperature complete in 30 min and purified by either column chromatography or HPLC. By column chromatography, it is found that ^{99m}Tc-MAG3-BPTI is eluted earlier (fraction 20–30), followed by free $^{99m}TcO_4^-$, (fraction 45-50) with confirmation by ITLC (ITLC-SG/MeOH, Whatman#1/acetonitrile). By HPLC (Fig. 10), the retention times of $^{99m}TcO_4^-$, ^{99m}Tc-tartrate, and ^{99m}Tc-BPTI-MAG3 are 21.86, 12.27, and 9.87 min, respectively.

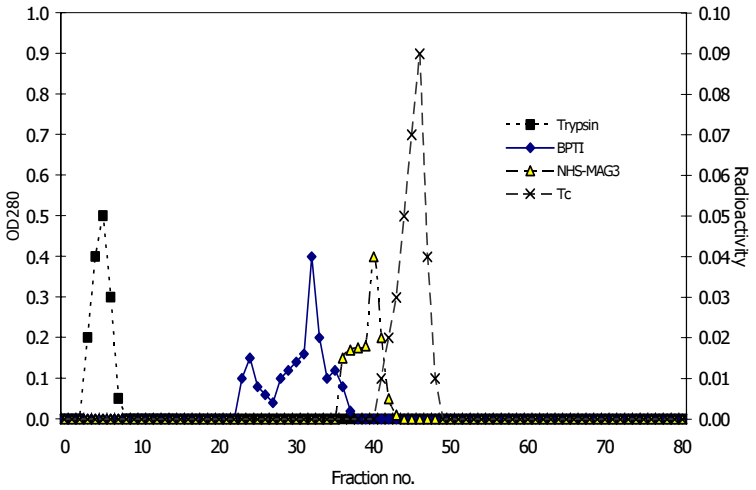


FIG. 8. Elution profile of trypsin, BPTI, NHS-MAG3, and ^{99m}Tc on Sephadex G-50 column.

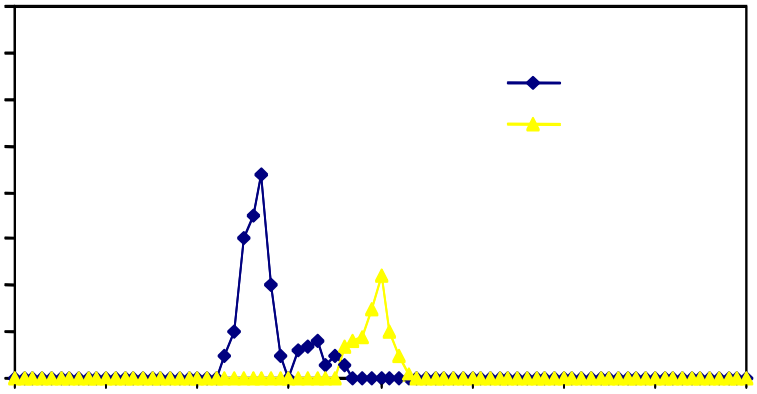


FIG. 9. Chromatogram of MAG3-BPTI and NHS-MAG3on Sephadex G-50 column.

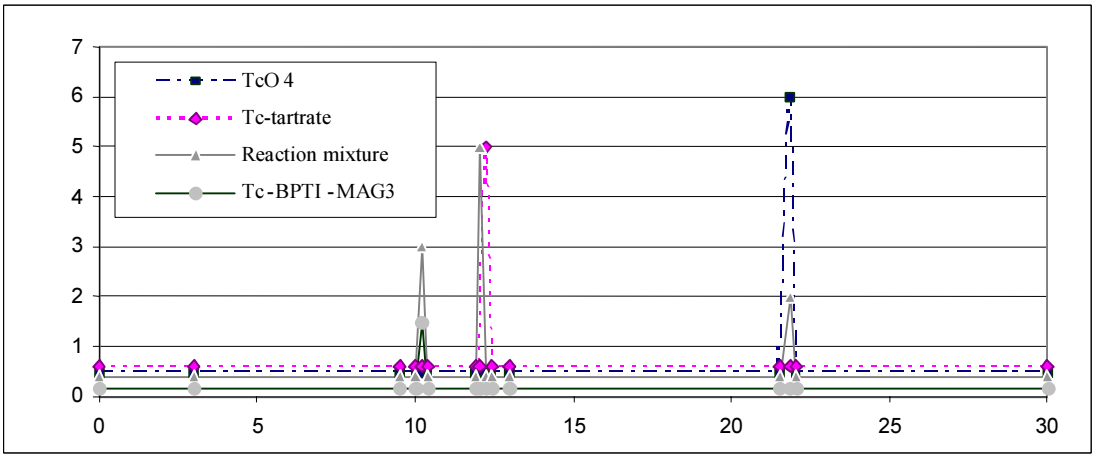


FIG. 10. HPLC radioactivity profile of reaction mixture of ^{99m}Tc-MAG-3BPTI.

The radiochemical purity determined by ITLC (ITLC-SG/acetone and ITLC-SG in 5:3:1 pyridine:acetonitrile:water) is greater than 90%. Study of quality control methods is shown in Table II.

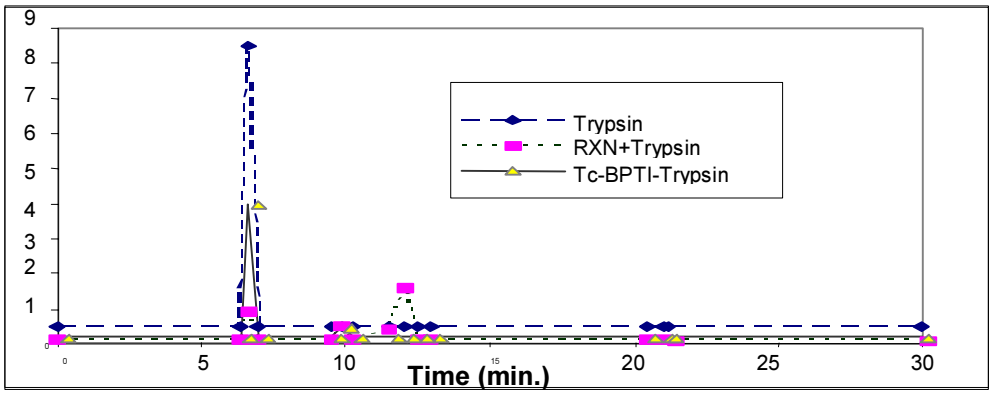


FIG. 11. Chromatograms of ^{99m}Tc-BPTI-MAG3 and Trypsin reaction mixtures: Trypsin (at 215nm.), reaction mixture of labelled sample added with Trypsin, and HPLC-purified ^{99m}Tc-BPTI-MAG3 added with Trypsin.

TABLE II. MIGRATION OF SAMPLES OF ^{99m}TC-MAG3-BPTI IN ITLC WITH VARIOUS SOLVENTS

Eluant	Tc-colloid	Tc-BPTI-MAG3	Tc-tartrate	$NaTcO_4$
Saline	Origin	origin	origin	solvent front
Acetone	Origin	origin	solvent front	solvent front
1 mM HCl	Origin	origin	solvent front	solvent front
50%EtOH/saline	Origin	solvent front	solvent front	solvent front
50%Etoh/water	Origin	solvent front	solvent front	solvent front
Pyridine:acetonitrile:water 5:3:1	Origin	solvent front	solvent front	solvent front

Affinity of the conjugated compound to trypsin is tested and verified on size-exclusion HPLC by the shift in retention time to 6.58 min (Fig. 11).

^{99m}Tc-MAG3-BPTI is stable up to 24 h in human serum while ~ 60% dissociation is noticed in cysteine solution (300:1).

5. DISCUSSION AND CONCLUSION

EB1 (40 μg) has been successfully labelled with 37 MBq of ^{99m}Tc in the presence of 1 μg $SnCl_2.2H_2O$. The labelled compound is confirmed by the shorter retention time of avidin-biotin complex (^{99m}Tc-EB1-avidin) compared to ^{99m}Tc-EB1 on P4 column and size exclusion column. The shift in retention time confirmed EB1 labelling to ^{99m}Tc. Even though ^{99m}Tc-EB1 is stable in human serum, dissociation is high with the increasing molar ratio of cysteine: EB1.

Direct labelling of UBI with ^{99m}Tc was studied. Radiochemical purity of ^{99m}Tc-UBI, determined by ITLC-SG in saline and 35% acetonitrile in 0.1%TFA/water, is greater than 90%. HPLC using reverse phase column can be used to identify ^{99m}Tc-UBI. The radiolabelling yield is not affected by the concentration of $Na^{99m}TcO_4$. However, ^{99m}Tc-UBI is unstable in human serum and cysteine solution. Affinity of ^{99m}Tc-UBI to bind the bacteria could be proved by very high binding with 10^9 CFU. Biodistribution at 2h post injection was encouraging for infection imaging.

High labelling efficiency kit for ^{99m}Tc HYNIC-UBI is formulated. ^{99m}Tc HYNIC-UBI shows lower ratio of uptake of target/nontarget than the product of direct labelling.

^{99m}Tc labelling efficiency of DTPA-UBI at pH about 5.2 and 7.6 are rather low but yield at pH 7.6 seem to be higher than at pH 5.2.

^{99m}Tc is labelled to BPTI by indirect method using MAG3 as bifunctional chelating agent. BPTI has been conjugated to NHS-MAG3 via active ester. The conjugation can be completed within 1 h. It can be purified by either by Sephadex G-50 or size exclusion HPLC. ^{99m}Tc labelling to MAG3- BPTI can be completed within 30 min. Radiochemical purity of ^{99m}Tc-MAG3-BPTI is greater than 90% by ITLC-SG in acetone and solvent mixture of pyridine:acetonitrile:water (5:3:1). Affinity measurement of ^{99m}Tc-MAG3-BPTI to trypsin proved that ^{99m}Tc is labelled to BPTI-MAG3. ^{99m}Tc-MAG3- BPTI is quite stable in both human serum and cysteine solution.

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^{99m}Tc RADIOPHARMACEUTICALS FOR INFECTION IMAGING: KITS DEVELOPMENT AND VALIDATION

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Abstract

This research concerns the preparation and evaluation of various radioactive agents for infection imaging. Our investigations concentrate on the development of a radiopharmaceutical for specific infection imaging. The molecules UBI 29-41, (TGRAKRRMQYNRR), EDTA-Biotin (EB1) and Human Neutrophil Elastase 2 (HNE2) were labelled with direct /or indirect methods with ^{99m}Tc by reducing pertechnectate. Quality control was done with chromatographic methods, including HPLC and Seppak. The binding studies to bacteria and biodistribution in normal and infected mice were performed. The labelling yield of UBI 29-41 was higher than 90% with a direct method. Target to non- target ratio of 2.1 at 1 h after injection of ^{99m}Tc-UBI 29-41 was obtained. For ^{99m}Tc-EB1 a yield higher than 90% was observed and the biological activity was illustrated by binding the radioactive compound to avidin. In the case of the HNE2 conjugation with cDTPA [Diethylenetriamineepntaacetic acid α, ω- bis (Biocytinamide)] was achieved.

1. INTRODUCTION

Various ^{99m}Tc labelled compounds have been developed for the scintigraphic detection of infection and sterile inflammation in man [1,2]. Unfortunately, these radiopharmaceuticals do not allow discrimination between infections with pathogens and sterile inflammatory processes [3], which is often of clinical importance. In an attempt to develop such a tracer, our research has been focussed on peptide antibiotics [4], which could be promising radiopharmaceuticals to distinguish between bacterial infections and sterile inflammatory processes [5].

Our group performed experiments with labelled ^{99m}Tc-IgG and peptide derivates from ubiquicidine (UBI). ^{99m}Tc-IgG is an agent that accumulates at sites of infection and inflammation whereas ^{99m}Tc-UBI has shown specific binding to bacteria. Our investigations were extended with studies on ^{99m}Tc- EB1 and ^{99m}Tc-HNE2. The latter compounds are known to accumulate at both sites of infection and inflammation. This report summarizes our findings as far as radiochemical and biological characteristics of these compounds are concerned with enphasis on a radiolabelled peptide derived from ubiquicidine: ^{99m}Tc- UBI 29-41.

2. MATERIALS AND METHODS

2.1. Direct ^{99m}Tc labelling and control of Ubiquicidin derivatives (UBI-29-41 and UBI-Scrambled)

The UBI 29-41 peptide, corresponding to residues 29-41 of a cationic α-helical domain of human ubiquicidin (TGRAKRRMQYNRR, 1,693 Da) was synthesized as described before [6]. A UBI scrambled peptide (Sc; KRNQRMARYRRGT, 1,693 Da) was included as a control [7,11].

The peptides were directly labelled according to an earlier developed method [5] by mixing 10 uL of the peptide solution (1 mM in 0.01 M acetic acid pH 4.0) with 4 uL of a solution of stannous pyrophosphate (0.5 mg/mL), 2 uL of a solution containing 10 mg/mL potassium borohydride in 0.1 M sodium hydroxide (both as reducing agents), and 0.1 mL of ^{99m}Tc-sodium pertechnetate (200-700 MBq/mL, CIS, Paris, France). Continuous and gentle agitation during 30 min at room temperature was done.

Physicochemical controls for determination of radiochemical species as well as stabiliy of the labelled peptides were carried out using the following methods:

Instant thin layer chromatography (ITLC-SG) using 0.9% NaCl, metyl ethyl ketone (MEK), or water-trifluoroacetic acid 0.1% (H_20/TFA 0.1%) as solvents.

Reverse-phase high performance chromatography (RP-HPLC) on a C 18 reverse phase column (Varian, Ireland) with 0.1% (w/w) trifluoroacetic acid in water (A) and 0.1% (w/w) trifluoroacetic acid in acetonitrile (B) as eluents. The run with a flow of 1 mL/min was performed as follows: 0-3 min 100% A, 3-13 min from 100% A to 100% B, 13-18 min 100% B, 18-23 min from 100% B to 100% A.

Sep-pak: extraction on a cartridge (Waters, Milford, Mass., USA) previously activated with 5 mL methanol, 5 mL water and 5 mL 0.1% acetic acid, and airdried. On the cartridge, 20 to 100 ul of sample was applied and the cartridge was then rinsed with 5 mL of 0.1% acetic acid and the trapped peptide was eluted with 5 mL of methanol.

Stability studies: the chromatographic system described above was used to assess the stability of labelled peptide at room temperature for 6 h. ITLC-SG was used to estimate the stability of the labelled peptides in (1/5) diluted human serum at 37ºC. at various time intervals (5, 30, 60, 120 min, and 24 hs). Around 30 µg (in 200 uL) of each radiolabelled peptide was added to 200 µL of diluted serum.

Cysteine Challenge: each labelled peptide was tested for instability towards sulfhydryl groups. A solution of 10 mg/mL cysteine was diluted in PBS 0.2 M, pH 7.0 to 1, 0.1, 0.01 and 0.001 mg/mL. Mixtures of 10 µL of each solution and 90 µL of radiolabelled peptide solution were incubated for 1 h at 37ºC and were compared with a blank sample. After this time the mixtures were controlled by chromatography.

In vitro binding of *S.aureus* and *S.aureus virulen:* studies were done with a modification of the techniques recommended by the group [8]. *S.aureus* ATCC 25923 (American type cell culture Catalog number 25923) and *S. aureus virulent* were used. Bacteria were thawed and let at RT for 10 min, and centrifugued for 5 min at 3500 rpm, 4º C. Two washings of the pellet were done resuspending each time in 10 mL of buffer A (PB 14 mM pH 7,5) and homogenizing (Politron). The final pellet was resuspended in 0.4 mL of the same buffer (contains about 10^9CFU in 0.4 mL). In order to prepare working preparations, made a 1:1solutions in Buffer A, serially seven times of the suspended pellet. To 0.1 mL of each dilution was added 0.1mL of labelled peptide (1nmol/tube) with 0,8 mL of a solution of 0.05% acetic acid and 0.005% Tween 80 in buffer A, incubated at 4ºC for 1 h, and total activity counted. Centrifuged in the same conditions as above and washed twice with 1 mL of the previous buffer and finally counted pellet activity. Specific binding of activity was calculated as activity in pellet minus activity in blank.

In vivo experiments: Biodisribution studies were performed in mice having a bacterial infection, sterile inflammation, or none. Infection was induced by an intramuscular injection of 0.1 mL containing around 10^8 CFU /mL of Staphylococcus aureus ATCC 25923 in the right hind leg of the mice, 24 h before the biodistribution study. Sterile inflammation was induced by intramuscular injection of 0.1 mL containing about 10^9 CFU /mL of heat killed multiresistant Klebsiella pneumoniae (K.p.R) (LPS). Labelled peptide (0.1 mL containing 20 to 70 MBq) was injected intravenously, and the activity uptake in different organs/tissues was determined after cervical dislocation at 1h and 2 h post injection. Data were expressed as % of injected dose/gram (%ID/g). In mice with an infection or with sterile inflammation target (infected/inflamed thigh muscle)-to-non-target (contralateral thigh muscle) ratios (T/NT) were calculated. The presence or absence of viable bacteria was determined microbiologically, in the muscle of sacrificiced mice from the biodistribution studies. These homogenized tissues were plate counted (both muscle inoculated and not inoculated, as blank). Finally, we calculated correlations between the T/NT ratio and the presence of viable bacteria/g tissue.

2.2. ^{99m}Tc labelling and control of BPTI and HNE-2 – Indirect method

According with the protocol suggested [8] we made the initial experiments with the control peptide Binding Pancreatic Trypsin Inhibitor (BPTI), with a view to tranfer the conditions standardized to the peptide of interest, HNE-2. Conjugation of BPTI with NHS-MAG3 (N-hydroxysuccinimide-mercaptoacetyltriglycine) was done as follows: Molar ratios of NHS-MAG3 / BPTI, were 20:1, 7:1 and 4:1, incubation of reagents during 1 h at RT, purifications of the conjugates by means of gel-filtration in a P4 column and elution with NH_4AcO 0.25 M, pH 5,2. Measurement of peptide concentration of the eluted fractions was done by UV spetrophotometry at 280 nm (extinction coefficient: 0.80 for BPTI [9]). Fractions with absorbances higher than 0,050 were stored at –80ºC for further use.

Labelling of the conjugates with ^{99m}Tc was done on 100-250 uL of the fractions with peptide concentration between 0.2-0.4 mg/mL, and adding 100-250 uL PBS 0.1 M, pH 7,2; 25-62 uL tartrate buffer 50 mg/mL, pH 9 (in sodium acid carbonate 0,5 M , ammonium acetate 0.25 M and ammonium hydroxide 0.175 M), 200-500 uL pertechnetate solution (1.5-4.4 MBq/mL) and 4-40 uL $SnCl_2.2H_20$ freshly prepared solution (1mg/mL) in 10 mM HCl.

The mixture was incubated for 30–60 min at RT. The labelled conjugate was purified using a P4 column with PBS 0.2 M (pH 7,2) as eluent. 1 mL Fractions were collected and activity measured in a dose calibrator.

The initial reaction mixture as well as all the fractions showing significant activities were evaluated using the following methods:

ITLC-SG with various solvent systems (NaCl 0.9%, MEK, ACN:H_20 60:40, 0.2 M Sodium citrate.

HPLC in two column systems: a) C18 Reverse Phase: TFA 0.1%/ H_20 (A), TFA 0.1%/- ACN (B), gradient from 100% A at 0 min, to 33% A– 7% B at 60 min. b) Superdex peptide column, sodium buffer phosphate 0,1 M, pH 7,2, isocratic mode.

Sep-pak (C18) previously activated with 10 mL ethanol and 10 mL HCl 0,001 M, washing with HCl 0.001 M to release pertechnetate and tartrate, and eluting with ethanol:saline (1:1) to release the labelled peptide, the $^{99m}TcO_2$ remained trapped in the cartridge.

Conjugation of HNE2 with α,ω-bis-(biocytinamide) acid and cDTPAa was done by the following assay conditions: molar ratio cDTPAa:HNE 25:1, incubation of reagents for 1 h at room temperature, purification of the conjugate by gel permeation in P4 column and elution with NH_4 AcO 0.25 M, pH 5.2. Peptide concentration was measured in the collected fractions by UV spectrophotometry at λ: 280 nm (extinction coefficient: 0.89 for HNE2 [9]. One peak was collected and stored at –80º C for further use.

Labelling with ^{99m}Tc of the conjugate was done on 250 uL of pooled fractions (peptide concentration 0.35 mg/mL), adding 45 uL tartrate buffer 50 mg/mL, pH 9 (in sodium acid carbonate 0,5 M, ammonium acetate 0.25 M and ammonium hydroxide 0.175 M), 250 uL pertechnetate solution (6.4 MBq/mL) and 10 uL of $SnCl_2.2H_20$ freshly prepared solution (1mg/mL) in 10 mM HCL. The mixture was incubated for 1 h at RT. The ^{99m}Tc conjugate was purified by P4 column, eluting with PBS 0,2 M, pH 7,2. Fractions of 1 mL each were collected. All fractions showing significant activities were controlled by ITLC-SG, HPLC and Seppak as was described for BPTI conjugate.

2.3. ^{99m}Tc labelling and control of EB1

According to the protocol recommended [10] the labelling of EB1 was done by mixing 100 uL of EB1 (10mg/mL in acetate buffer 1 M, pH 6), 1000 uL(0.1-0.6 MBq/mL) of $^{99m}TcO_4^-$, and 10, 35, 100 uL of $SnCl_{2.2}H_20$ freshly prepared solution (1mg/mL) in 10 mM HCL, incubated either at RT for 30 min, heating in a water bath at 60-65ºC 15 min; 90-95ºC, 5 min or immediately heating at 90–95ºC for 5 min. Physicochemical methods for determination of radiochemical purity of the ^{99m}Tc-EB1 were evaluated: ITLC-SG in 0,9% NaCl and MEK and HPLC in TSK G3000 SW, UV at λ: 280nm and radiometric on line detection, isocratic elution with phosphate buffer 0.1M, pH: 7, at flow 0.5 mL/min.

Stability at RT until 48 hs was evaluated by chromatography in saline and MEK.

Stability in human serum: Incubation of 200 uL labelled peptide with 200 uL diluted human serum (1/5) at 37ºC for 5´,30´, 60´ 120´ and 24 hs, controlling with the chromatographic systems.

Cysteine challenge: Incubation of 1000 uL of different solutions of cysteine with concentrations of 10mg/mL, 1mg/mL, 0,1 mg/mL, 0,01 mg/mL and 0,001 mg/mL, with 4,5 uL of labelled peptide for 1 h at 37ºC, and controlling by chromatographic systems. The highest molar ratio was 500/1 (cysteine:EB1).

Specific binding of ^{99m}Tc-EB1 to avidin was evaluated by gel permeation (PD10) using phosphate buffer 0,05 M , pH 7.2, SAB 0,2 % and NaCl 0.9%, as eluent. One mL fractions were collected and counted for activity. Molar ratio of ^{99m}Tc-EB1 and ^{99m}Tc-EB1 to avidin was 1:50.

3. RESULTS

3.1. Direct ^{99m}Tc labelling and control of Ubiquicidin derivatives (UBI-29-41 and UBI-Scrambled)

According to ITLC analysis the labelling efficiency for ^{99m}Tc-UBI-29-41 was 92,0±7.7% (n=11) and 92.5±7.4% (n=11) determined using MEK and 0.9% NaCl respectively. For ^{99m}Tc-UBI-Scrambled the results were 87.7±7.7% (n=6) and 88.8±3.4% (n=7) determined using MEK and 0.9% NaCl respectively. For analysis using 0.1% TFA as eluent in a number of experiments results were not significantly different than those determined with MEK and 0.9% NaCl.

HPLC profiles for both labelled peptides showed peaks (gamma detection) with similar retention times: 9,2 min for ^{99m}Tc-UBI-Scrambled and 9,5 for ^{99m}Tc-UBI 29-41 min, and area higher than 95%. Recovery of eluted activity was 83±19% for both labelled peptides. The retention time of the unlabelled species was 8,8 min.

The recovery of the radioactivity fractions eluted from the Sep-pak cartridge, corresponding to labelled peptides, were 69.8±5.0% (n=5) for ^{99m}Tc-UBI 29-41 and 68.8±1.6% (n=2) for ^{99m}Tc-UBI-Scrambled, respectively. In addition, the acidic elution contained about 2.7% of the applied radioactivity that corresponds with free ^{99m}Tc-pertechnetate and the remaining activity on the cartridge was 27.5% of the total activity.

Stability studies: Using the same chromatographic systems as described above, ^{99m}Tc-labelled UBI 2941 showed no release of ^{99m}Tc-pertechnetate up to 6 h post labelling indicating good stability of the labelled peptide. Stabilty of ^{99m}Tc-UB 29-41 in human serum as depicted in Fig. 1 shows no release of radioactivity during the first hour of incubation.

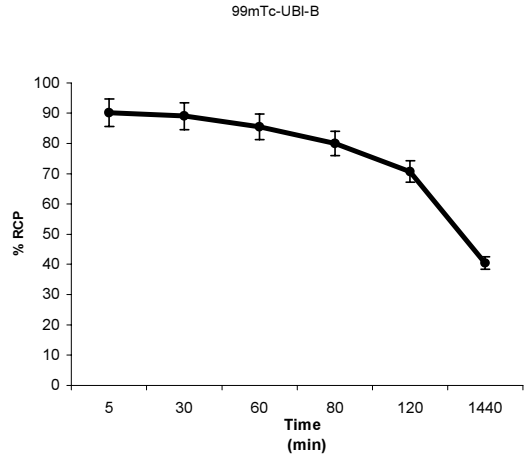

FIG. 1. Stability of ^{99m}Tc-UBI 29-41 in human serum.

Cysteine Challenge: As shown in Fig. 2, more than 10% of the label has been transchelated to cysteine at concentrations higher than 0.01mg/mL. ^{99m}Tc-UBI-Scrambled showed similar results under the same conditions (Fig. 3).

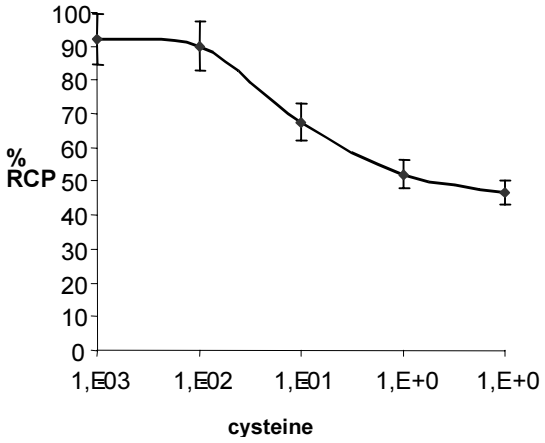

FIG.2. Cysteine challenge of 99mTc-*UBI 29-41*

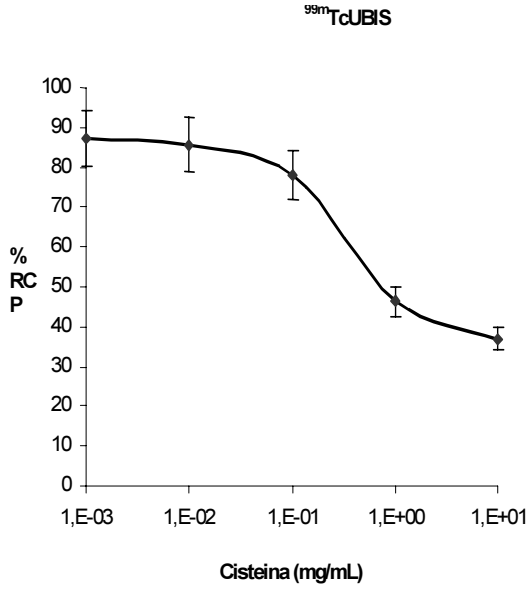

FIG. 3. Cysteine challenge of ^{99m}Tc-UBI-scrambled.

In vitro binding to bacteria showed an increase in specific binding as function of number of bacteria for both strains of *S. aureus* (Fig. 4). In the conditions of assay, specific binding of ^{99m}Tc-UBI 29-41 was 38.1% for *S. aureus Virulent* (1.47E+06 06 CFU/tube), and 32.7% for *S. aureus* (5.08E+06 CFU/mL).

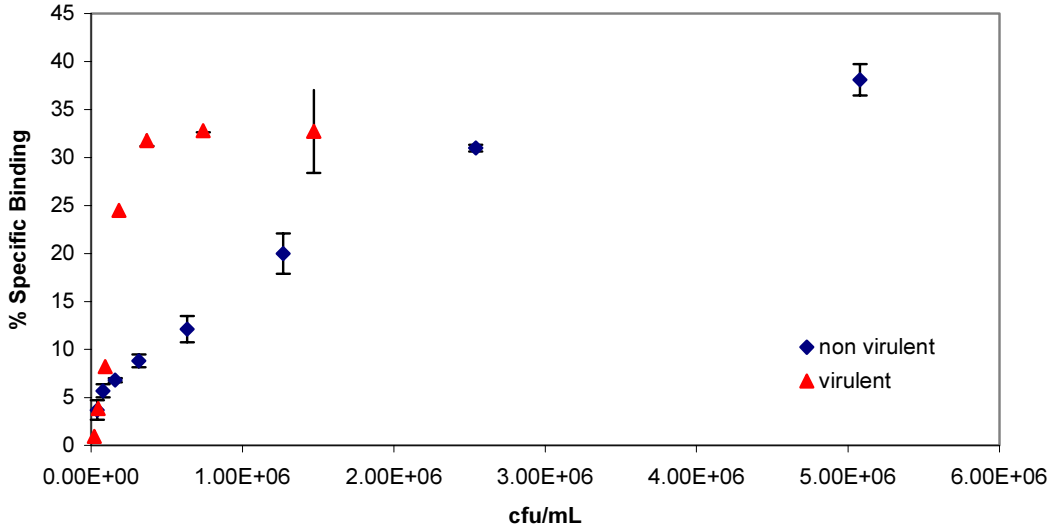

FIG. 4. In vitro binding ^{99m}Tc-UBI 29-41 to S.a.

In vivo studies: Macroscopic examination of both hind legs of mice showed positive nodules in the infected group which was, as determined by microbiological assays, associated with bacterial growth.

Biodistribution studies showed that the highest accumulation of radioactivity (about 60% ID) of both labelled peptides was located in the urinary system (Fig. 5). Uptake in the different organs and tissues is shown in Table I. T/NT ratios determined at 1 h after injection was: 2.07±0.97 (n=11) and 2.39 ±1.36 (n=3) for ^{99m}Tc-UBI 29-41 and ^{99m}Tc-UBI-Scrambled respectively. Two hour post-injection ratio was 2.18±0.42 (n=2) for ^{99m}Tc-UBI29-41. In mice injected with LPS the ratio was 1.2±0.4 (n=2).

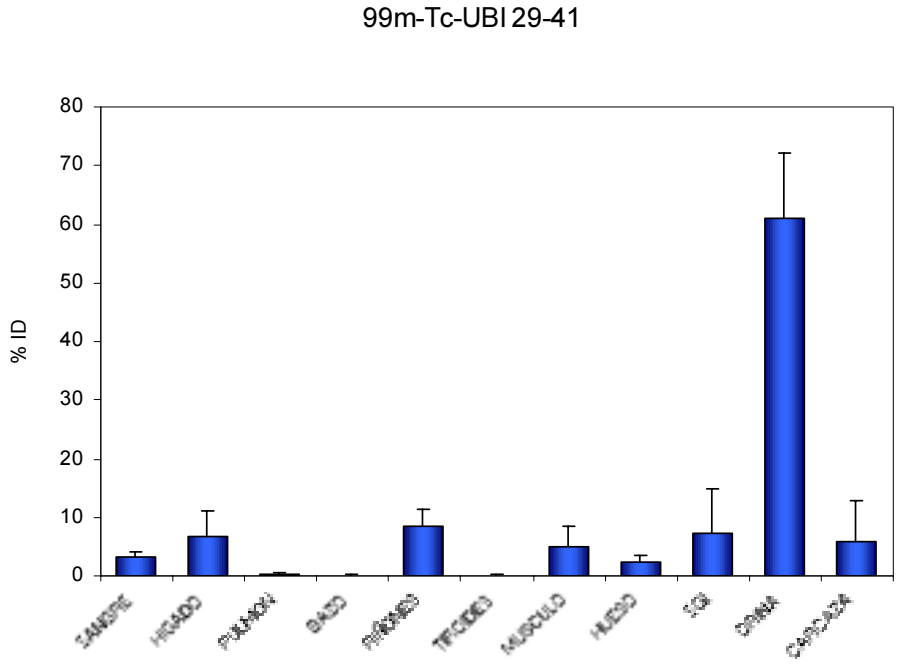

FIG. 5. Biodistribution in infected mouse.

TABLE III. BIODISTRIBUTION OF ^{99m}Tc-UBI-29-41 AND OF ^{99m}Tc-UBI-29-41-SCRAMBLED IN INFECTED MICE WITH S.A. (%ID/gm, MEAN ± ST. DEV)

Peptide	^{99m}Tc-UBI-29-41	^{99m}Tc-UBI-29-41	^{99m}Tc-UBI-29-41-scrambled
Distribution time	1 h	2 h	1 h
Blood	1.6 ± 0.7	0.6 ± 0.2	2.6 ± 1.0
Liver	3.4 ± 2.0	4.3 ± 2.9	5.9 ± 2.0
Lung	1.3 ± 1.1	0.5 ± 0.1	2.1 ± 0.4
Spleen	1.5 ± 2.0	2.3 ± 2.6	1.9 ± 2.5
Kidney	13.7 ± 3.2	14.9 ± 1.9	13.0 ± 3.8
Thyroid	1.9 ± 1.8	0.4 ± 0.1	3.1 ± 2.3
Normal muscle	0.3 ± 0.3	0.12 ± 0.01	0.6 ± 0.2
Infected muscle	0.5 ± 0.2	0.25 ± 0.04	1.2 ± 0.5
Bone	0.7 ± 0.4	0.41 ± 0.06	1.6 ± 0.6
Stomach + intestine	2.6 ± 2.9	1.92 ± 0.45	6.3 ± 5.0
Urine+ bladder	345 ± 162	128 ± 21	360 ± 219
Carcass	0.6 ± 0.3	0.19 ± 0.01	1.1 ± 0.6
T/NT	2.07 ± 0.97	2.2 ± 0.4	2.4 ± 1.4
n	11	2	3

Infection was confirmed by determining the number of viable bacteria in the group inoculated with *S. aureus* and injected with either ^{99m}Tc-UBI 29-41 and ^{99m}Tc-UBI-Scrambled; in both the number of viable bacteria was between 10^3 and 10^{10} CFU/g. Finally, correlation between number of viable bacteria recovered from the infected thigh muscles and T/NT ratio for ^{99m}Tc-UBI 29-41 is shown in in Fig. 6.

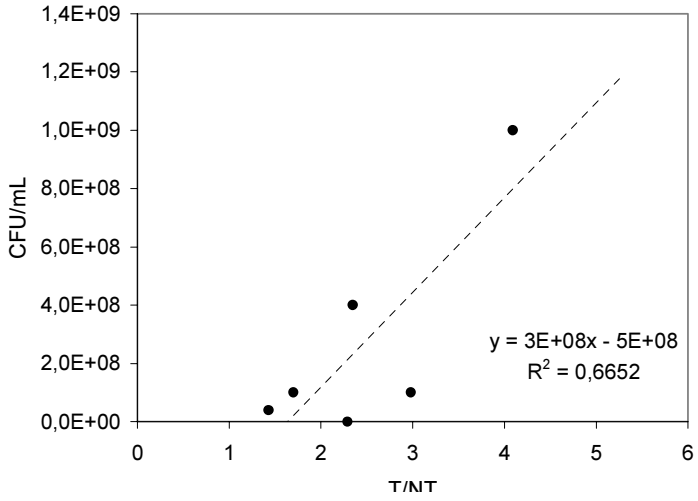

FIG. 6. Target to non-target ratio for ^{99m}Tc-UBI 29-41 in mice infected with S. Aureus.

3.2. ^{99m}Tc labelling and control of BPTI and HNE2

Following the techniques described above, a single peak was obtained from the purification profile when using a molar ratio of BPTI:NHS-MAG3 of 1:4 (83%, 5th mL) and two peaks when using molar ratios of 1:7 (40%, 5th mL and 60%, 10th) and 1:20 (35%, 5th mL and 65%, 12 th mL). The fractions labelled and purified by gel permeation P4 showed two peaks of activity: 1th (5,5 mL and 2nd (23 mL).

ITLC-SG: At 1 to 2 h post-labelling showed 44±3%, (n=5), determined by MEK and 40±2% (n=5) by 0.9% NaCl, respectively, in a sample of the initial reaction vial. The control on the fractions collected after purification on P4 column, at 2–5 h post labelling gave an RCP of 89±5% (n= 4) determined by MEK and 90±5% (n=5) by 0.9% NaCl chromatography.

Sep-pak C18: RCP determined as percent activity in the ethanolic fraction was 60-74%. When using this same procedure for control of the fractions collected at the gel permeation on P4 column, radiochemical purities of 78-95% were achieved.

HPLC: The radiochemical purity determined by HPLC of the initial reaction mixture was 61%, RT: 14.76 min (Superdex peptide Column) and 58-63%, RT: 27.75 (C 18 Column). After purification by gel permeation on P4 column, an increase to 93%, RT: 14.8 min (Superdex Column) of radiochemical purity was achieved.

The results obtained with HNE2 are: Labelling yield determined by ITLC-SG in MEK was 50% (Rf 0.0). The purification on P4 column, showed an expanded peak from 5 to 15 mL (10 fractions) which, upon chromatographic control in ITLC-SG using MEK gave 90-100% of activity at Rf 0.0.

Sep-pak: Fraction 6 from P4 purification (corresponding to an eluted volume of 5.8 mL) and fraction 10 (corresponding to an eluted volume of 10.3 mL) gave 58% and 71.1% of total activity collected in the ethanolic fractions respectively.

3.3. ^{99m}Tc labelling and control of EB1

The results obtained for the various labelling conditions tested are shown in Table II. The technique of labelling was using a molar ratio 100:1 EB1/$SnCl_2.2H_20$, with immediate heating at 90-95ºC, for 5 min.

Chromatographic control in Whatman1 and MEK gave RCP: 92.6±5% (n=3).

Stability evaluation by chromatographic control in MEK showed slight decrease in RCP up to 4 h at RT as can be seen in Fig. 7.

Stability in human serum evaluated as percentage at origin in chromatography in MEK (Whatman 1) showed no significant change for 2 h.

Challenge to cysteine showed no tranchelation of ^{99m}Tc-EB1 to cysteine up to molar ratio 1/50 EB1/Cys (1mg/mL).

Specific binding of ^{99m}Tc-EB1 to avidin was evidenced by gel permeation (Fig. 8**).** Specific Binding of ^{99m}Tc-EB1 to *S.aureus* and *S.aureus Virulent* were 1,4±0.3 (n=2) and 1,5±0.1 (n=2) respectively.

TABLE IV. LABELLING OF ^{99m}TC-EB1 UNDER DIFFERENT INCUBATION CONDITIONS

Molar ratio Sn/EB1	Incubation Time (min)	Temperature	% Origin MEK	% Origin Saline	pH
1/10	30	20	61,4	64,1	6,5
1/10	180	20	85,2	51,7	6,0
1/10	10	65	85,1	39,4	7,0
1/10	10	96	86,4	23,7	7,0
1/10	15	96	86,0	42,2	6,5
1/30	30	20	69,8	49,5	7,0
1/30	15	96*	91,7	14,8	6,5
1/50	5	96*	91,8	23,2	6,5
1/50	15	96*	91,8	23,2	6,5
1/100	5	96*	85,6	2,8	6,5
1/100	5	96*	94,8	ND	6,5
1/100	5	96*	96,4	4,3	6,5
Mean ± SD			**92,3 ± 5,8**	**3,6 ± 1,1**	
1/1000	5	96*	27,5	1,9	6,5

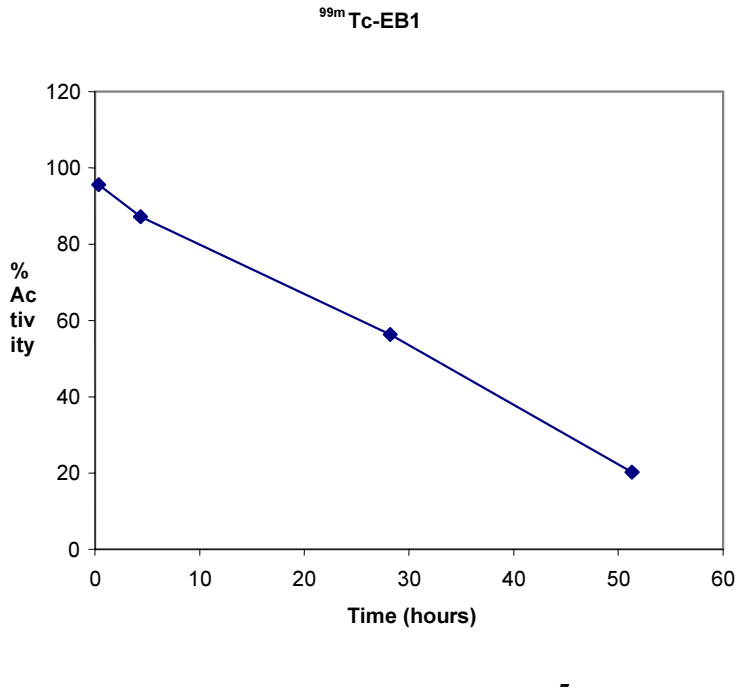

FIG. 7. In vitro stability of ^{99m}Tc-EB1.

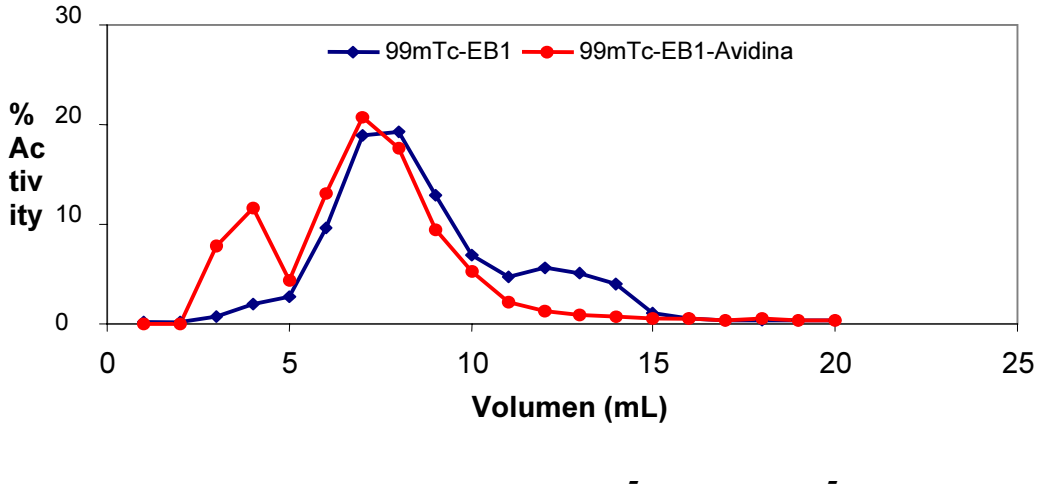

FIG. 8. Gel permeation in PD10 column of ^{99m}Tc-EB1 and ^{99m}Tc-EB1-avidin.

4. DISCUSSION AND CONCLUSIONS

4.1. ^{99m}Tc UBI 29-41 and ^{99m}Tc UBI-Scrambled

This study shows that the derivate UBI 29-41 can be labelled rapidly with ^{99m}Tc resulting in a stable tracer suitable for the selective detection of bacterial infections in mice.

RP-HPLC analysis revealed that the both UBI 29-41 and UBI-Scrambled unlabelled peptides eluted with the same retention time indicating the same lipophilicity. For both labelled peptides, we observed a later retention time with respect to unlabelled ones indicating a higher lipophilicity. This is probably due to the fact that some hydrophilic groups in the molecule are involved in the coordination sphere of Tc.

Radiolabelled peptides are stable with respect to cysteine challenge. Our results reveal that at the highest cysteine/peptide molar ratios, there is some degree of transchelation, that could be due to lower stability in Tc-O and/or Tc-N bonds rather than with respect to Tc-S bonds in the methionine residue. [5].

The calculated accumulation in the confirmed infected tissues, as expressed by a T/NT ratio, was statistically higher than those for inflamed tissues but standard deviations among different animals tested gave a poor significance between populations. Under the experimental conditions of the mild infections by pathological bacteria (less than 10^6 CFU/g in infected muscle) there was a slight but not significantly higher T/NT ratio for ^{99m}Tc UBI 29-41 compared with ^{99m}Tc UBI-Scrambled peptide which tends to increase with the level of infection. These data are in concordance with earlier observations seen at 1 hr after injection [7] as we could observe no differences macroscopically and microbiologically from infected muscle with respect to the number of viable bacteria in both the groups injected with either ^{99m}Tc UBI 29-41 or ^{99m}Tc UBI-Scrambled. As a possible explanation we could think that at early intervals such differences could not be observed because of the relatively high amounts of absolute radioactivity accumulating in the infected tissues that does not allow us to depict differences in accumulation depending on charge or receptor interactions. Nevertheless, though there is small number of experiments in this study, according to our findings we expect to obtain higher

T/NT ratios by increasing the number of viable bacteria in the inoculum that would give us a clearer view on the differences in accumulation of both tracers at early intervals.

In the in vitro binding studies we observed a high specific binding for both *S.aureus* strains with ^{99m}Tc-UBI 29-41 (38.1% for *S. aureus* and 32.7% for *S. aureus Virulent).*

4.2. ^{99m}Tc HNE2 and BPTI

^{99m}Tc labelling MAG3-BPTI was achieved with high yield as evidenced by HPLC control on reverse phase column with radiometric and UV detection. However we could not show that the ^{99m}Tc – BPTI had intact biological properties because we could not evaluate its activity in the presence of trypsin.

Through the analysis of the labelling of various conjugates and radiochemical purity attained, the best molar ratio for the conjugate production was determined as 1:7. Therefore future work will be stressed on optimizing this step.

Gel permeation through P4 column represents an effective separation method even though it is time consumming.

4.3. ^{99m}Tc-EB1

The direct labelling of EB1 was simple, quick and efficient. ^{99m}Tc-EB1 had good stability for at least 4 h. ^{99m}Tc-EB1 didn't show good binding to *S. aureus*. and *S. aureus Virulent.* So it is not a promising tracer for differential bacterial infection diagnosis.

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