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DEVELOPMENT OF RADIOIMMUNOMETRIC ASSAYS AND KITS FOR NON-CLINICAL APPLICATIONS

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FOREWORD

Immunoassays are versatile analytical techniques that had a leading role in various clinical applications, during the last four decades. The studies carried out by Rosalyn Yalow, Solomon Berson and Roger Ekins in the 1960s gave a breakthrough in the development of this novel analytical method. Sensitivity up to femtomolar concentrations, high specificity and universal application to different classes of molecular species made immunoassay a very useful tool in analytical investigation. The expertise acquired by immunochemists in producing antibodies against any antigen and the ability of radiochemists in labeling the antigens with ^{125}I without affecting the active site are the two main factors responsible for the above development.

There are concerns about the safety and health of humans due to the high-level contamination of environment by pesticides, industrial compounds and metals, anabolic steroids in milk and meat products, and presence of mycotoxins in food and feed attributable to improper storage of agricultural commodities. Accordingly, the need to rapidly and inexpensively monitor contaminants has gained prime importance. Hence, there is a need for low-cost, rapid and automated analytical systems. Immunoassay procedures satisfy the above requirements and provide a useful adjunct to traditional methods of analysis.

The knowledge of the levels of various chemicals or other contaminants in food, milk, soil, water and other significant matrices will help in improving quality of livestock and crops, in regulating anabolic steroids and monitoring of toxic compounds in food products. The immunoassay technique, which was introduced for clinical applications, has now traversed to non-clinical fields such as food industry, environmental surveillance, industrial hygiene, drug research, veterinary science and forensic science.

The International Agency for Research on Cancer (IARC) has categorized aflatoxin B_1 as the most potent carcinogen among mycotoxins. Many countries have imposed restrictions on the levels of aflatoxin B_1 present in food and food products. Accordingly there is a need to have reliable analytical techniques with high sample throughput. The US Department of Agriculture (USDA) and the US Food and Drug Administration (FDA) have investigated the applicability of immunoassays for detection of residues in food, both from the perspectives of inspection as well as prevention of contamination.

Leptin, a protein secreted by the adipose tissue has been implicated in the regulation of food intake, energy expenditure and whole body energy metabolism. A multi-species radioimmunoassay for leptin hormone measurements in laboratory animals, livestock animals as well as in humans is of interest in nutrition research and food industry.

Considering the growing universal need to measure the level of presence of a variety of antigens that have an impact on the quality and safety of food, livestock husbandry and the environment in general, the International Atomic Energy Agency (IAEA) initiated a Coordinated Research Project (CRP) on Development of Radioimmunometric Kits and Assays for Non-Clinical Applications. The overall objective of this CRP was to help in building capabilities in development of assays for progesterone, aflatoxin B_1 and atrazine. These analytes are small molecules and the development of radioimmunoassays for them poses considerable difficulties and challenges. Due to the various applications of leptin measurement, development of an immunoassay for it was also taken up.

This report reviews major achievements in the preparation and characterization of key reagents for the identified analytes. The quality control aspects and the importance of right choice of sample matrix for the assay development are discussed. The work reported here is the culmination of the efforts of participants from various countries.

The IAEA wishes to thank all the participants in the CRP for their valuable work and scientific contributions. The IAEA officer responsible for this publication was M.R.A Pillai of the Division of Physical and Chemical Sciences.

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SUMMARY

1. Introduction

Radioimmunoassay (RIA) and related methods are attractive analytical tools for determination of a wide variety of molecules present in complex matrices in view of the high degree of specificity of the antigen antibody reaction, the high sensitivity offered by radioactivity measurement, simplicity of the technique, reliability and the requirement for only modest equipments and facilities. The technique first introduced in clinical chemistry for measurement of circulating hormones present in sub-microgram levels was later extended to many other substances such as tumor markers, viral antigens, drugs, etc. that are useful in clinical management. The role of RIAs in establishing the usefulness of measurement of these hormones and antigens in clinical practice is very significant. Even though these assays were largely used initially in clinical applications, their applications are not limited to clinical use. Other areas, which have benefited include veterinary science, food industry and drug industry.

Veterinary science is one of the early beneficiaries of the immunoassay technique. Progesterone assay in milk is a very good indicator for identifying luteal phase defects in cattle and detecting missed conception or embryonic death. It provides information both on problems in breeding management and livestock improvement as well as in artificial insemination services. An assay for anabolic steroids in food products could also be used for estimating steroids contamination of meat and meat products. The presence of residual antibiotics in animal foods could lead to development of drug resistant microorganism that may pose harmful effects to humans. Hormones and related compounds are used to accelerate growth in animals. But these agents could possibly cause cancer in humans and hence it is necessary to monitor the antibiotic residues and hormones in animal tissues.

Immunoassays are also developed for a variety of substances present in food. These include flavor constituents, nutritionally important components, contaminants in food, indicators of spoilage of food and food crops, indicators of diseases in animal feed and microorganisms encountered in food processing. Immunoassays are also conveniently used for quantification of pathogenic microorganisms and their toxins. Mycotoxins, which are low molecular weight compounds produced by certain groups of fungi, are a widespread problem faced by food supplies universally. As a result, many countries have enacted regulations to control the level of mycotoxins in the food supply both for their internal use as well as that moving in international trade. The International Agency for Research on Cancer has classified aflatoxin B_1 as the most potent carcinogen among mycotoxins. Various countries have imposed limits on aflatoxin B_1 contamination in food and food products. RIA can be a reliable analytical technique with high sample throughput for estimation of aflatoxin B_1. The U.S. Department of Agriculture and the U.S. Food and Drug Administration have investigated immunoassays for use in the detection of residues in food both from an inspection perspective as well as from a contamination prevention perspective.

In the 1970s, pesticide chemists realized the potential benefits of immunoassay methods for use in compounds that were difficult to analyze by gas chromatography. This transition of the technology has extended to the methods of analysis of soil, water, food and other matrices of environmental and human exposure significance. The utility of RIA and enzyme immunoassays for environmental investigations was recognized in the 1980s by the U.S. Environmental Protection Agency with the initiation of an immunoassay development program. In addition to use in pesticide estimation, immunoassays have also been used for detection of several industrial compounds including polychlorinated biphenyls pentachlorophenol and explosives such as trinitrotoluene. Immunoassays for metals based on metal ion-EDTA chelates or metal conjugates have been introduced with somewhat limited applicability. In medical research, RIA methods are presently the primary choice for new compounds, e.g. recently discovered growth-related factors IGF-1, leptin and Ghrelin.

The IAEA convened a consultants meeting in Vienna in 2000 to take stock of the present status of the application of immunoassays in general and particularly the role of radioimmunometric assays in non-clinical areas and to advise the IAEA in formulating possible future programmes of assay development in these areas. The meeting recognized that several national nuclear centres have built up significant RIA capabilities through IAEA programmes implemented over many years. They are able to prepare

and distribute RIA kits for thyroid hormones and protein hormones that are radioiodinated relatively easily, for wider use. Development of radioiodinated tracers for molecules such as steroids needs preparation of suitable derivatives, specific radioiodination methods and purification procedures. These involve interesting radiochemical techniques which when mastered can considerably enhance the capabilities of these laboratories and lead them to new applications of their RIA capability thus enabling them in serving different sectors in their respective countries.

Small molecules, even though antigenic, do not stimulate antibody production. The most important parameter, which governs the sensitivity and specificity of an immunoassay, is the quality of the antibody. Developing an antibody for such small molecules will need the preparation of a protein conjugate of these molecules prior to their administration in animals. The quality of the antibody, whether monoclonal or polyclonal, can be considerably influenced by the chemical modifications introduced in the molecule and the link through which it is coupled to the protein.

Preparation of a radiotracer for RIA of small molecules is also a challenging task. Molecules such as steroids have to be derivatised with appropriate groups that can be labeled with ^{125}I. Since different positions in the molecules can be used for derivatising, different moieties can be introduced for preparing the tracer and different approaches to iodination and purification can be adopted, it is essential that the appropriate method be adopted at each stage.

The meeting recognized the urgent and growing need of measuring the levels of chemicals that have an impact on the environment, agriculture, livestock husbandry and the quality and safety of food. The meeting recommended formulating a CRP in this field. Groups were selected based on the following criteria:

(i) Expertise in development of RIA reagents and kits.
(ii) Research and Development orientation.
(iii) Availability of synthetic chemistry specialists.
(iv) Active working relationship with user laboratories that will be beneficiaries of the RIA techniques to be developed.
(v) Links with institutions having national mandate for production and distribution of RIA reagents and kits for different applications.

2. Objective

The overall objective of the CRP was to develop immunoassays for small molecules with a molecular weight less than 1000D. Three target areas of applications were identified as veterinary science, food technology and environmental science. The group of molecules identified for the proposed CRP consisted of steroids, mycotoxins and triazines. Assays of these molecules have applications in livestock improvement programmes, ensuring food safety and in estimation of pesticide residues. Immunoassays for these molecules are highly complex and they require synthetic modifications for both radioiodination and immunogen preparation.

With the above objective in view, the CRP aimed at developing RIA/IRMAs for progesterone, aflatoxin B_1 and atrazine using locally produced primary reagents including radiotracer, antibodies and appropriate calibrators and validating them. Considering the importance of leptin measurement in obesity management and nutrition research, It was decided to include leptin as part of the CRP.

Progesterone
Progesterone assay in milk is a good indicator for identifying luteal phase defects in cattle and detecting missed conception or embryonic death. It provides information vital to the solutions of problems encountered in breeding management and livestock improvement as well as in artificial insemination (AI) services. Progesterone assay can detect animals that have not conceived by AI within 21 days and alert farmers the need to have these animals closely observed for their oestrus and re-insemination at the appropriate time.

Aflatoxin B_1

Aflatoxins are a group of toxic secondary metabolites, most commonly produced by certain strains of Aspergillus flavus and all strains of Aspergillus parasiticus. Crops that contain high levels of starch and lipids such as peanut, corn, cottonseed, dryfruits, etc. are found to be contaminated by aflatoxins in the fields during harvest and upon storage thereafter. Aflatoxins exhibit both acute and chronic toxicity. It is a major threat to animals particularly poultry livestock. Prolonged exposure to subacute levels of aflatoxins is of serious concern in human as it leads to hepatotoxicity and liver cancer. It has also been found to be teratogenic in animals. Among the aflatoxins, aflatoxin B_1 has been classified as the most potent carcinogen. Thus the contamination of aflatoxin B_1 in agricultural commodities has become a subject of serious concern at national and international levels. Regulations governing the aflatoxin B_1 levels in food and feed have become stringent. Exportable commodities face rejection due to the aflatoxin B_1 contamination. This may lead to heavy economic losses to farmers and livestock producers. To face these challenges, a simple, sensitive, specific and quick analytical method for the estimation of aflatoxin B_1 levels is essential.

Atrazine

Atrazine is the most widely used herbicide in the world and is used as a selective herbicide for controlling weed in corn, wheat and several other crops. Atrazine can be released into the environment both via effluents from manufacturing sites and at the user end. Atrazine in the soil can leach into the ground water processing plant. It belongs to the group of triazine pesticides. To protect the natural water resources from the unwanted load of pesticides, it is desirable to check water samples for the content of these contaminants. Immunoassays were recommended for use in the environmental monitoring as it reduces the total number of costly laboratory analyses.

Leptin

Leptin is a 16 KDa polypeptide hormone encoded by the obese gene (*ob*) and secreted by adipose tissue. Leptin is studied by many research groups worldwide in the last decade and is now understood to be a molecule that plays a significant role in basic metabolic processes. It affects food intake, bodyweight regulation, neuroendocrine, hematopoetic and fertility processes.

3. Scientific achievements

Working immunoassays were developed for all the analytes identified in the original proposal. All participants demonstrated the expertise necessary for synthesizing the appropriate immunogens and conjugates for radiolabeling. More than half the groups produced the antibodies in their own laboratories.

In general, the project developed the confidence and experience necessary to initiate research projects in the areas under study. Over the period of the CRP, there was extensive collaboration and exchange of procedures between the groups.

4. Research outputs

All the six groups involved in progesterone assays developed workable assay systems. For progesterone, there were a great variety of methodologies. However, all the assays gave comparable performance characteristics. All groups succeeded in producing radiolabeled progesterone tracer locally.

Three groups worked on the development of assay for aflatoxin B_1. Both RIA and EIA were developed for aflatoxin B_1. Aflatoxin B_1-oxime was synthesized and used for radiolabeling. An enzyme labeled second antibody conjugate was used as tracer in EIA. Both assays displayed similar characteristics in terms of sensitivity and dynamic range.

Two groups developed radioimmunoassay system for atrazine, one with a liquid phase and the other with a solid phase, while one group mainly synthesized the required derivatives and carried out immunization. The production of radiolabeled tracer in both groups demonstrated adequate expertise

in chemical synthesis. Both assays were suitable for application and one in particular was more sensitive than what is generally available.

One group developed a specific, sensitive and accurate method for the quantitative determination of human leptin in serum samples. There is a core sequence comprised of eight amino acids (GLDFIPGL) that is totally conserved in leptin molecules from all the evaluated species and hence the experience obtained in this development could be used for developing a multi-species immunoassay.

4.1. Progesterone

Immunogen and antibody

Progesterone is a small and non-immunogenic molecule and hence it was necessary to conjugate it to a carrier protein to make it immunogenic. Almost all groups used BSA as the carrier protein to prepare the immunogen. Regarding the position on the progesterone molecule that was selected for the formation of bridge, all the groups reported that the most successful strategy was to use the 11 position, since immunogen conjugated at 3 position provided antibodies with low affinity. All groups recommended progesterone11α-hemisuccinate as the most appropriate derivative for the preparation of the immunogen. Most of the participants raised polyclonal antibodies in rabbits. Two of the participants used commercially available polyclonal antibodies from ORION, and IZOTOP, whereas one participating group preferred the use of monoclonal antibodies from BiosPacific.

Tracer

Since progesterone cannot be radiolabeled directly, it was necessary to couple it to a molecule that can be iodinated. There were several approaches to prepare different conjugates for radioiodination. The essential details are summarized as follows:

(i) Coupling was carried out at either 3 or 11 position of progesterone.
(ii) Three molecules viz. tyrosyl-methyl-ester (TME), histamine (Hist) and gamma globulin (γ glob), were found to be satisfactory for iodination.
(iii) The type of chemical bridge for conjugation is important as far as progesterone assays are concerned. Hemisuccinate (HS), carboxymethyloxime (CMO) and phthalate (Phth) bridges were tested.

Consensus was that satisfactory results were achieved with the following conjugates:

(i) Progesterone-11α-HS-TME
(ii) Progesterone–3-CMO-Hist
(iii) Progesterone-11α-HS- γ glob.

All the groups used the conventional chloramine-T procedure with carrier-free Na-^{125}I-iodide for radioiodination. In some groups, iodination preceded conjugation and in others the conjugates were prepared and purified before iodination. Although both procedures gave satisfactory tracers, it was appreciated that the latter approach was more efficient in terms of yield and minimal radioactive waste. HPLC, TLC and gel chromatography were the different techniques that were used for the purification of the radiolabeled tracers. All the three methods were found to be satisfactory.

Standards

Standards were based on the addition of chemically pure progesterone (Sigma or Steraloids) to a variety of matrices. All the groups made an initial stock solution in methanol. The concentration was calibrated spectrophotometrically. Working standards were prepared in either phosphate buffer containing protein or diluted milk powder (fat free) or whole milk from cows in estrous or in bull serum. These give different results and the choice of matrix depends on the type of assay.

TABLE I. SUMMARY OF ACHIEVEMENTS OF PARTICIPATING COUNTRIES

COUNTRY	ANALYTE	LOCAL PRODUCTION	OTHER ACHIEVEMENTS
CHINA	Progesterone	Polyclonal Ab, Conjugates, Tracer, Milk standards, RIA	Validation in progress 41 samples evaluated
	Aflatoxin B_1	Polyclonal Ab, conjugates, Tracer, ELISA	Validation in progress
	Atrazine	Polyclonal Ab, Conjugates, Tracer, RIA	Validation in progress
CUBA	Progesterone	Conjugates, Tracer, Milk standards, RIA	Validation complete, Studies of animal samples in progress
GREECE	Progesterone	Conjugates, Tracer, Full fat milk Standards, RIA	Validation complete
HUNGARY	Leptin	Polyclonal Ab, Tracer, Second Ab, RIA	Validation complete, Applied in Research
INDIA	Progesterone	Polyclonal Ab, Conjugates, Tracer, Serum standards, RIA in liquid phase and coated tubes.	Validation complete for liquid phase assay Validation in progress for coated tubes
	Aflatoxin B_1	Polyclonal Ab, conjugates, Tracer RIA in liquid phase and coated tubes, ELISA	Validation complete for liquid phase assay Validation in progress for coated tube and ELISA
INDONESIA	Progesterone	Polyclonal Ab, Conjugate for immunogen, Tracer, Milk Standards, RIA	Validation is in progress
POLAND	Progesterone	Conjugate for tracer, Tracer, Defatted milk matrix, Standards in buffer, RIA	Validation complete, 60 milk samples evaluated
THAILAND	Atrazine	Conjugate for tracer, Tracer RIA	Validation complete, Sample analysis in progress
URUGUAY	Aflatoxin B_1	AfB_1 conjugates, polyclonal Ab	Further studies in progress
	Atrazine	Conjugates, Polyclonal Ab, HPLC purified derivatives	Further studies in progress

Assay formats

Both solid and liquid phase assays were developed during the project. Most of the participants recommended the use of coated tubes for the separation of the bound from the free fractions. Some of the participants immobilized the anti-progesterone specific antibody directly on the solid phase. The directly coated tubes were either prepared in-house or supplied by ORION. Another approach that was followed, involved the immobilization of second antibody on the solid support, prior to the immuno-adsorption of the specific anti-progesterone antibody. One of the participants used commercially

available streptavidin coated tubes as an alternative to tubes coated directly or indirectly with antibody. Apart from the coated tubes, commercially available magnetic particles from IZOTOP were also proposed as an alternative solid support. Although the majority of the participants suggested the use of solid phase assays, some of the participants proposed that a well-established liquid phase assay system based on the use of second antibody and polyethylene glycol for the precipitation of the immunocomplex could also be employed for the development of progesterone assays.

Assay buffer
All the participants recommended the use of phosphate buffer, pH 7.5 either with or without the addition of sodium chloride as the most appropriate assay buffer. Some of the groups suggested the addition of bovine serum albumin (BSA) at concentrations ranging from 0.2 to 0.5 % (w/v), while the addition of gelatin at concentration ranging from 0.1 to 0.2 % (w/v) could be also used as an alternative carrier protein.

Incubation
All the participants proposed the addition of all reagents in one step. The incubation time of the reaction mixture ranged from one to three hours at room temperature either with or without shaking. One group suggested that the incubation of the immunoreagents should be performed at 4^{o}C.

Washing
Washing of coated tubes did not seem to be a critical parameter since most of the participants proposed the exclusion of this step from the assay protocol. However, a few of the participants proposed the inclusion of two washing steps in the assay protocol in an attempt to reduce the non-specific binding. The washing buffer used in the assay was phosphate buffer containing 0.05 to 0.1% (v/v) Tween-20.

Assay validation
The participating groups optimized the assay parameters so as to achieve maximum sensitivity, optimal dynamic range and high reliability. Almost all the participating groups found that the assay procedure developed had acceptably low variations. In general, the intra-assay variation ranged between 0.6 and 10.8%, whereas the inter-assay variation ranged between 2.3 and 12%. The sensitivity of the developed assays ranged between 0.06 nmol/L (0.02 ng/ml) to 7.3 mol/L (2.3 ng/ml).

The variety of the dynamic ranges of the developed assays as demonstrated in the country reports could be ascribed to the different reagents employed, the matrices used for the preparation of standards and the assay format adopted. Most of the participants performed recovery and parallelism tests in milk samples. The assays exhibited good parallelism and the recovery ranged from 83 to 115%. In general, the antibodies used in the assay were highly specific and only minor cross reactivity values were found with compounds structurally similar to progesterone. However, these compounds are not normally encountered in milk samples and therefore are not expected to influence the developed assay.

Samples and sample processing
Most of the participants used milk samples to study the performance of their assays. Three participants included full-fat milk in their studies. Two of the participating groups used fat-free milk samples prepared from whole milk samples by centrifugation. In addition, one participant analyzed bovine serum samples. More than two hundred samples were analyzed and were correlated with the physiological condition of the cow. Two of the participating groups found that their results correlated well with the commercially available progesterone RIA assay from ORION. One group found good correlation of values measured using the developed progesterone RIA and an in-house enzyme immunoassay.

Further investigations
Results from the progesterone studies need to be compared to results from different veterinary projects. These assays could be applied to other biological samples eg. saliva and faeces, which would be significant for wild animal studies.

TABLE II. ASSAY FOR PROGESTERONE

Assay parameter	CHINA	CUBA	GREECE	INDIA	INDONESIA	POLAND
Antibody	Local in rabbit	Commercial polyclonal	Commercial monoclonal	Local in rabbit	Local in rabbit	Commercial polyclonal
Immunogen bridge	11-HS	11-HS	11 position	11-HS and		
3-CMO	11-HS	Not available				
Tracer	Local, synthesized	Local, synthesized	Local, synthesized	Local, synthesized	Local, synthesized	Local, synthesized
Tracer bridge	11-HS	11-HS	11-HS	3-CMO	11-HS	3-CMO
Iodinating group	Histamine	TME	Γ- globulin	Histamine	TME	Histamine
Tracer stability	60 days	60 days	45 days	60 days	60 days	60 days
Standard matrix	Buffer	Fat-free milk	Whole milk	Serum	Skimmed milk powder	Fat free milk with buffer
Sample vol.	10 µL	50 µL	300 µL	50 µL	200 µL	50 µL
Buffer	0.2% gelatin, PBS	0.2% gelatin, PBS	0.1% gelatin, PBS	0.2% BSA, PB	0.1% BSA, PBS	0.5% BSA, PBS
Incubation	18h at 4°C & 1h at 37°C	2h at RT	2h at RT	1h at RT	3h at RT	2h at RT
Separation	Ab2-PEG	Magnetic particles	Coated tube	Ab2-PEG	Coated tube	Coated tubes
Sensitivity	0.015 ng/ml	1.2 nmol/L*	0.1 ng/mL	0.4 ng/mL	2.3 ng/ml	0.8 nmol/L*
Dynamic range	0.03-30 ng/ml	3-300 nmol/L	0.2-40 ng/mL	0.4-25 ng/mL	10-80 ng/ml	1-200 nmol/L
Precision	In progress	<12 %	<7.8 %	<10 %	In progress	2.4-5.5 %
Recovery test	In progress	80-110 %	84-105 %	85-115 %	In progress	94-104 %
Linearity	In progress	85-110 %	R = 0.975	87-110 %	In progress	87-104 %
Validation	In progress	Comparison with ORION kit	Comparison with in-house ELISA	Correlated with clinical condition	In progress	Comparison with ORION kit
Samples	41	85	30	20	In progress	60

* 1nmol/L of progesterone = 0.314 ng/mL

4.2. Aflatoxin B_1

Aflatoxin B_1 being a small molecule is not immunogenic and has no direct group for introduction of an iodine atom. Three groups worked on the development of radioimmunoassay and enzyme immunoassay for measurement of aflatoxin B_1. Out of two groups that used solid phase method of separation, one group used immunogen coated microtitre plates whereas the other group used antibody-coated tubes. In the ELISA format, the aflatoxin B_1-BSA conjugate coated on the tubes and the aflatoxin B_1 in the standard or sample compete for the antibody. The antibody bound on the solid phase is determined using an enzyme labeled second antibody

Immunogen and Antibody
All the three groups succeeded in the production of antibody by administration of the commercially available aflatoxin B_1-oxime-BSA conjugate. All the groups followed a common pattern for the immunization schedule, which was carried out in rabbits with the conjugate emulsified in Freund's adjuvant. The primary injection was intradermal at multiple sites with 100-500µg of the conjugate per rabbit. Booster injections of 100-200µg of the emulsion were given at monthly intervals in case of two groups while the third group used a single injection with one booster injection after four months. The tested antisera had titres from 1:20000 to 1:50000 with no cross-reactivity with other aflatoxins.

Tracer
The aflatoxin B_1 has no suitable functional group to couple an iodinating moeity and therefore it was necessary to derivatise the aflatoxin B_1 for the introduction of a carboxyl group. Aflatoxin B_1-oxime was prepared by two groups by refluxing aflatoxin B_1 with aminoxy acetic acid at 55ºC for two hours. The product was isolated and purified by silica column chromatography and characterised by TLC and ^{1}H-NMR.

^{125}I-Aflatoxin B_1 was successfully prepared by one group by coupling radioiodinated histamine to aflatoxin B_1-oxime. Aflatoxin B_1-oxime was activated using isobutyl chloroformate and tributyl amine in dry dioxane. Histamine was radioiodinated using the chloramine T method and coupled to the activated oxime. The tracer was purified by solvent extraction followed by preparative TLC. In the ELISA format, horseradish peroxidase (HRP) was coupled to the second antibody and used as the tracer. The enzyme was coupled to the aflatoxin B_1-oxime by the periodate method.

Standards
All the three groups used a common format to prepare the standard. Stock solutions were prepared in methanol and calibrated spectrophotometrically. Working standards were prepared in PBS containing 0.2% BSA and 0.1% sodium azide. In the case of the ELISA method, sodium azide was not added to the standards.

Assay format
One group worked on radioimmunoassay using both liquid and solid phase assay systems. In liquid phase, standards or samples, antibody and tracer were incubated for 3h at room temperature. The separation was carried out by the addition of second antibody aided PEG. In solid phase assay, the antibody was coated to the polystyrene tubes through rabbit gamma globulin and second antibody. The assay involved addition of standard or sample and the tracer to the coated tubes and incubation for 3h at room temperature. At the end of incubation, the tubes were decanted and counted. In the ELISA format, the aflatoxin B_1-BSA conjugate coated on the microtitre plate was incubated with standard or sample and antibody for 1h at 37ºC. The wells were washed and the HRP labeled second antibody was added and further incubated for 30 min at 37ºC. The wells were washed and the tetramethyl benzidine solution was added to develop colour. After addition of 1M sulphuric acid, the optical density was measured at 450 nm.

Validation
The liquid phase assay has been validated to give 90-110% recovery, parallelism (90-110%) and precision with inter-assay <15% and intra-assay <10%. The sensitivity of the assay was 0.1 ng/mL and the assay range was 0.5-10 ng/mL. Specificity studies for the antiserum indicated no cross-reactivity

for aflatoxins B_2, G_1 and G_2. For solid phase assay and the ELISA, validation is to be completed. About 40 samples were analysed and compared with TLC method. The results show that the values were in correlation.

TABLE III. ASSAY FOR AFLATOXIN B_1

	INDIA	CHINA	URUGUAY
Antibody	Polyclonal (Inhouse)	Polyclonal (inhouse)	Polyclonal (Inhouse)
Immunogen	AflaB1-CMO-BSA	$AflaB_1$-CMO-BSA	AflaB1-CMO-BSA
Tracer	$AflaB_1$-CMO synthesized $AflaB_1$-CMO-^{125}I-hist	HRP-Ab2	Synthesis of $AflaB_1$-CMO
Standard	Aflatoxin B_1 (Sigma)	Aflatoxin B_1 (Sigma)	Aflatoxin B_1 (Sigma)
Matrix for standard	0.2% BSA in 0.05M $P0_4^-$, 0.1% azide	0.2%BSA in 0.05M $P0_4^-$	-
Incubation	3h at 25ºC	1h at 37ºC +30 min at 37ºC	-
Separation	Liquid phase Solid phase	Solid phase	-
Assay range	0.5-10 ng/mL	0.5-100ng/mL	

Processing of the samples

The non-uniform nature of aflatoxin B_1 contamination makes the sampling process extremely important. 2 to 40 kg lot were mixed and sub samples of 25 to 50 g were selected. 5 g of the samples were finely ground, extracted with 25 mL of methanol/water (80/20) and filtered. The filtrate was stored at 4ºC for analysis.

Further investigations

Extensive fieldwork with different types of agricultural commodities using the developed system remains to be carried out. An assay with a color response will be easier and ideally suited for analysis to be carried out in the field

4.3. Atrazine

Development of an assay for atrazine by immunoassay and formulation into kits based on locally developed reagents would give routine service a practical approach in solving the problems. Three groups worked on the development of assay for atrazine.

Immunogen and antibody

Atrazine is a small molecule and therefore is not a good immunogen. Hence, it needs to be coupled to a carrier protein to elicit antibodies on administration into animal species. Atrazine thiopropanoic acid-BSA conjugate was synthesized by M. H. Goodrow's method. Immunization was carried out by multiple injections of the immunogen on the back of male New Zealand rabbits with 500 μg at the first injection and monthly boosters of 250 μg. Antibodies obtained had titres ranging from 1:5000 to 1:70000 as well as adequate specificities for the triazine group. One group used sheep anti-atrazine polyclonal antibody from Guildhay, U.K.

Tracer
Atrazine conjugate for radioiodination was synthesized according to the method of M.H. Goodrow *et al*. Either tyramine or histamine were used for iodination by chloramine-T method. Purification was carried out by HPLC on reverse phase C18 column with an isocratic flow of 1mL/min using acetonitrile: water (70:30).

Standards or calibrators
Atrazine was obtained from commercial sources. Stock solutions were prepared in methanol or acetonitrile and working standards were prepared in phosphate buffer pH 7.4 with saline containing 0.2% BSA.

Assay format
One group developed a liquid phase (second antibody) format and another group developed a solid phase method using antibody coated tubes method. Incubation times were 3h at 37°C and 45 min at room temperature. Both the systems gave promising results for the range of 0-100 ppb with sensitivities of 0.1 ppb and 0.02 ppb, respectively.

Validation
One group had imprecision less than 10% in the range of 0.1 to 100 ppb and recovery ranged from 105–114 %. Validation is to be completed by the other groups

TABLE IV. ASSAY FOR ATRAZINE

	China	Thailand	Uruguay
Tracer			
Bridge	thiopropanoic acid	thiopropanoic acid	
Iodinating group	tyramine	histamine	
Antibody	Rabbit anti-atrazine (in-house)	Sheep anti-atrazine (Guildhay,U.K.)	Rabbit anti-atrazine (in-house)
Standards			
Matrix	0.05M PB pH 7.4. with 0.2% BSA	Water with 0.1% azide	
Range	0.5-100 ppb	0-100 ppb	
Optimization			
Assay system	Liquid phase (second antibody)	Coated tubes	
Incubation	3 hr, 37^{o}C	45 min, RT	
Validation			
Sensitivity	0.1 ppb	0.02 ppb	
Imprecision	In progress		
within assay		3.9 – 6.5% C.V.	
between assay		5.4 – 7.5% C.V.	
Recovery	In progress	106 – 115%	

Further investigations
- Field assessment with water samples should be performed
- Comparison with conventional methods (HPLC & GC/MS) should be carried out
- Cross reactivity with other triazine members should be determined.

4.4. Leptin

Leptin studied by many research groups worldwide during the last decade is now understood to be a molecule that plays a significant role in basic metabolic processes. It affects food intake, bodyweight regulation, neuroendocrine, hematopoetic and fertility processes. Because of the potential application of leptin measurement in obesity and nutrition research as well as in live stock development, one participant took efforts to develop a leptin radioimmunoassay kit.

Immunogen and antibody
Human recombinant leptin purchased from a commercial source with a molecular weight of 16 KDa was sufficiently large to be immunogenic. Polyclonal antisera was produced by direct immunization of two rabbits. 200 µg human recombinant leptin was initially injected intradermally in multiple sites and 3 booster doses were given at three weeks intervals with 100 µg of leptin.

Tracer
Human recombinant leptin was labeled with ^{125}I using the chloramine-T method. Purification was carried out by gel filtration to give a radiochemical purity of >98% and the tracer was stable at 4ºC for 6 weeks. Specific activity of 2.8–3.3 MBq/µg was achieved.

Standards
Leptin standards were prepared by diluting rhleptin in sterile horse serum. They were freeze-dried and stored at 4°C. The optimized format used a liquid phase reaction with a second antibody-PEG precipitation method. The liquid phase system was also compared with a solid phase system using magnetic separation method. Both were satisfactory and the former adopted for convenience.

Assay format
For the assay, 100 µL of standards, antiserum, tracer and unknown samples were used. The antigen–antibody reaction time was found to be optimum at 18 h (overnight) at room temperature or 4°C. Separation of bound and free fractions in the liquid phase assay was achieved by the addition of 1mL of a mixture of anti-rabbit IgG (second antibody raised in sheep) and 8% PEG-solution with an incubation of 20 min at room temperature.

Validation
Coefficients of variation in intra-assay and inter-assay precision tests were <6% and <8%, respectively and the recovery ranged from 88-106%. The specificity of antisera was checked with 10 different antigens and found to have no cross-reactivity. The sensitivity of the developed assay was 0.5 ng/ml with a range of 0.5-100 ng/mL.

Samples and sample processing
Preliminary research data are presented from a total of 350 serum samples from approximately 90 human subjects. Levels of leptin in lean, overweight and obese have been estimated. Overweight and obese human patients, both women and men, were checked before treatment and follow-up after treatment for leptin concentrations. The levels were found to correlate well to physiological features, treatment response and some other parameters such as bodyweight decrease, BMI, and other laboratory parameters including total cholesterol and triglycerides.

Further investigations
A significant interest in leptin hormone measurements in serum samples of different mammals is shown by basic researchers, veterinary and medical doctors. There is a core sequence comprised of eight amino acids (GLDFIPGL) that is totally conserved in leptin molecules from all the evaluated species. On this basis, the future plan is to develop a multi-species radioimmunoassay, which is available for leptin hormone measurements in laboratory animals, companion (pet) and livestock animals as well as in humans. More details can be found in the country report.

TABLE V. ASSAY FOR LEPTIN

	HUNGARY
Tracer	
Antigen	Human recombinant leptin (rhLeptin, Sigma)
Iodination	Chloramine-T method
Antibody	Polyclonal rabbit anti-leptin (local production)
Standards	
Matrix	Horse serum (local production)
Range	0.5–100 ng/mL
Optimization	
Assay system	Liquid phase (second antibody)
Incubation	18 h (overnight), 4°C or at room temperature
Validation	
Sensitivity	0.5 ng/mL
Imprecision intraassay	< 6% (CV)
Imprecision interassay	< 8% (CV)
Recovery	88 – 106%

5. Conclusions

(i) A number of different methods were found to be satisfactory for progesterone.
(ii) Difficulties were encountered in milk samples presumably due to variations in fat content. This needs to be investigated in detail.
(iii) The assays developed for progesterone could be applied to other biological samples eg. saliva and faeces, which would be significant for wild animal studies.
(iv) Results from the progesterone studies need to be compared to results from other veterinary project.
(v) Satisfactory immunoassays were developed for aflatoxin B_1, which need to be evaluated by reference HPLC systems for a range of samples.
(vi) A very sensitive assay for atrazine was developed which is suitable for all environmental screening studies and is under consideration for official adoption.
(vii) Specificity of the atrazine assays needs to be assessed with reference to standardized materials from appropriate authorities.
(viii) Leptin RIA system was shown to be suitable for human serum samples. Furture work is to raise an antibody for leptin using an octapeptide sequence which is common, in rats, rabbits, swine and mice and to develop an assay for measuring leptin levels in different animal species.

BIBLIOGRAPHY

General

BUTT, W.R., (Ed.) Practical Immunoassays, Marcel Dekker Inc., New York (1984).

DER BALIAN, G.P., GOMEZ, B., MASINO, R.S. AND PARCE, J.W., A fluorometric method for determination of degree of biotinylation of proteins. J. Immunol. Methods **126 (**1990) 281-285.

DIAMANDIS, E.P., CHRISTOPOULOS, T.K., The biotin-(strept) avidin system: principles and applications in biotechnology (Review) Clin. Chem. **37** (1991) 625-636.

ERLANGER, B.F., BOREK, F., BEISER, S.M AND LIEBERMAN S., Steroid-protein conjugates II Preparation and characterization of conjugates of bovine serum albumin with progesterone, deoxycortisone and estrone. J. Biol Chem. **234** (1959) 1090.

PETROU, P.S., KAKABAKOS, S.E., KOUPPARIS, M.A AND CHRISTOFIDIS, I., Antibody coating Approach involving gamma globulins from nonimmunised animal and second antibody antiserum. J. Immunoassay **19** (1998) 271-293.

PILLAI, M.R.A. AND BHANDARKAR, S.D., Radioimmunoassay, Principles and Practice, Third edition, Bhabha Atomic Research Centre, Mumbai, India, 1998.

SAMUEL, G., VENKATESH, M., BALAKRISHNAN, S.A AND SARMA, H.D., Preparation of ^{125}I-testosterone derivatives for use in radioimmunoassay J. Radioanal. Nucl. Chem. **233** (1997) 83-87.

MASSAGLIA, A., BARBIERI, A AND UPATKAM, C.S., Preparation of ^{125}I-labeled steroid derivatives for radioimmunoassay. Int. J. Appl. Radiat.Isotopes. **24** (1973) 455-462.

Progesterone

BULMAN, D.C. AND LAMMING, G.E., Milk progesterone levels in relation to conception, repeat breeding and factors influencing acyclicity in dairy cows. J. Reprod. Fert. **54** (1978) 447-458.

CAVESTANY, D. AND FOOTE, R.H., The use of milk progesterone and electronic vaginal probes as aids in large dairy herd reproductive management. Cornell Vet. **75** (1985 441-453.

FOLMAN, Y., ROSENBERG, M., HERZ, Z. AND DAVIDSON, M., The relationship between plasma progesterone concentration and conception in post-partum dairy cows maintained on two levels of nutrition. J. Reprod. Fert. **34** (1973) 267-278.

FRANCOS, G., Association between milk progesterone concentration after first insemination and conception in dairy cattle. Israel. Vet. Record.**142** (1998) 63-64.

GOWAN E.N, ETCHES, R.J., BRYDEN, C. AND KING, C.J., Factors affecting accuracy of pregnancy diagnosis in cattle. J. Dairy Sci. **65** (1982) 1294-1302

KANCHAN KOTHARI, RAMJI LAL AND PILLAI, M.R.A., Development of a direct radioimmunoassay for serum progesterone. J. Radioanal. Nucl. Chem, Articles, **196** (1995) 331-338.

KASTELIC, J.P., BERGFELT, D.R. AND O.J. GINTHER, Relationship between ultrasonic assessment of the corpus luteum and plasma progesterone concentration in heifers. Theriogenology **33** (1990) 1269-1278.

KI ARNSTAD AND CLEERE, W.F., Enzyme-immunoassay for determination of progesterone in milk from cows. Journal of Reproduction and Fertility **62** (1981) 173-180.

KOTHARI, K., AND PILLAI, M.R.A., Preparation and Characterization of ^{125}I labeled progesterone derivatives for the development of a radioimmunoassay for progesterone. J. Radioanal. Nucl. Chem, Articles **177** (1994) 261-269.

KOTHARI, K. AND PILLAI, M.R.A., Direct radioimmunoassay of serum progesterone using heterologous bridge tracer and antibody. J. Radioanal. Nucl. Chem. **231** (1998) 77-82.

MARKUSFELD, (NIR) O., ADLER, H., NAHARI, N. AND KESSNER, D., Impaired summer fertility associated with lower milk progesterone levels in dairy cows. Isr. J. Vet. Med.**46 (**1991) 54-56.

MARKUSFELD, (NIR) O., NAHARI N AND ADLER, H. A., Routine 21 to 23/24 postservice milk progesterone monitoring for reducing the interinsemination interval in dairy cows. Isr. J. Vet. Med. **44** (1989) 111-119.

NAKAO, T., SUGIHASHI, A., SAGA, N., TSUNODA, N. AND KAWATA, K., Use of milk progesterone enzyme immunoassay for differential diagnosis of follicular cyst, luteal cyst, and cystic corpus luteum in cows. Am. J. Vet. Res. 44 (1983) 888-890.

NOSEIR, M.B., GYAWU, P. AND POPE, G.S., Progesterone concentration in defatted milk of dairy cows in early pregnancy. Br. Vet. J. **148** (1992) 45-53.

PIERRE RIOUX AND DENIS RAJOTTE, Progesterone in milk: a simple experiment illustrating the estrous cycle and enzyme immunoassay Advan. Physiol. Edu. **28** (2004) 64-67.

RAJAMAHANDREM, R., BURTON, B. AND SHELFORD, J., A field study on usefulness of milk progesterone determination to confirm estrus and pregnancy of dairy cows in Fraser Valley area of British Columbia. Can. Vet. J. **34** (1993) 349-352.

SREENAN, J.M. AND DISKIN, M.G., Early embryonic mortality in the cow, its relationship with progesterone concentration. Vet. Record. **112 (**1983) 517-521.

WIMPY, T.H., CHANG, C.H., ESTERGREEN, V.L. AND HILLERS, J.K., Milk progesterone enzyme immunoassay: Modification and a field trial for progesterone detection in dairy cows. J. Dairy Sci. **69** (1986) 1115-1121.

Aflatoxin B_1

SHARMA, A. AND M.R.A. PILLAI, Immunological Techniques for detection and analysis. Encyclopedia of Food Technologies, Academic Press, (1999) 1532-1539.

BHATTACHARYA, D., BHATTACHARYA, R. AND GAUR, T.K., A novel Signal Amplification technology for ELISA based on catalyzed reporter deposition. Demonstration of its applicability for measuring aflatoxin B_1. J. Immun. Methods. **230** (1999) 71-86.

CHU, F.S., STEPHEN HSIA, M. AND PIERA, S.S., Preparation and characterization of aflatoxin B_1-1-(O-carboxymethyl) oxime. J. Assoc. off. Anal.chem. **60 (**1977) 791-794

CHU, F.S. AND UENO, I., Production of antibody against aflatoxin B_1. J. Applied and Environmental Microbiology. **33** (1977) 1125-1128.

CHU, F.S., Chromatography of crude aflatoxins on adsorbsil-5. J. Assoc. Off. Anal. Chem. **54** (1971) 1304-1306.

CHU, F.S., Development and use of immunoassays in the detection of ecologically important mycotoxins. In the handbook of Applied Mycology, Deepak Bhatnagar, Eivind B. Lillehoj and Dilip K. Arora, Eds, Marcel Dekker, INC. New York.**5** (1992) 87.

CHU, F.S., Immunoassays for analysis of Mycotoxins. J. Food Protection. **47** (1984) 562-569.

DORNER, J.W., Mycotoxins in Food: Methods of Analysis In Handbook of Food Analysis Ed. Leo M.L. Nollet, Marcel Dekker, Inc. New York, **2** (1996).

GROOPMAN, J.D., CAIN, L.G. AND KENSLER, T.W., Aflatoxin exposure on Human population: measurements and relationship to cancer. CRC Crit. Rev.Toxicol. **19** (1988) 113-114.

KORDE, A., BANERJEE, S., PILLAI MRA, VENKATESH, M., Preparation and evaluation of ^{125}I–aflatoxin B_1 J. Radioanal. Nucl. Chem. **250** (2001) 1231-1237.

LANGONE, J.J. AND VAN VUNAKIS, H., Aflatoxin B_1 :specific antibodies and their use in radioimmunoassay. J. National Cancer Institute **56** (1976) 591-595.

LAU, H.P., GAUR, P.K. AND CHU, F.S., Preparation and characterization of aflatoxin B_{2a}–hemiglutarate and it's use for the production of antibody against aflatoxin B_1. J. Food Safety. **3** (1981) 1-13.

NEWSOME, W.H., Potential and Advantages of Immunochemical Methods for Analysis of Foods. J. Assoc. Off, Anal. Chem**. 69** (1986) 919-923.

PESTKA, J.J., GAUR, P.K. AND CHU, F.S., Quantitation of Aflatoxin B_1 and Aflatoxin B_1 antibody by an enzyme immunosorbent Microassay Appl. Enviro. **40** (1980) 1027-1031.

POHLAND, A.E., CUSHMAC, M.E. AND ANDRELLOS, P.J., Aflatoxin B_1-hemiacetal. J. Assoc. Office Anal. Chem. **51** (1968) 907-910

SINHA, K.K. AND BHATNAGAR, D.D., (Eds.) (1998) Mycotoxins in Agriculture and Food Safety, Marcel Dekker, Inc. New York.

Atrazine

BUSHWAY, R.J., PERKINS, B., SAVAGE, S.A., LEKOUSI, S.J. AND FERGUSON, B.S., Determination of Atrazine Residues in Water and Soil by Enzyme Immunoassay. Bull. Environ. Contam. Toricol**. 40 (**1988), 647-654.

CHLAEPPI, J.M., WERNER, F. AND RAMSTEINER, K., Hydroxy atrazine and Atrazine Determination in Soil and Water by Enzyme- Linked Immunosorbent assay using Specific Monoclonal Antibodies. J. Agric. Food. Chem. **37 (**1989) 1532-1538.

CHOI, M.J., JO, Y., CHOI, J., KANG, C.Y. AND HANG, C.T., Production and characterization of Monoclonal Antibodies specific to Atrazine Group Compounds; effects of Coating Ligand Structure on the variation of Sensitivity and Specificity. J. Immunoassay **20** (1999) 57-77.

DUNBAR, B.D., NISWENDER, G.D. AND HUDSON, J.M., Antibody for the Detection and Quantification of Atrazine. US. Patent **4,530** (1985) 786,.

GOODROW, M.H., HARRISON, R.O. AND HAMMOCK, B.D., Hapten Synthesis, Antibody Development And Competitive Inhibition Enzyme Immunoassay For S-Triazine Herbicides J. Agric Food Chem. **38** (1990) 990-996.

HARRISON, R.O., GOODROW, M.H. AND B.D. HAMMOCK, Competitive Inhibition ELISA for the S-Triazine herbicides: Assay Optimization and Antibody Characterization. J. Agric. Food Chem., **39** (1991) 122-128.

HAYES, M.C., JOURDAN, S.W. AND HERZOG, D.P., Determination of Atrazine in Water by Magnetic Particle Immunoassay: Collaborative Study. Journal of AOAC International **79** (1996) 529-537

HEINZOW, B.G.J. AND MCLEAN, A., Critical Evaluation of Current Concepts in Exposure Assessment. Clin. Chem. **40** (1994) 1368-1375.

HUBER, S.J., Improved Solid-Phase Enzyme Immunoassay System in the ppt Range for Atrazine in Fresh Water. Chemosphere **14**, (1985) 1795-1803.

JUNG, F., GEE, S.J., HARRISON, R.O., GOODROW, M.H., KARU, A.E., BRAUN, A.L., LI, Q.X. AND HAMMOCK, B.D., Use of Immunochemical Techniques for the Analysis of Pesticide Residues. Pestic. Sci., **26** (1989) 303-317.

SAMARAJEEVA, U., WEI, C.I., HUANG, T.S. AND MARSHAL, M.R., Application of radioimmunoassay in food industry, CRC Critical reviews in Food Sci. & Nut. **29 (**1991) 403-434.

SCHLAEPPI, J., FORY, W. AND RAMSTEINER, K., Hydroxyatrazine and Atrazine Determination in Soil and Water by Enzyme-Linked Immunosorbent Assay Using Specific Monoclonal Antibodies. J. Agric. Food Chem. **37** (1989) 1532-1538.

STEINHEIMER, T.R., HPLC Determination of Atrazine and Principal Degradations in Agricultural soils and Associated Surface and ground water. J. Agric Food Chem. **41** (1993) 588-595.

VAN EMON, J., SEIBER, J.N., HAMMOCK, B.D., Immunoassay Techniques for Pesticide Analysis. In Analytical Methods for Pesticides and Plant Growth Regulators: Advanced Analytical Techniques; Sharma, J., Ed.; Academic Press: New York, **XVII** (1989) 217-263**.**

WITTMAN, C. AND HOCK, B., Improved Enzyme Immunoassay for the Analysis of s-Triazines in Water Samples. Food Agric. Immunol. **4**, (1989), 211-224.

Leptin

IMAGAWA, K., MATSUMOTO, Y., NUMATA, Y., MORITA, A., KIKUOKA,S., TAMAKI, M., HIGASHIKUBO, C., TSUJI, T., SASAKURA, K., TERAOKA, H., MASUZAKI, H., HOSODA, K., OGAWA, Y. AND NAKAO, K., Development of a sensitive ELISA for human leptin, using monoclonal antibodies. Clin. Chem. **44** (1998) 2165-2171.

KADOKAWA, H., BLACHE, D., YAMADA, Y. AND G.B. MARTIN., Relationships between changes in plasma concentrations of leptin before and after parturition and the timing of first post-partum ovulation in high-producing Holstein dairy cows Reproduction, Fertility and Development **12** (2000) 405-411.

KAUTER, K., BALL, M., KEARNEY, P., TELLAM, R. AND MCFARLANE, J.R., Adrenaline, insulin and glucagon do not have acute effects on plasma leptin levels in sheep: development and characterization of an ovine leptin ELISA J. Endocrinology, **166** (2000) 127-135.

MAFFEI, M., HALAAS, J., RAVUSSIN, E., PRATLEY, R.E., LEE, G.H., ZHANG, Y., FEI, H., KIM, S., LALLONE, R., RANGANATHAN, S., KERN, P.A. AND FRIEDMAN, J.M., Leptin levels in human and rodent: measurement of plasma leptin and ob RNA in obese and weight-reduced subjects. Nature Med. **1** (1995) 1155-1161.

MEASUREMENT OF PROGESTERONE IN MILK BY RIA

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Abstract
The locally available progesterone RIA kit used for clinical diagnosis was modified to estimate the level of progesterone in milk. The modified assay has an overnight pre-incubation of antibody and standard or sample at 4°C followed by a 1h incubation with tracer at 37°C. The sensitivity of the modified assay was less than 0.015 ng/mL and the range of assay was 0.03–30 ng/mL. The level of progesterone in milk from 41 cows was assayed by the modified progesterone RIA kit. The accuracy of detection of pregnancy was 76.5% according to Eastman's cut off value.

1. INTRODUCTION

Reproduction is a major factor affecting the production and economic efficiency of dairy herds. Some dairy farmers increase the size of their herds, and thereby the milk production, by artificial insemination of the cow as soon as possible after calving. Ideally, the calving interval should average one year, but this can only be achieved if the success of pregnancy and detection of estrous rates are high and the interval between parturition and first service is less than 90 days. It is possible to determine whether a cow is pregnant or is in estrus based on the progesterone levels in milk. Progesterone is a di-keto steroid secreted chiefly by the corpus luteum and the placenta and is secreted into the blood and subsequently into the milk. Progesterone levels are lowest at the time of estrus and higher when the corpus luteum matures or the animal is in pregnancy.

The progesterone RIA kit produced in our laboratory has been used in clinical diagnosis for many years, but was not suitable for estimation of the level of progesterone in milk due to its lower sensitivity. We improved the sensitivity of the kit by optimizing the dilution of antibody and tracer. The modified kit could thus be used for the estimation of progesterone level in milk.

2. MATERIALS

Buffer A: 0.05M disodium hydrogen phosphate-citric acid buffer, pH 6.0.
Buffer B: Buffer A containing 0.01% 8-anilino-1-naphthalene-sulphonic acid (ANS)
Buffer C: Buffer A containing 0.1% gelatin
Buffer D: 0.05M, PO_4^{-3}, pH 7.4 with 0.1% gelatin, 0.1% NaN_3 and 1.9% EDTA.
Progesterone RIA kits were supplied by China Institute of Atomic Energy.

3. METHODS

3.1. Improvement of progesterone RIA kit

The antibody solution supplied in kit was diluted with buffer D to 1 to 24 fold, and tracer with buffer B to 1 to 4 fold. A series of progesterone standards viz. 0.03, 0.1, 0.3, 1, 3, 10, 30 ng/mL respectively was prepared in buffer C.

3.2. Milk samples processing

Milk was collected from dairy cows at the 12^{th} and 24^{th} day after artificial insemination. Samples were collected into 15mL plastic tubes and stored at –20ºC. Before assay, samples were thawed and incubated for 60 min in water bath at 25ºC, and diluted 1 to10 with saline.

3.3. Assay procedure

The assay was designed as an imbalance competitive inhibition assay. 100μL of progesterone antibody was mixed with 100μL of standards or milk samples and incubated overnight at 4ºC. 200μL tracer was dispensed into each tube, mixed and incubated for 1h at 37ºC. 500μL of the separating reagent was added to each tube except total "T", mixed and separated by centrifugation at 3500g for 15min. The supernatant was discarded and the counts were measured in a γ counter.

4. RESULTS AND DISCUSSION

4.1. Improvement of progesterone RIA kit

We improved the sensitivity of the assay by changing the dilution of antibody working solution and tracer supplied in progesterone RIA kit for human serum. If the dilution of antibody working solution alone was changed, the range of standard curve was 0.2–30 ng/mL as shown in Figure 1. If the dilution of antibody and tracer were changed, the range of standard curve was 0.03-30 ng/mL as shown in Figure 2. The sensitivity of assay is less than 0.015 ng/mL.

4.2. Inspection result

The levels of progesterone in milk from 41 cows were assayed by the improved progesterone RIA kit and the results are shown in Table I and II. According to Eastman's research [1–3], the level of progesterone in whole milk of dairy cattle on Day 24 is the main index point to classify cows as pregnant and non-pregnant. Cows with levels greater than 5 ng/mL are pregnant and less than 5 ng/mL are non-pregnant. From the above results, the accuracy of prediction of pregnancy was 76.5% and that of non-pregnancy was 85.7%.

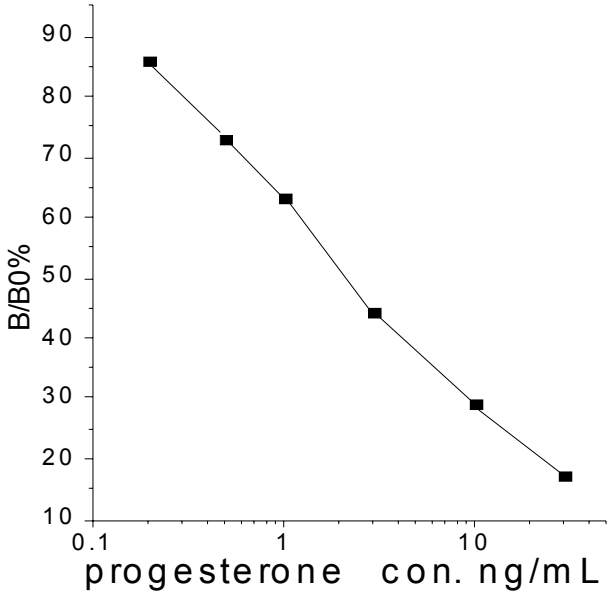

FIG.1. Progesterone RIA standard curve
(Changing the dilution of antibody work solution only)

TABLE I. PROGESTERONE LEVEL IN MILK IN RELATION TO PREGNANCY

Cow Number	12^{th} Day (ng/mL)	24^{th} Day (ng/mL)	Possibly pregnant	Pregnant
00090	1.8	1.8		
00112	2.6	1.9		
0021	2.4	4.2		
0062	2.7	2.4		
01009	1.6	1.7		
01032	3.3	6.9	☆	★
01057	0.4	5.1	☆	
01070	5.9	5.3	☆	
01088	3.5	4.3		
01103	2	2.4		
01129	4.5	0.5		
01144	2.6	1.7		
01145	1.8	5.6	☆	
01166	1.8	2.2		
01171	7.7	8.3	☆	★
01185	3.5	0.7		
01188	5.5	0.3		
01196	4.8	6.2	☆	★
01209	3.2	7.5	☆	★
01217	5	0.1	☆	
01218	5.6	11.4	☆	★
01220	7.4	8.6	☆	★
02011	1.9	11.5	☆	★
02069	2.8	5		
20144	3.6	10.4	☆	★
7003	3.4	0.9		
7032	3.1	4.4		
8092	2.4	6.1	☆	★
9095	1.5	3.4		

TABLE II. PROGESTERONE LEVEL IN MILK IN RELATION TO PREGNANCY

Cow Number	12th Day (ng/mL)	24th Day (ng/mL)	Possibly pregnant	Pregnant
94045	1.3	1.7		
96008	1.7	0.3		
97105	7.3	6.6	☆	★
97302	2.6	6.3	☆	★
98146	2.9	4.9		
98210	11.7	8.8	☆	★
99095	5.9	1.7		
99127	5.6	4.6		
99135	2.9	3.9		
99161	1.6	0.4		
99193	2.6	0.6		
99198	2.6	8.6	☆	★
	Total		17#	13#
Accuracy of pregnancy （13/17） x 100 =76.5%				

Considering the samples from Table I and II

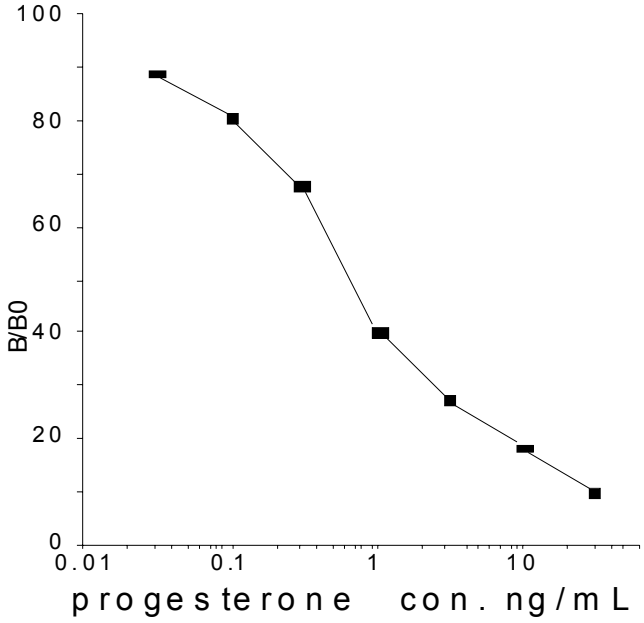

FIG. 2. Progesterone RIA standard curve
(Changing the dilution of antibody and tracer)

5. CONCLUSION

The progesterone RIA kit used for clinical diagnosis was improved in order to detect the level of progesterone in milk. The levels of progesterone in milk from 41 cows were assayed by improved progesterone RIA kit. The accuracy of detection of pregnancy is 76.5% according to Eastman's cut off value.

REFERENCES

[1] LAING, J.A., EASTMAN, S.A.K. AND BOUTFLOWER, J.C., The Use of Progesterone Concentrations in Milk and Plasma for Pregnancy Diagnosis in Cattle Br. Vet. J. **135** (1979) 204-209.

[2] EASTMAN, S.A.K., Methods of Improving the Accuracy of Positive Results from Milk Progesterone Pregnancy Test. Br. Vet. J. **135** (1979) 489-490.

[3] LAING, J.A., GIBBS, H.A. AND EASTMAN, S.A.K., A Herb Test for Pregnancy in Cattle Based on Progesterone Levels in milk Br. Vet. J. **136** (1980) 413-415.

DEVELOPMENT OF A DIRECT RADIOIMMUNOASSAY FOR PROGESTERONE IN WHOLE MILK

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Abstract
A system for the measurement of progesterone in whole milk by radioimmunoassay has been developed and validated. The assay uses a radioiodinated tracer purified by HPLC and standards prepared in fat-free milk. The detection limit of the assay is 1.2 nmol/L, calculated from the maximum binding minus two standard deviations and the precision was satisfactory (<12% CV). The recovery obtained with two different whole milk samples was 96–97% and the parallelism test indicated good linearity. The assay is simple, rapid and enables the measurement of progesterone in whole milk without sample dilution. The cyclic changes of this hormone that reflect the ovarian activity in the animals can be determined using the developed assay.

1. INTRODUCTION

Progesterone is a hormone secreted by the luteal tissue of the ovaries and its levels in serum and milk increase or decrease in response to the maturation or regression of this ovarian structure. A functioning corpus luteum and active progesterone secretion are associated with gestation, the period of cycle between heats, pathological conditions that inhibit the luteal regression, as well as inflammatory processes of the uterus. Absence of corpus luteum and baseline progesterone concentrations are found in estrus, purpural acyclicity, and non-luteal pathological conditions such as anoestrus and cystic ovarian disease. It is important to understand that the level of progesterone in milk and serum increases slowly after ovulation. Peak levels of progesterone are found between the 10^{th} and 17^{th} days of the cow's reproductive cycle. In the non-pregnant cow, levels of progesterone will begin to diminish fairly sharply from day 18 or 19. During this time, estrogen levels begin to increase as the cows begin to cycle through another estrus period. Measurement of total progesterone in whole milk is an important tool for assessing the ovarian function and determining fertility parameters such as pregnancy diagnosis. The main objective of this work was to develop a simple, sensitive and radioimmunoassay (RIA) method for the direct determination of progesterone concentration in whole milk.

2. MATERIALS

Progesterone, succinic acid anhydride, tyrosine methyl ester hydrochloride, bovine serum albumin (BSA), chloramine-T (CL-T), sodium metabisulphite (MBS) were purchased from Sigma Chem Co, USA. Carrier free ^{125}I as sodium Iodide (specific activity 15–17 mCi/μg, radioactive concentration 100 mCi/mL) was obtained from IZOTOP, Hungary.. The primary biotinylated polyclonal antibody and magnetic immunosorbent (MIS) were obtained from IZOTOP-Hungary. The milk samples used were received from Animal Improvement Center (CIMA). The commercial kit, RIA Progesterone Veterinary SPECTRIA from ORION Diagnostica, were used for comparison. All the other reagents were of AR grade. HPLC column C_{18}, 5μm, from Knauer was used for purifying the radiolabeled progesterone. Gamma counter, Berthold, from Germany, was used for identifying the radioactive zones

3. METHODS

3.1. Progesterone-11αhemisuccinate (HS)

300mg 11αOH-progesterone and 500mg succinic anhydride were dissolved in 5mL pyridine under heating and kept at room temperature for 17h. The solvent was removed by distillation under vacuum, the residue obtained was mixed with 15mL of hydrochloric acid (5%) and cooled to 0°C. The mixture was extracted with 30mL of ethyl acetate and the organic extract was washed twice with distilled water. The compound was extracted with diethyl ether. Hydrochloric acid (5%), pH 3–4 was added, and extracted with 30mL of ether, the ether layer was washed with water and the solvent was removed

under vacuum. The residue was dissolved in benzene and a few drops of heptane were added. The product crystallized when cooled to 4–8°C. The crystals of progesterone-11α-hemisuccinate (progesterone-11α-HS) were filtered under vacuum.

3.2. Progesterone-11αHS-TME

15mg of progesterone-11αHS was dissolved in 1mL of dioxane alongwith 12µL of tri-n-butylamine. To this was added, 5µL of isobutyl chloroformate under stirring and cooled to 8°C, and the mixture was kept in a refrigerator for 45 min. 7.8mg of tyrosine methyl ester was dissolved in 350µL mixture of dioxane/water (3:5) with two drops of NaOH (0.2M) The mixture was cooled to 4°C and was added to the activated progesterone-11αHS. The solution was kept in a refrigerator for 12h. The product was extracted with 10mL of dichloromethane and the organic layer washed with distilled water. The solvent was removed by distillation under vacuum. 12mg of progesterone-11αHS-TME was obtained.

3.3. Radioiodination of progesterone-11αHS-TME

20µL (2µg) of progesterone-HS-TME, 1mCi (37MBq) of ^{125}I and 10µL of 0.25M phosphate buffer, pH 7.4 were added in an eppendorf tube. 20µL (2 mg/mL) of chloramine T dissolved in 0.05M phosphate buffer, pH 7.4 was added and the reaction was stopped after 45 sec by the addition of 20µL (2 mg/mL) sodium metabisulphite in 0.05M phosphate buffer, pH 7.4. The crude reaction mixture was purified by high performance liquid chromatography (HPLC) column (nucleosil RP-18) at room temperature using methanol: water (70:30) as mobile phase. The flow rate was 0.8 mL/min. The stability of the tracer was studied for 18 weeks by estimating the non-specific binding as well as the binding of the tracer with the antibody.

3.4. Standards, controls and unknown milk samples

Working standards covering the concentration range from 0 to 300nmol/L were prepared by appropriate dilution with zero fat-free milk. Control samples were also prepared using fat-free zero milk. The control samples were dispensed in 500µL and kept at –20°C.

3.5. Assay procedure

Various parameters were optimized to arrive at a suitable assay system. In the optimised assay, 50µL of standard, control and unknown samples, 100µL of tracer and 100µL of biotinylated antiserum were added into the tubes (12 x 75 mm), mixed and incubated at room temperature for 2h. 500µL of magnetic immunosorbent (MIS) was added and incubated for 15 min. The rack was placed on the magnetic separator base for 5 min and the MIS particles were allowed to settle. Placing the rack on the separator, the tubes were decanted and measured for radioactivity.

4. RESULTS AND DISCUSSION

4.1. ^{125}I- progesterone-hemisuccinate derivative (Progesterone-11αHS-TME-^{125}I)

The iodinated conjugate progesterone-11α-HS-TME-^{125}I was purified by HPLC and the radioactive profile obtained is shown in Figure 1. The peak observed at a retention time (T_R) of 16 min (tube 23) was attributed to progesterone-11α-HS-TME-^{125}I (peak 2) since Na^{125}I and ^{125}I-TME were eluted just after the solvent (peak 1). As observed, progesterone-11α-HS-^{125}I-TME is well separated from these impurities as well as from other unknown radioactive contaminants (peak 3) as shown in the radioactive profile.

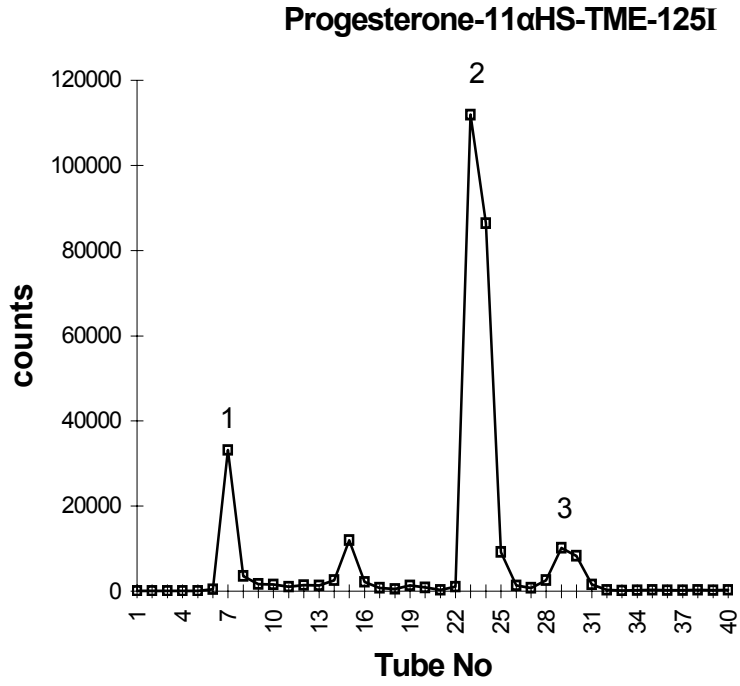

FIG. 1. HPLC profiles obtained for progesterone-11αHS-TME-^{125}I, methanol: water (70:30) as mobile phase, flow rate 0.8 mL/min, room temperature.

TABLE I. STABILITY OF ^{125}I- PROGESTERONE (READY-TO-USE AT 2–8°C)

Parameter	Time of storage (weeks)						
		0	4	8	12	16	18
NSB (%)	≤ 5	2.7	3.1	3.9	4.2	4.8	5.1
B_0 (%)	30–60	45.3	40.7	39.4	42.6	40	38.9
Control low (nmol/L)	5.4 (4.62–6.18)	5.3	4.9	6.0	5.9	4.9	4.9
Control medium (nmol/L)	86.5 (71–102)	80.7	79.7	95.1	90.5	81.2	76.6
Control high (nmol/L)	156 (126 –186)	158.8	144.9	171.6	155	145.8	140.6

Stability of progesterone tracer

The stability of the tracer was evaluated for 18 weeks by estimating the non-specific binding as well as the binding of the tracer. It was observed that there was a slight increase in non-specific binding (3% to 5%) while the immunoreactivity remained unchanged upto a period of four months, indicating the high stability of the product (Table 1).

4.2. Determination of optimum assay conditions

Effect of shaking and incubation time on % binding

The effect of shaking and incubation time on the percent binding of zero progesterone standard was studied by carrying out incubations for 1, 2, 3 and 4h with and without shaking at room temperature. The results are shown in Figure 2 and it was observed that the optimum time for the assay was two hours without shaking.

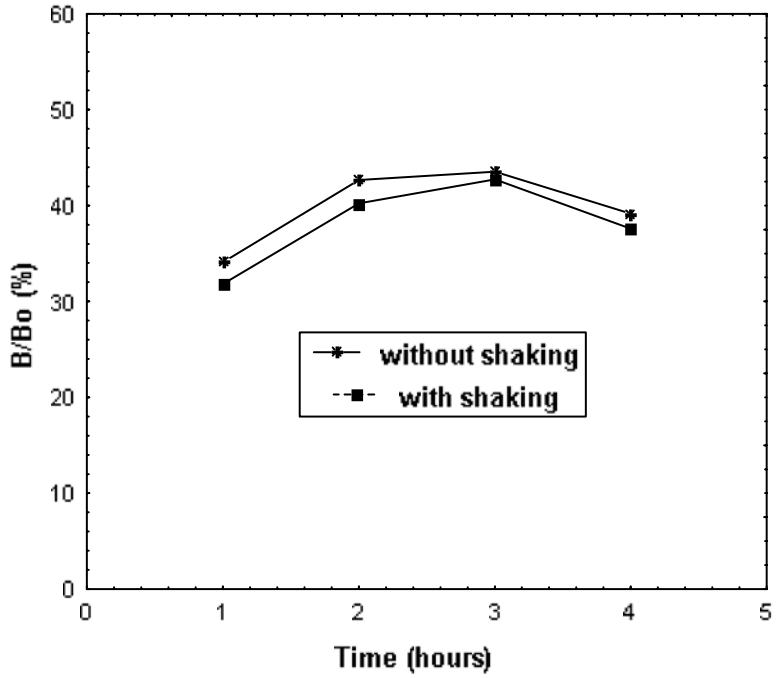

FIG. 2. Effect of shaking and incubation time at room temperature on % binding at zero concentration of progesterone.

4.3. Analytical characteristics of RIA kit for progesterone in whole milk

Precision (imprecision)

Milk controls of various concentrations (low, medium and high) were assayed to determine intra-assay variation and inter-assay variation. The coefficients of variation obtained are presented in Tables II and III.

Sensitivity

The sensitivity of this assay as determined from the dose at 2SD away from zero standard was 1.2 nmol/L and has been obtained by assaying 15 replicates of the zero standard. The sensitivity of commercial kit (ORION Diagnostica) is 1.0 nmol/L.

TABLE II. INTRA-ASSAY PRECISION

Control samples (nmol/L)	Number of replicates	Mean value	CV (%)
Control low 5.4 (4.62 – 6.18)	10	4.9	10.8
Control medium 86.5 (71 – 102)	10	81.3	6.8
Control high 156 (126 –186)	10	157.9	6.1

TABLE III. INTER-ASSAY PRECISION

Control Samples (nmol/L)	Number of replicates	Mean value	CV (%)
Control low 5.4 (4.62 – 6.18)	10	5.6	11.6
Control medium 86.5 (71 – 102)	10	85.9	7.1
Control high 156 (126 –186)	10	157.9	6.6

TABLE IV. RECOVERY TEST

Sample + Standard (1:1)	Expected (nmol/L)	Observed (nmol/L)	% Recovery
Sample A	—	5.8	—
A + 0	2.9	2.40	82.9
A + 3	4.4	4.22	95.9
A + 15	10.4	9.80	94.2
A + 50	27.9	26.11	93.6
A + 150	77.9	68.88	89.7
A + 300	152.9	144.49	94.5
Sample B	—	17.6	—
B + 0	8.8	9.74	110.7
B + 3	10.3	9.83	95.4
B + 15	16.3	15.42	94.6
B + 50	33.8	31.50	93.2
B + 150	83.8	88.07	105.1
B + 300	158.8	164.52	103.6
	% Recovery		96.1

TABLE V. PARALLELISM TEST

Samples	Dilution	Expected (nmol/L)	Observed (nmol/L)	% Recovery
Sample 1	Undiluted	—	111.8	—
	2x	55.90	56.46	101
	4x	27.95	26.27	94
	8x	13.98	13.56	97
	16	6.99	6.64	95
Sample 2	Undiluted	—	95.7	—
	2x	47.85	50.24	105
	4x	23.93	23.45	98
	8x	11.96	11.01	92
	16	5.98	5.74	96
Sample 3	Undiluted	—	70.4	—
	2x	35.20	35.90	102
	4x	17.60	16.19	92
	8x	8.80	7.74	88
	16	4.40	3.92	89

Recovery

Two samples (sample A and sample B) of whole milk progesterone concentrations were mixed in equivalent volumes with each of the progesterone standards. The obtained results and the recovery percent are shown in the Table IV. Recovery estimated by analyzing samples in the presence of different concentrations ranged from 82 to 110 %. The mean recovery for added progesterone was 96.1 %.

Parallelism testing

Parallelism test was carried out by diluting three milk samples containing high concentration of progesterone with milk of zero standard. The results of parallelism test are shown in the Table V. The results suggest that the matrix for standards and samples are identical. A correlation between expected values and observed values for three cow whole milk samples is showed in the Figure 3

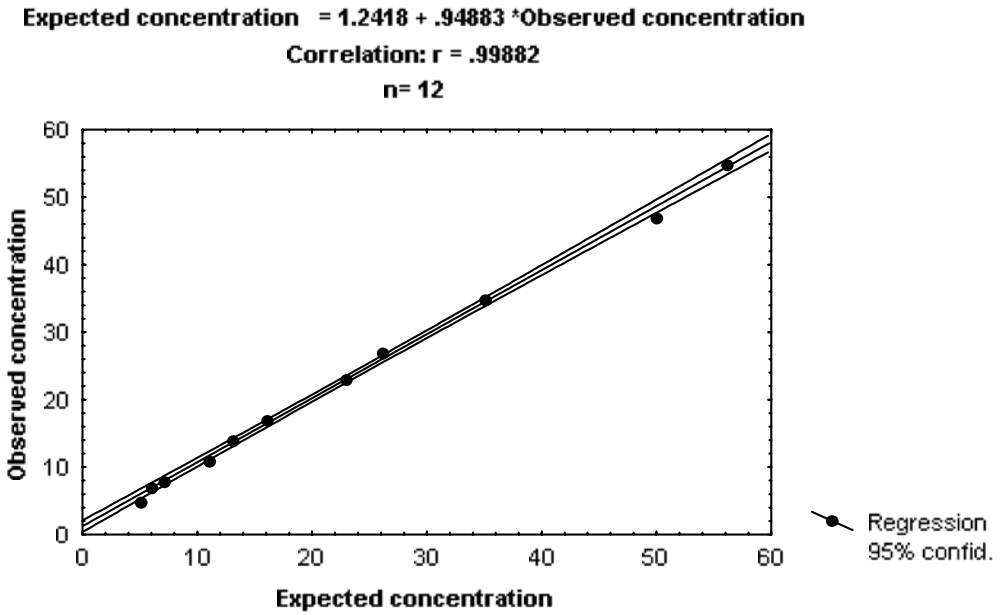

FIG. 3. Correlation of the expected progesterone values with observed progesterone values for three whole milk samples.

Comparison of CENTIS Progesterone kit with the ORION Diagnostica commercial kit

Progesterone levels in 85 whole milk progesterone samples from pregnant and non-pregnant cows were determined using in-house CENTIS progesterone kit and ORION Diagnostica kits. The results of regression analysis is showed in the Figure 4. A good correlation of 0.993 was obtained with the present method and commercial kit (ORION Diagnostica = -0,1270 + 1,072X CENTIS Progesterone, at n = 85). A comparison between CENTIS and ORION was carried out in order to determine whether there are statistically significant differences between the two methods.

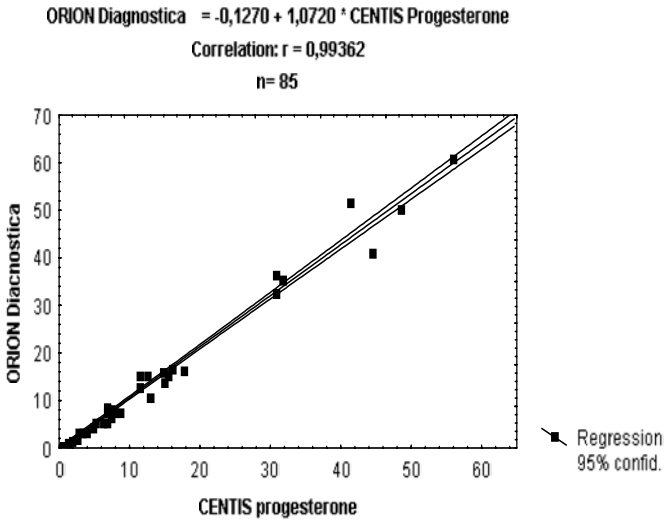

FIG.4. Correlation of progesterone values in whole milk samples using in-house CENTIS progesterone with ORION Diagnostica.

The analysis show there was no statistically significant differences in the values obtained by the two methods (appendix).

5. CONCLUSION

The preparation of a progesterone conjugate for radiolabeling has the advantage of direct radiolabeling as well as the storage of the cold progesterone conjugate for a longer time. The use of HPLC for progesterone-11α–HS-TME-^{125}I purification allows a rapid preparation of the tracer and offers the advantages of speed, reliability and reproducibility.

The RIA kit that had the required sensitivity, accuracy, and precision for direct determination of progesterone in whole milk was developed. This kit could be used to determine the cyclic changes of this hormone that reflect the ovarian activity in the animals. The results obtained from the optimized assay showed good correlation with the kit obtained commercially.

BIBLIOGRAPHY

THORELL, J.I., EKMAN, R., MALMQUIST, Technical aspects of the production and application of iodinated steroids for radioimmunoassay, Proceedings of a Symposium on Radioimmunoassay and Related Procedures in Medicine. IAEA,Vienna (1982) 147-160.

HUNTER, W.M., NARS, P. AND RUTHERFORD, F., Preparation and behaviour of ^{125}I-labeled radioligands for phenolic and neutral steroids. Proceedings: Steroid Immunoassay 5th Tenovus Workshop, April 1974, Alpha Omega Alpha, Cardiff (1975) 141-152.

LEE, J.M., KIM, S.G., JEONG, S.G. AND JUNG, J.K., Studies on the early pregnancy determination in cows by using the enzyme-immunoassay and radio-immunoassay in milk, Australian-Asian J. of Animal Sciences**. 9** (1996) 299-302.

HRUSKA, K. Milk progesterone determination in dairy cows, Reprod Dom Anim. **31** (1996) 483-485.

ABRAHAM, G.E. Solid phase radioimmunoassay for estradiol-17β, J. Clin Endocrinol Metab. **29** (1969) 886-870.

KOTHARI, K., LAL, R. AND PILLAI, M.R.A., Development of a direct radioimmunoassay for serum progesterone. J. Radioanal. Nucl. Chem. Articles. **196** (1995) 331-338.

VON STETTEN AND SCHLETT, R., Purification of ^{125}I-labeled compounds by high-performance liquid chromatography with on line detection. J. Chromatography, **254** (1983) 229-235.

Comparison of Standard Deviations

	CENTIS progesterone	ORION
Standard deviation	11.5992	12.5139
Variance	134.541	156.598
Df	84	84

Ratio of Variances = 0.859147

95.0% Confidence Intervals
Standard deviation of CENTIS progesterone: [10.0793,13.663]
Standard deviation of ORION: [10.8742,14.7405]
Ratio of Variances: [0.558539,1.32154]

F-test to Compare Standard Deviations

Null hypothesis: sigma1 = sigma2
Alt. hypothesis: sigma1 NE sigma2
F = 0.859147 P-value = 0.488117

The StatAdvisor

This option runs an F-test to compare the variances of the two samples. It also constructs confidence intervals or bounds for each standard deviation and for the ratio of the variances. Of particular interest is the confidence interval for the ratio of the variances, which extends from 0.558539 to 1.32154. Since the interval contains the value 1.0, there is not a statistically significant difference between the standard deviations of the two samples at the 95.0% confidence level.

DEVELOPMENT OF RADIOIMMUNOASSAY SYSTEM FOR THE DETERMINATION OF PROGESTERONE IN COW MILK

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Abstract

A reliable solid-phase radioimmunoassay for the quantitative determination of progesterone in cow milk was developed using two different assay formats. The first format was based on immobilization of a progesterone-protein conjugate on a solid-support, which competes with the progesterone present in the sample for binding with a progesterone specific antibody. The binding on the solid support was detected using a ^{125}I labeled mouse monoclonal anti-progesterone antibody. The second approach was based on immobilization of second antibody on a solid-support. The progesterone specific antibody was immunoadsorbed to the second antibody. The progesterone in the sample or the standard and the ^{125}I-labeled progesterone-protein conjugate competed for the specific antibody immobilized on the solid support. The second assay format provided better analytical characteristics. The assay developed had a detection limit of 0.1 ng/mL and a dynamic range of 0.2–40 ng/mL. The average percent recovery was 93.3±8.5% and there was a good correlation between the expected and the determined values in the linearity dilution experiment (r = 0.975). The progesterone values of full fat milk samples determined with the developed radioimmunoassay correlated well with those determined by the respective enzyme immunoassay for progesterone.

1. INTRODUCTION

Progesterone is a steroid hormone produced mainly by corpus luteum. Progesterone plays an important role during menstrual cycles in controlling associated organs, preparing the endometrium for implantation of fertilized ovum, supporting pregnancy and preparing mammary glands for milk secretion [1]. Progesterone level in blood is minimum just before and during ovulation and become maximal on the fifteenth day. If fertilization and implantation does not occur, the corpus luteum degenerates, the concentration of progesterone returns to its initial level and a new ovarian cycle begins. The progesterone concentration in blood correlates closely with the corresponding concentration of the hormone in the milk. In absolute values, progesterone concentration in milk is higher than that in serum due to its solubility in milk fat. The changes of the hormone levels observed in the different phases of the cycle follow the same pattern in milk and in serum. Accordingly, the determination of progesterone concentration in milk samples instead of that in serum samples can be used in evaluating the reproduction condition of dairy animals. The determination of progesterone in milk has the advantage of simplicity of the sample collection and the direct use of milk sample for estimation unlike blood samples, which require the isolation of the serum before estimation.

Several immunochemical methods have been developed based on isotope, enzyme and fluorescent markers for the determination of progesterone levels in serum and milk [2–6]. The fact that progesterone circulates in blood mainly bound to proteins, such as corticosteroid binding globulin (CBG), sex hormone binding globulin (SHBG) and albumin with only 2–10% of the hormone circulating as free hormone causes a major problem in the development of immunoassays for determination of progesterone. Accordingly, the determination of total blood progesterone demands the release of the hormone from these proteins using appropriate reagents. In the case of milk samples, the trapping of progesterone in the micelles formed by the milk fat had to be overcome.

In this context, the main objective of our work was to develop a simple, rapid and reliable immunoassay for the determination of progesterone in full milk samples. Two different assay formats were evaluated. The first involved the use of solid-phase immobilized progesterone-protein conjugate and labeled antibody, while the second involved immobilized second antibody and labeled progesterone-protein conjugate. Both assay formats were used to develop enzyme immunoassays as well as radioimmunoassays. The enzyme immunoassays were developed in order to evaluate the reagents required for carrying out the assay without the limitations arising mainly from the short life of

radiolabels. Several parameters had to be optimized when the enzyme label was replaced with a radioactive label in order to establish a reproducible and sensitive radioimmunoassay. Other issues including tracer stability were also addressed.

2. MATERIALS

4-Pregnen-3,20-dione (progesterone) and 4-pregnen-11α-ol-3,20-dione hemisuccinate (progesterone-11-hemisuccinate) were obtained from Steraloids Inc, Newport, RI, USA. The mouse monoclonal anti-progesterone antibody (A25010045P), developed using progesterone-11-BSA as immunogen, and rabbit anti-progesterone antiserum (D2436), developed using progesterone-11-HSA as immunogen, were obtained from BiosPacific, Emeryville, CA, USA. The goat anti-mouse IgG antibody (41-GM25) used for coating was procured from Fitzgerald Industries International Inc., Concord, MA, USA. Bovine γ-globulins, bovine serum albumin, horseradish peroxidase labeled goat anti-mouse IgG antibody (A4416), N,N'-carbonyldiimidazole (CDI), 2,2' azino-biz (3ethylbenzylthiazoline-6-sulfonic acid (ABTS) and (17α)-Pregna-2,4-dien-20-yno[2,3-d]isoxazol-17-ol (danazol) were purchased from Sigma Chemical Co, St Louis, MA, USA. Sulfosuccinimidyl 6-(biotinamido)hexanoate (sulfo-NHS-LC-biotin) was procured from Pierce Chemical Co, Rockford, USA. Carrier-free $Na^{125}I$ (specific radioactivity 17 mCi/μg, radiochemical purity 99.9%, iodate < 2%) obtained from Nordion Europe S.A. (Belgium) was used for radiolabeling. Sephadex was obtained from Pharmacia, Sweden. All other reagents were from Merck, Darmstadt, Germany, except as otherwise indicated. Full fat milk obtained from animals in estrus was kindly donated by Dr. Kaloyiannis, Agricultural University of Athens. Microtitration wells (F8, flat bottom, MaxiSorp) were purchased from NUNC A/S, Roskilde, Denmark. Polystyrene tubes (75 x 12 mm) for RIA were obtained from VIVE Co. Athens, Greece. ELISA plate reader was procured from Labsystems, Finland. A 12-well gamma-counter was purchased from Berthold Technologies GmbH & Co, Germany.

3. METHODS

3.1. Preparation of progesterone bovine γ-globulin conjugate (BgG-Prog)

Progesterone-11-hemisuccinate derivative was dissolved in anhydrous dimethylsulfoxide (DMSO) to a final concentration of 16.3 μmol/mL. To 1.4mL of this solution, 0.9mL of a 30.8 μmol/mL carbonyl diimidazole in anhydrous DMSO was added. The mixture was incubated for 15min at room temperature and 0.15mL were transferred in another glass test tube containing 1.0mL of 0.6 μmol/mL bovine γ-globulin in 0.2M Na_2CO_3, pH 9.1. The reaction mixture was incubated overnight at room temperature and transferred to a dialysis tube. The mixture was dialyzed against 0.005 M 2-(N-morpholino)ethanosulfonic acid (MES) buffer, pH 6.0, containing NaCl with several changes of buffer.

3.2. Biotinylation of progesterone bovine γ-globulin conjugate

Biotinylation of the progesterone bovine γ-globulins conjugate was performed following a protocol proposed by S.E. Kakabakos *et al* [4]. In brief, 1.5mL of the conjugate solution (1 mg/mL) was mixed with 0.5mL of 1M carbonate buffer, pH 9.1. To this solution, 30μL of a 100 mg/mL sulfo-NHS-LC-biotin in DMSO was added and the reaction was allowed to proceed for 2h at room temperature. The reaction mixture was transferred to a dialysis bag and dialyzed against 0.05M $NaHCO_3$ buffer, pH 8, containing 9 g/L NaCl and 0.5 g/L NaN_3 with several changes of buffer. After dialysis, the content of the dialysis bag was recovered and the conjugate was stored at 4°C until use.

3.3. Preparation of progesterone standards

Standard solutions of progesterone were prepared in progesterone-free serum, in 0.01M phosphate buffer, pH 7.0, containing 9 g/L NaCl, 0.1 g/L NaN_3, 5 g/L BSA and 12.5 g/L casein, and in full fat milk collected from animals in estrus. Initially, a stock solution of progesterone at a concentration of 4mg/mL was prepared in absolute ethanol. The stock solution was further diluted to 0.4mg/mL using

50% ethanol in water and further to 0.04 mg/mL using 10% ethanol solution. Progesterone solutions with concentration of 4 μg/mL were prepared in all the three matrices after appropriate dilution. These solutions remained overnight at 4ºC and were used for the preparation of standard solutions. The final concentrations of the standards were 0.1, 0.5, 2, 5, 10, 20 and 40 ng/mL.

3.4. Enzyme immunoassay for progesterone

3.4.1. Enzyme immunoassay for progesterone in wells coated with progesterone bovine γ-globulin-conjugate

Microtitration wells were coated overnight at RT with 100μL of a 2 μg/mL progesterone bovine γ-globulin conjugate in 0.05M carbonate buffer, pH 9.2. The wells were washed twice with 300μL of 0.05M phosphate buffer, pH 7.4, containing 0.9% NaCl (wash buffer) and 300μL of blocking solution (10g/L BSA in 0.1M $NaHCO_3$) were added per well. After 2h incubation at RT, the wells were washed as previously. To each well, 50μL of progesterone standards and 100μL of a 50 ng/mL mouse monoclonal anti-progesterone antibody solution in 0.1M phosphate buffer, pH 7.0, with 0.1% gelatin, 0.9% NaCl, 0.1% (v/v) Tween 20 and 0.05% NaN_3 were added. The wells were incubated for 1h at RT under shaking and washed four times with 300μL of washing buffer containing 0.05% (v/v) Tween 20. To each well, 100μL of a peroxidase labeled anti-mouse IgG diluted 1 to 1000 fold in 0.15M Tris/HCl buffer, pH 8.25, with 0.5% BSA, 0.05% bovine IgG and 0.05% NaN_3 were added. The wells were incubated for 1h at RT under shaking and washed four times as before. To each well, 100μL of peroxidase substrate (1.9 mM ABTS/0.03 % H_2O_2) (2,2' azino-biz (3ethylbenzylthiazoline-6-sulfonic acid) was added and the wells were incubated in the dark under shaking. After 30 min, the absorbance at 405 nm was measured using the Multiscan RC ELISA plate reader.

3.4.2. Enzyme immunoassay for progesterone in wells coated with anti-mouse IgG antibody

Microtitration wells were coated overnight at RT with 100μL of a 5 μg/mL goat anti-mouse IgG solution in 0.05M carbonate buffer, pH 9.2. The wells were washed twice with wash buffer and 300μL of 1% BSA in 0.1M $NaHCO_3$ was added as blocking solution to each well. The wells were incubated for 2h at RT and were washed as before. To each well, 20μL of standard, 50μL of a 100 ng/mL biotinylated progesterone bovine γ-globulin conjugate in 0.05M Tris-HCl, pH 8.25, containing 0.1% gelatin, 0.9% NaCl, 0.1% (v/v) Tween 20 and 0.05% NaN_3, and 50μL of a 40 ng/mL mouse monoclonal anti-progesterone antibody in the same buffer were added. The wells were incubated for 1h at room temperature under shaking and were washed four times with wash buffer containing 0.05% (v/v) Tween 20. To each well, 100μL of a 250 ng/mL streptavidin-HRP solution in 0.05M phosphate buffer, pH 6.5, containing 0.1% BSA, 0.9% NaCl and 0.02% thiomersal, were added and incubated under shaking for 30 min at RT. After washing the wells four times, 100μL of peroxidase substrate (1.9 mM ABTS / 0.03% H_2O_2) was added per well and incubated in the dark under shaking. After 30 min, the absorbance at 405nm was measured using the Multiscan RC ELISA plate reader.

3.5. Radioimmunoassay for progesterone

3.5.1. Radioiodination of progesterone bovine γ-globulin conjugate

The radioiodination of progesterone bovine γ-globulin conjugate was performed by a modification of the method of Greenwood *et al*. [7]. Briefly, 20μL of a 1 mg/mL conjugate in 0.25M phosphate buffer, pH 7.4, (radioiodination buffer) was mixed with $Na^{125}I$ (0.5mCi) and 5μL of 1.775M chloramine-T solution in the same buffer. After 30s, the reaction was terminated by adding 10μL of a 0.14 % (v/v) mercaptoethanol solution in radioiodination buffer. The labeled protein was separated from free iodide on a 30x1cm Sephadex G-75 column eluted with 0.1M phosphate buffer, pH 7.4, containing 1 mg/mL of BSA, and 0.5 mg/mL of NaN_3. The radiolabeling yield was ~65%. Fractions with high radioactivity were pooled and diluted with 0.1M PBS, pH 7.4, containing 0.1% gelatin, 0.9% NaCl, 0.05% NaN_3 and 0.05% (v/v) Tween 20. Radioactivity measurements were performed in a 12-well gamma-counter.

The fractions collected from the Sephadex G-75 column were diluted with 0.33M phosphate buffer, pH 7.4, containing 0.33% BSA and 0.16 % NaN_3. The tracer was aliquoted as 3mL per vial, lyophilized and stored at 4ºC. The tracer was reconstituted with 10mL of distilled water containing 0.05% (v/v) Tween 20 before use. 100µL of this solution provided ~100,000 cpm.

2.3.3.5.1. Assay procedure

Polystyrene tubes were coated with anti-mouse IgG by incubating overnight at RT with 500µL of a 5 µg/mL anti-mouse IgG (Fc specific) in 0.05M carbonate buffer, pH 9.2. The tubes were washed three times with 1.0mL of 0.01M PBS buffer, pH 7.4, and blocked by incubation with 1.0mL of 0.1M $NaHCO_3$ buffer, pH 8.5, containing 1% BSA and 0.05% NaN_3, for 2h at RT. The tubes were washed as previously and 100µL of a 50 ng/mL mouse monoclonal anti-progesterone antibody in 0.1M PBS buffer, pH 7.4, containing 0.1% gelatin, 0.9% NaCl, 0.05% NaN_3 and 0.05% (v/v) Tween 20, 300µL of standard solution in full fat progesterone-free milk or sample and 100µL of tracer were added per tube and were incubated for 2h at RT under shaking. The tubes were aspirated and washed with 0.01 M PBS, pH 7.4, containing 0.05% Tween 20. The radioactivity bound to the solid phase was measured in the gamma-counter.

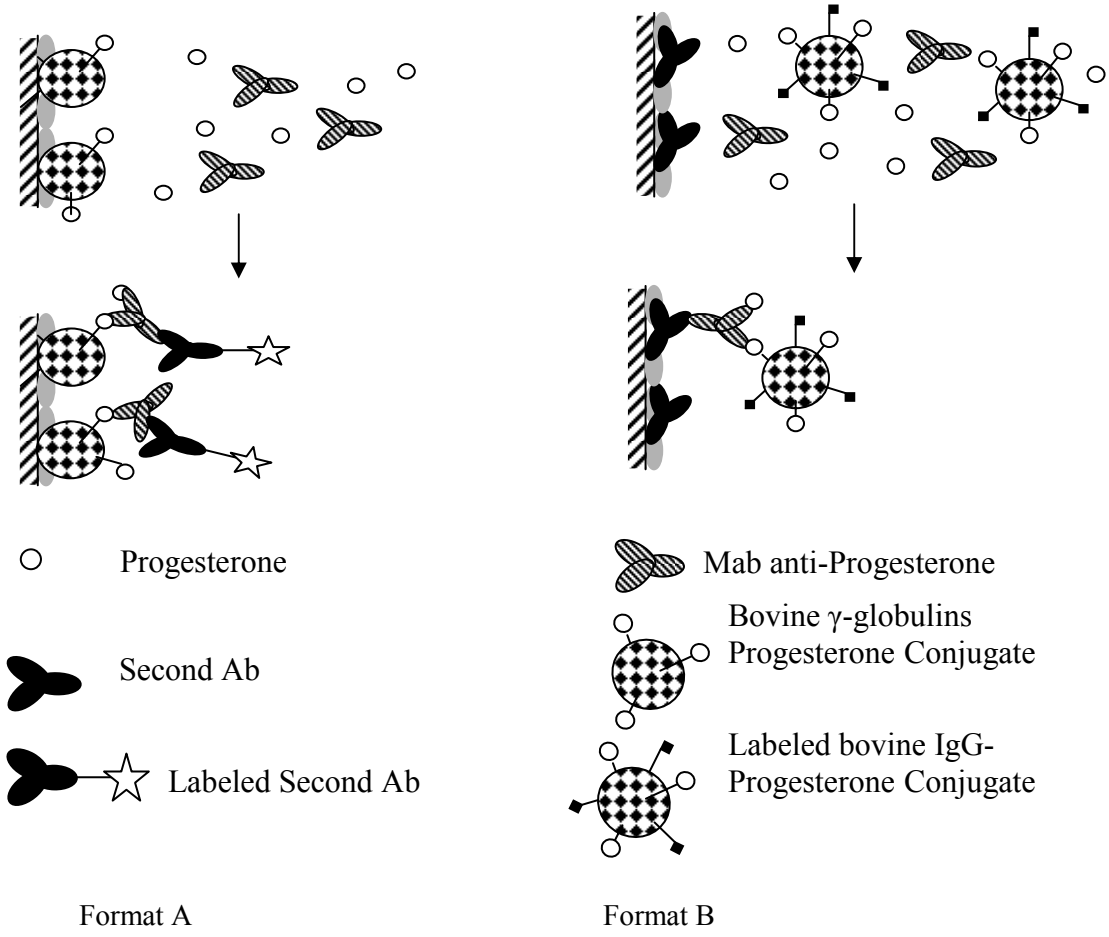

FIG.1. Schematic representation of the assay formats involved in the development of enzyme immunoassay for progesterone: (A) assay format based on immobilized progesterone-protein conjugate and labeled second antibody and (B) assay format based on immobilized second antibody and labeled progesterone-protein conjugate.

4. RESULTS AND DISCUSSION

The two assay formats followed for the development of either enzyme immunoassays or radioimmunoassays are schematically depicted in Figure 1. The first one is based on the use of a progesterone-protein conjugate as solid-phase reagent and a labeled second antibody, whereas the second format is based on solid-phase coated second antibody to which the specific progesterone antibody was immunoadsorbed and use of a labeled progesterone-protein conjugate as label.

Both assay formats required the preparation of progesterone-protein conjugates. Two progesterone derivatives, namely the progesterone-3-carboxymethyl oxime and the progesterone-11-hemisuccinate, were conjugated to bovine γ-globulins following different conjugation methods. In particular, the progesterone-3-carboxymethyloxime derivative was conjugated to bovine γ-globulins by the mixed anhydride, the carbodiimide and the imidazole method. The progesterone-11-hemisuccinate derivative was conjugated to bovine γ-globulins by the imidazole method. In all cases, the molar ratio of progesterone to amino-groups of protein was 2.5:1. These conjugates were used as solid-phase reagents in the assay format A. As shown in Figure 2, the conjugate prepared using the progesterone-11-hemisuccinate derivative provided the highest signal and was therefore further investigated.

Studies were carried out using both progesterone free human serum as well as casein solution in PBS buffer as the matrices for the standard preparation. When serum was used for the preparation of standards, the addition of an appropriate blocker such as danazol was necessary in order to release the protein bound progesterone. In case of casein solution in PBS buffer that contained this protein in concentration similar to that in milk, it was found that the presence of danazol had little effect on the signals and the calibration curve. Therefore, full-fat milk from animals in estrus that was essentially progesterone-free was adopted for the preparation of standards. Progesterone standards were prepared

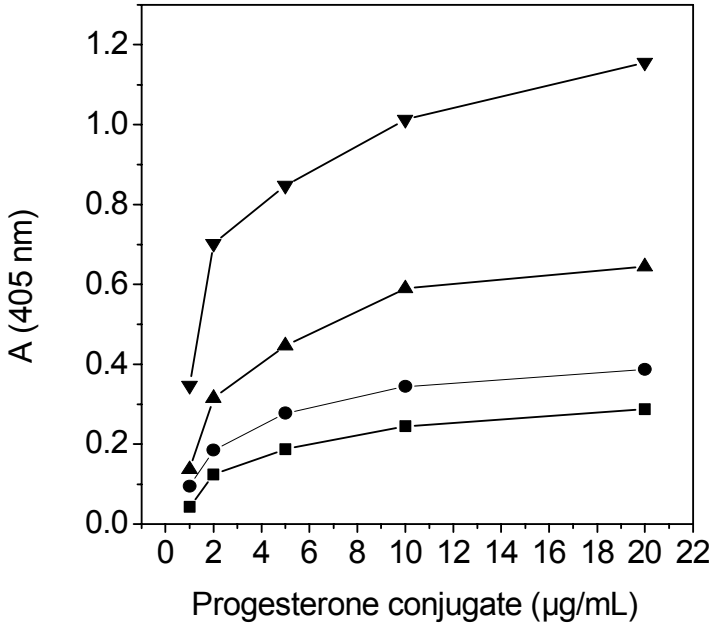

FIG. 2. Effect of concentration of progesterone-bovine γ-globulins (Prog-BIgG) conjugate used for coating on the analytical signal (A 405 nm). The progesterone-bovine γ-globulins conjugates used were prepared either by employing the progesterone-3-carboxymethyloxime derivative following the carbodiimide (■), the mixed anhydride method (●), the imidazole method (▲) or the progesterone-11-hemisuccinate derivative following the imidazole method (▼). In all cases a 25 ng/mL concentration of anti-progesterone mouse monoclonal antibody was used.

by adding known amount of exogenous progesterone to the progesterone free milk. Using these standard solutions, the final assay parameters were determined for each of the above-mentioned assay formats.

Format A

Initially the concentration of the progesterone bovine γ-globulins conjugate used for coating on the solid surface as well as the concentration of the mouse monoclonal antibody was optimized. Several assay parameters, such as concentrations of the immunoreagents, reagents, the incubation conditions, composition of buffers, the concentration of the blocker, the volume of the standard solution in the reaction mixture, were further optimized. As shown in Figure 3, in order to obtain adequate signal, concentration of progesterone bovine γ-globulins conjugate should be equal to or higher than 5 μg/mL in combination with mouse monoclonal anti-progesterone antibody concentration equal to or higher than 25 ng/mL.

The final selection of the concentration of these two reagents was based on the sensitivity of the calibration curve obtained. Several combinations of progesterone bovine γ-globulin conjugate and anti-progesterone mouse monoclonal antibody concentrations that provided adequate signal were compared with respect to the IC_{50} values obtained. As shown in Table 1, the combination that provided high signal and adequate sensitivity was 5μg/mL of progesterone bovine γ-globulin conjugate and 50ng/mL mouse monoclonal anti-progesterone antibody. Thus, this combination of immunoreagent concentration was selected for further studies.

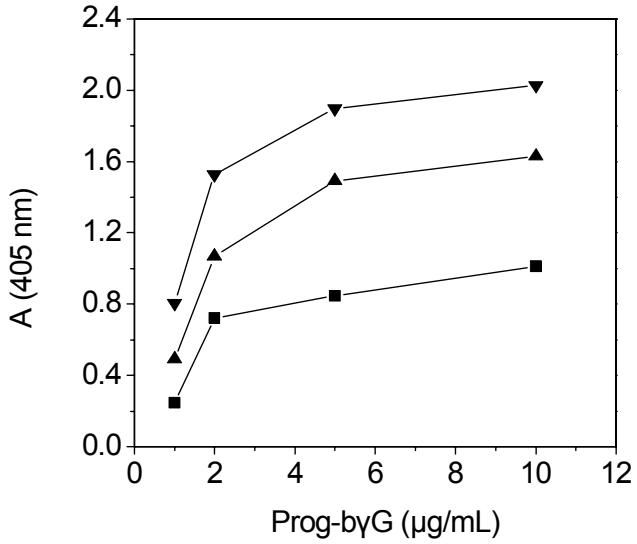

FIG.3. Effect of progesterone bovine γ-globulin conjugate concentration on the signal obtained for anti-progesterone mouse monoclonal antibody of 25(■), 50(▲), 100(▼) ng/mL.

TABLE I. ZERO SIGNAL AND IC_{50} VALUES AT VARIOUS COMBINATIONS OF PROG-IgG CONJUGATE AND ANTI-PROGESTERONE MOUSE MAb.

Progesterone Mab (ng/mL)	Prog-IgG (μg/mL)	A (405 nm) at Zero concentration	IC_{50}* (ng/mL)
50	2	1.069	7.9
50	5	1.491	9.1
50	10	1.630	9.4
100	2	1.528	11.7

**IC_{50} is the dose at 50%B/Bo*

Format B

Several assay parameters such as the concentration of second antibody used for coating, mouse monoclonal anti-progesterone antibody and biotinylated progesterone bovine γ-globulin conjugate were optimized to arrive at the most suitable assay. As shown in Figure 4, maximum plateau values were obtained for second antibody concentration equal to or higher than 5 μg/mL.

It may be noted that when direct adsorption of mouse monoclonal anti-progesterone antibody onto the solid-phase was attempted, a 50 times higher antibody concentration was required in order to achieve a signal similar to that obtained when the specific anti-progesterone antibody was immobilized through immunoadsorption on the solid-phase second antibody.

For the development of an enzyme assay following format B, two different options were evaluated viz. simultaneous incubation of the immobilized second antibody with a mixture of progesterone specific antibody and biotinylated progesterone-bovine γ-globulins conjugate and sample, or incubation of the second antibody with the antigen specific antibody, prior to addition of a mixture of biotinylated progesterone-bovine γ-globulins conjugate and sample. In Figure 5, the signal obtained for the one step and two-step incubations are presented. As shown, the one step assay provided high signal using half the amount of anti-progesterone antibody compared to the concentration of anti-progesterone antibody required to obtain the same signals in the two-step assay format. The one step assay format is also preferred since it has the advantage of reduced assay time.

The combinations of mouse monoclonal anti-progesterone antibody and biotinylated progesterone-bovine γ-globulins conjugate concentrations that provided adequate signal were determined (Figure 6), and calibration curves were obtained in order to define which of them provided the most sensitive calibration curve. As shown in Figure 7, the most sensitive calibration curve was obtained using a 50 ng/mL mouse monoclonal anti-progesterone antibody solution in combination with a 100 ng/mL biotinylated progesterone-bovine γ-globulins conjugate solution.

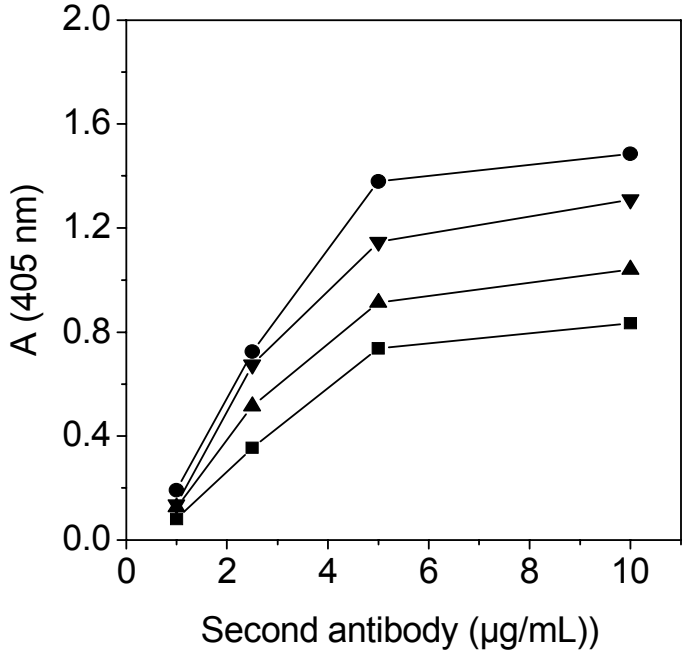

FIG. 4. Effect of second antibody concentration on the analytical signal for mouse monoclonal anti-progesterone antibody concentrations of 25(■), 50(▲), 100(▼) and 200 (●) ng/mL. The biotinylated progesterone-bovine γ-globulin conjugate was 5 μg/mL.

Using the selected combination of reagents, other assay parameters were determined for both assay formats. Among them the composition of the assay buffer, pH, the ionic strength and the protein content were investigated. As shown in Table II with assay format A, the best results in terms of analytical signal and assay sensitivity were provided using as assay buffer 0.1M PBS, pH 7.0, whereas for assay format B, the best results were obtained using 0.05M Tris-HCl, pH 8.25 as assay buffer.

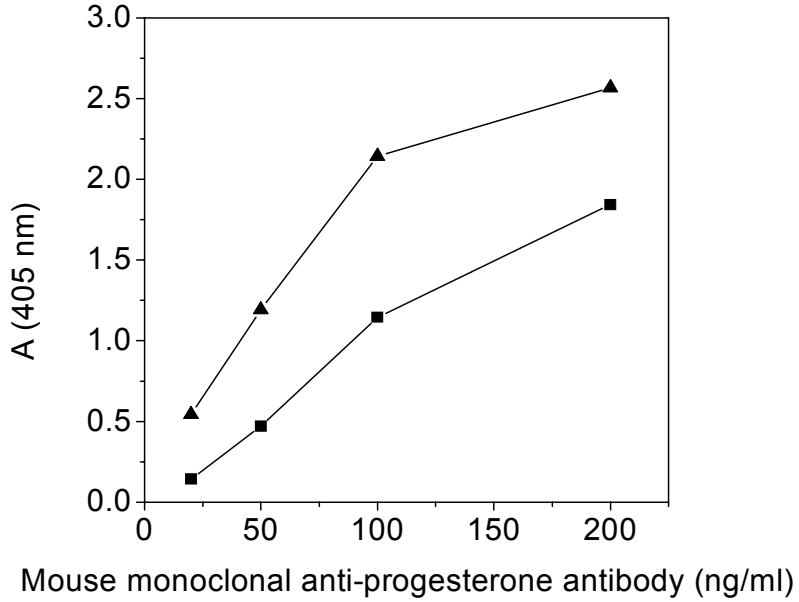

FIG. 5. Effect of the concentration of mouse monoclonal anti-progesterone on the analytical signal when the incubation with the immobilized second antibody, the biotinylated bovine γ-globulins (Bγg) conjugate and the sample was performed in one step (◢) or in two steps (■). The biotinylated Bγg conjugate concentration used was 200 ng/well.

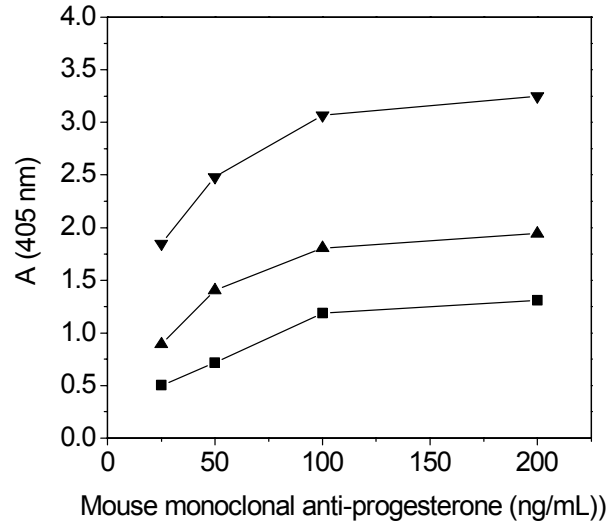

FIG. 6. Effect of mouse monoclonal anti-progesterone antibody concentration used for coating on the signal for progesterone-bovine γ-globulins conjugate concentrations of 50(■), 100(▲) and 200 ng/mL (▼)

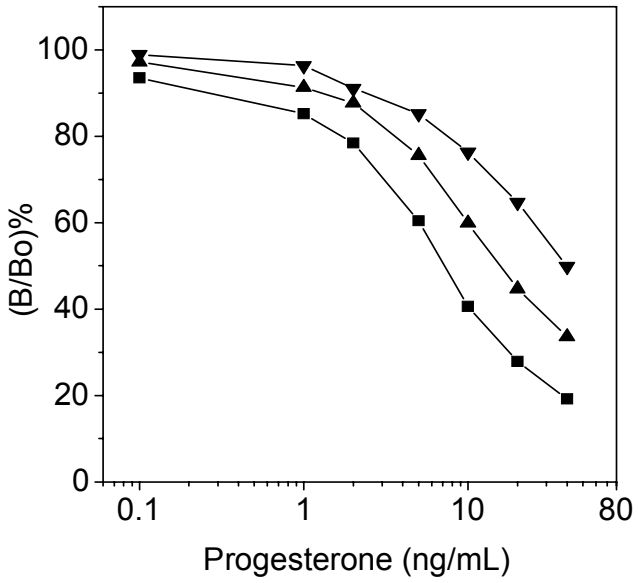

FIG. 7. Progesterone calibration curves obtained using mouse monoclonal anti-progesterone antibody and biotinylated progesterone-bovine γ-globulins conjugate concentrations of 50-100(■), 100-150(▲) and 200-250 ng/mL (▼), respectively.

TABLE II. COMPOSITION OF ASSAY BUFFER

BUFFER	FORMAT A		FORMAT B	
	A 405nm	IC_{50}(ng/mL)	A 405nm	IC_{50}(ng/mL)
0.1 M PBS, pH 6.5	1.033	5.3	2.472	7.0
0.1 M PBS, pH 7.0	1.080	3.6	2.044	6.3
0.1 M PBS, pH 7.4	0.999	4.1	1.883	5.6
0.05 M Tris-HCl, pH 7.2	1.188	4.8	2.087	6.0
0.05 M Tris-HCl, pH 7.8	1.067	4.0	1.956	5.0
0.05 M Tris-HCl, pH 8.25	0.986	3.9	1.930	4.7

For both assay formats, the addition of gelatin in the assay buffer at a concentration of 0.1% (w/v) provided higher signals and lower IC_{50} values compared with other proteins or different concentrations of gelatin (Table III). Another parameter that was optimized was the concentration of Tween 20 in the assay buffer. Although the addition of Tween 20 did not affect significantly either the analytical signal or the calibration curve sensitivity, it was found that it substantially improved the recovery of exogenous added progesterone in serum samples and was therefore included in the assay buffer. The concentration that was found to provide the best results was 0.1% for both assay formats.

The optimum sample volume was also determined for both assay formats. In Figure 8, for assay format A, the sensitivity of the calibration curve was significantly improved by increasing the sample volume from 10μL to 50μL and therefore 50μL were adopted in the final protocol. On the other hand, for the.

TABLE III. EFFECT OF PROTEIN CONTENT OF THE ASSAY BUFFER ON THE ASSAY SENSITIVITY

PROTEIN	FORMAT A		FORMAT B	
	A 405nm	IC_{50}	A 405nm	IC_{50}
Gelatin 0.05 %	0.882	4.0	1.057	5.3
Gelatin 0.1 %	**1.103**	**3.8**	**1.133**	**4.6**
Gelatin 0.2 %	0.887	4.2	1.127	4.9
Gelatin 0.5 %	0.896	4.7	1.119	5.1
BSA 0.5 %	0.784	4.1	0.907	6.0
BSA 1.0 %	0.795	4.9	0.876	6.4
BSA 2.0 %	0.826	5.2	0.813	6.6
Casein 0.5 %	0.727	8.1	0.797	7.0
Casein 1.0 %	0.508	9.4	0.600	7.5
Casein 2.0 %	0.347	16.4	0.361	17.3

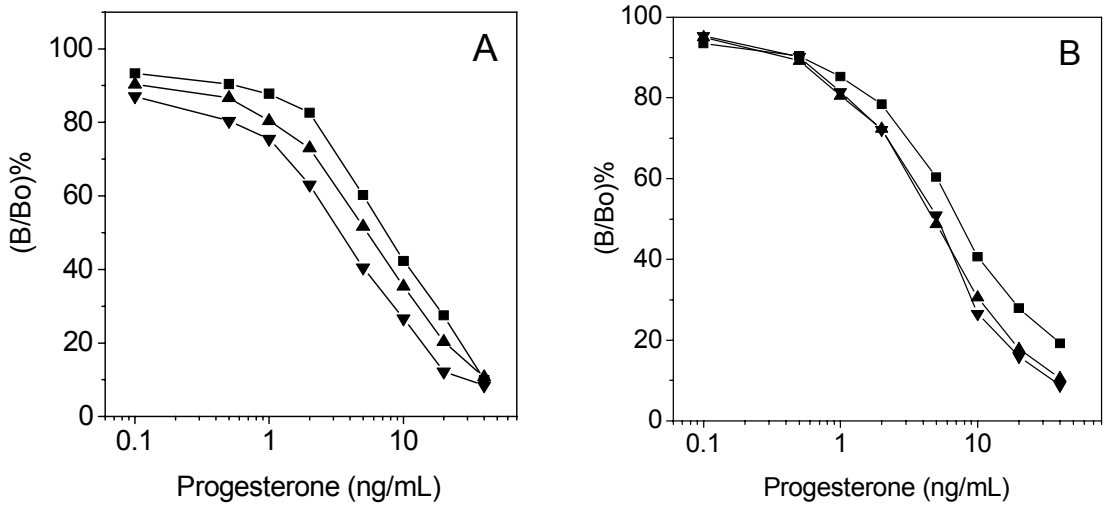

FIG. 8. Progesterone calibration curves obtained using both formats and sample volumes of 10(■), 20(▲) and 50µL(▼).

assay format B, the use of 20µL or 50 µL of sample provided essentially the same calibration curve. In this case, a sample volume of 20µL was adopted in the final protocol

The calibration curves obtained with both assay formats following the final assay protocols as described under Methods are presented in Figure 9. The analytical characteristics of the developed progesterone enzyme immunoassays are presented in Table IV. The format B assay was more sensitive than the format A. However, both assays provided reproducible and accurate results as indicated from the results of the recovery and dilution linearity experiments conducted with samples prepared in full fat milk after addition of known quantities of progesterone. The specificity of the assays developed was evaluated by cross-reactivity studies with substances with structure homologies. The results are presented in Table V. Amongst the substances tested, 11α-hydroxy-progesterone had a cross-reactivity value that reached approximately 90%. This was expected since the mouse monoclonal anti-progesterone antibody was developed using progesterone-11α-BSA conjugate. However, since this progesterone derivative is not present in milk, the high cross-reactivity would not

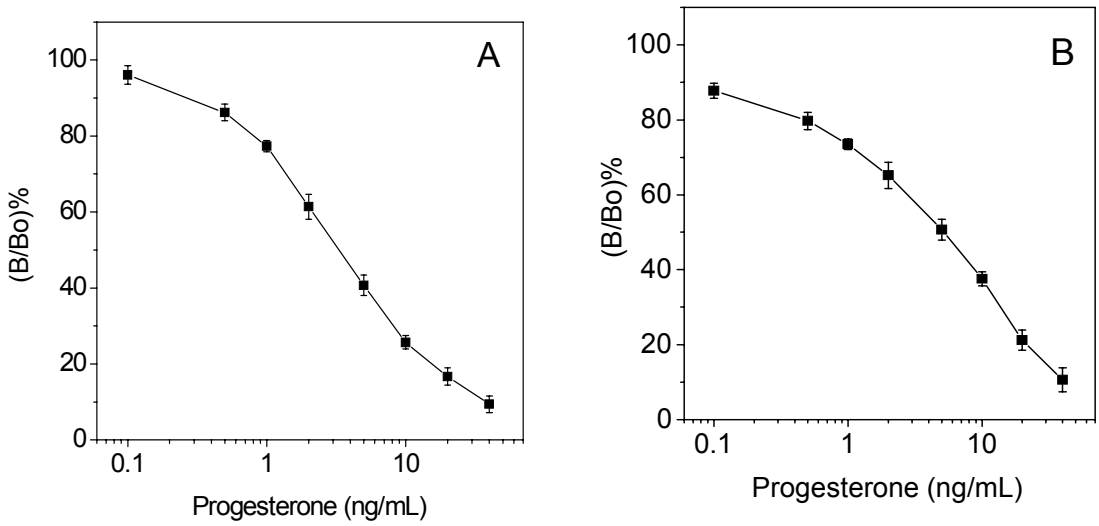

FIG. 9. Progesterone calibration curves obtained with standard solutions prepared in milk, following the assay format presented in Fig. 1A and 1B, respectively. Each point is the mean value of triplicate determinations ± S.D.

TABLE IV. ANALYTICAL CHARACTERISTICS OF PROGESTERONE ENZYME IMMUNOASSAY

ASSAY CHARACTERISTICS	FORMAT A	FORMAT B
Detection limit (ng/mL)	0.08	0.04
Dynamic range (ng/mL)	0.2 - 40	0.1 – 40
Intra-assay %CV	1.5 – 5.4	1.3 – 4.8
Inter-assay %CV	2.2 – 6.1	2.5 – 5.7
% Recovery	76.9 – 107	79.1 – 105
Dilution linearity (r) [Determined value] =a x [Expected value] + b	0.998	0.996
a	1.078	0.959
b	- 0.02	+ 0.07

cause any problems during progesterone determination in milk samples with the assays developed. Relatively high cross-reactivity value was observed with 17α-hydroxy-progesterone, but will not affect the assay since the concentrations of this related steroid in milk samples is much lower than the corresponding concentrations of progesterone.

The two formats were comparable with respect to the repeatability and accuracy of the determination, although the assay format B provides a more sensitive calibration curve compared with that obtained by the assay format A. In order to determine which assay format will be more appropriate for the development of a radioimmunoassay for progesterone, preliminary experiments were performed both with ^{125}I-mouse monoclonal anti-progesterone antibody as label in assay format A as well as with ^{125}I-progesterone bovine γ-globulins conjugate as label in assay format B.

In assay format A, it was found that in order to achieve a bound/total ratio of 18–22%, a high specific activity tracer was required, viz. ^{125}I/antibody molar ratios higher than 1. Such a tracer, however, degraded in about 10 days when kept at 4ºC in solution whereas lyophilization of the antibody resulted in almost complete loss of its reactivity. On the other hand, the application of assay format B, using

^{125}I-progesterone-bovine γ-globulin conjugate as tracer, provided higher bound/total values and the tracer was more stable, especially after lyophilization. Therefore, the development of radioimmunoassay using format B was further investigated. Different assay parameters such as the coating volume, the radiolabeling ratio of ^{125}I-progesterone-bovine γ-globulins conjugate, the anti-progesterone antibody concentration and the sample volume were optimized.

TABLE V. CROSS-REACTIVITY DATA

Compounds	% Cross reactivity	
	Format A	Format B
Progesterone (4-pregnen-3,20-dione)	100	100
Pregnenolone (5-pregnen-3β-ol-20-one)	3.7	4.6
Corticosterone (4-pregnen-11β,21-diol-3,20-dione)	1.1	0.9
21-Hydroxy-progesterone (4-pregnen-21-ol-3,20-dione)	4.4	4.3
17α-Hydroxy-progesterone (4-pregnen-17-ol-3,20-dione)	10.2	7.7
Cortisol (4-pregnen-11β,17,21-triol-3,20-dione)	2.3	1.7
11α-Hydroxy-progesterone (4-pregnen-11α-ol-3,20-dione)	85.1	90.1
17β-Estradiol (1,3,5(10)-estratrien-3,17β-diol)	< 0.01	< 0.01
Cortisone (4-pregnen-17,21-diol-3,11,20-trione)	0.2	0.2
Dehydroepiandrosterone SO4 (5-androsten-3β-ol-17-one sulphate)	4.6	2.5

Several coating volumes (0.2-1.0mL) were tested in order to achieve adequate binding of the radiotracer. It was found that the most appropriate coating volume in terms of binding capacity and coating reproducibility was 0.5mL. Several batches of ^{125}I- progesterone bovine γ-globulins conjugate with labeling ratios ranging from 0.1 to 2 were prepared. The preparations with relatively high ^{125}I incorporation (higher than 0.5) degraded quickly due to auto-radiolysis and thus were not useful for the assay. The labeling ratio that provided adequate stability, binding capacity and acceptable calibration curve was the tracer with a ^{125}I/progesterone bovine γ-globulins conjugate molar ratio of 0.25. Figure 10 presents a typical elution pattern of the iodination reaction mixture from the Sephadex G-75 column, used to isolate the ^{125}I-progesterone-bovine γ-globulins conjugate from the free ^{125}I. The yield of radioiodination was 65%

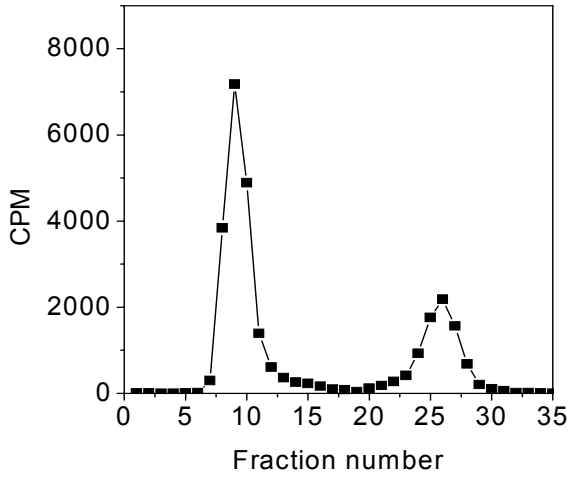

FIG. 10. Elution pattern of the radioiodination reaction mixture containing ^{125}I-progesterone bovine γ-globulins and free ^{125}I on Sephadex G-75 column.

Studies were carried out on the stability of the tracer, which was stored diluted in assay buffer as well as stored after lyophilization from a solution that contained BSA instead of gelatin, which can be more easily reconstituted with water. Figure 11 shows that the lyophilized tracer was stable for at least 45 days after its preparation, whereas the tracer kept in solution degraded very quickly. Thus, tracer preparations with ^{125}I/progesterone bovine γ-globulins conjugate molar ratio of ~0.25 were used for further experiments and assay optimization. In addition, it was found that the use of conjugate with lower progesterone to protein ratios for the preparation of the radiotracer could improve the assay sensitivity without affecting significantly the binding capacity values. Thus, for the final application, a progesterone bovine γ-globulins conjugate prepared with the imidazole method using a 1:1 molar ratio of progesterone-11-hemisuccinate to protein was selected for the preparation of the radiotracer.

The assay was performed in tubes coated with mouse monoclonal anti-progesterone antibody immunoadsorbed on immobilized goat-anti-mouse IgG antibody. The sample and the tracer were added and incubated with the solid-phase immobilized antibody. The concentration of mouse monoclonal anti-progesterone antibody that could provide satisfactory binding capacity and assay sensitivity was determined. As shown in Figure 12, maximum plateau binding capacity values were obtained using mouse monoclonal anti-progesterone antibody concentration equal to or higher than 50 ng/mL. However, the best curve in terms of both sensitivity and dynamic range was obtained using the mouse monoclonal anti-progesterone antibody at a concentration of 50 ng/mL. Therefore this concentration was selected for further experiments.

The sample volume was also optimized with respect to the calibration curve sensitivity and dynamic range. Increasing the standard solution volume did not affect the percent binding value obtained with the zero standard. However, it affected the sensitivity and dynamic range of the calibration curve. Thus, the sample volume was increased from 100μL to 400μL in order to improve the assay sensitivity. In addition, it was found, that pre-incubation of the immobilized monoclonal anti-progesterone antibody with the standard solution and subsequent addition and incubation with the tracer solution could further improve the assay sensitivity and detection limit (Figure 13).

In order to simplify the assay procedure, the assay format that was adopted in the respective enzyme immunoassay, was also tested. According to this format, the mouse monoclonal anti-progesterone antibody, the tracer and the standard solution or milk sample were added to the polystyrene tube coated with second antibody. This assay format has the advantage that the time required for the preparation of the tubes is significantly decreased. In addition, the immobilized second antibody preserves its reactivity, after drying, for longer periods of storage (at least 3 months) than monoclonal anti-progesterone antibody (less than 12 weeks).

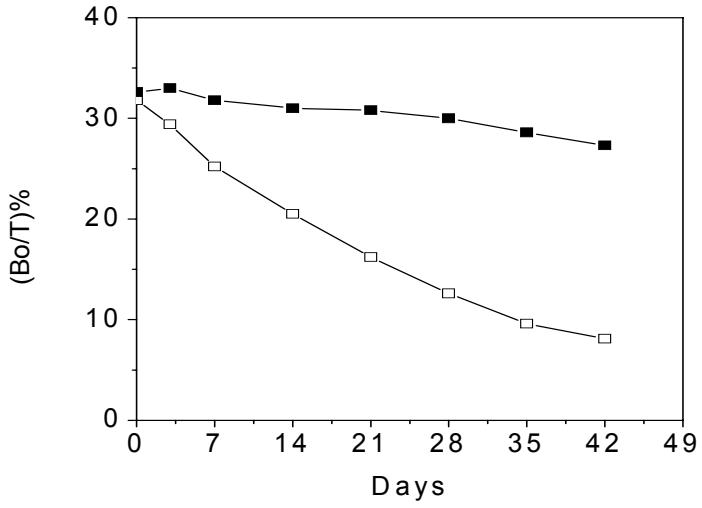

FIG.11. Stability of the ^{125}I labeled progesterone-bovine-γ-globulin conjugate either lyophilized (■) or in solution (□).

The monoclonal anti-progesterone antibody concentration and volume, the volumes of tracer and standards as well as the duration of the assay were optimized with respect to the binding capacity and the sensitivity of the calibration curve. Briefly, to perform the assay in tubes coated with 500µL of a 5 µg/mL anti-mouse IgG (Fc specific) solution, 100µL of a 50 ng/mL mouse monoclonal anti-progesterone antibody, 300µL of standard solution in full fat progesterone-free milk or sample and 100µL of tracer solution were added and incubated for 2h at room temperature under shaking. The calibration curve obtained using this protocol was compared with that obtained following the protocol with the pre-immobilized solid-phase antibody. Figure 14 indicates the calibration curve obtained with tubes coated with second antibody and simultaneous incubation with the anti-progesterone antibody, the standard and the tracer, which also has adequate sensitivity for the determination of progesterone in milk samples.

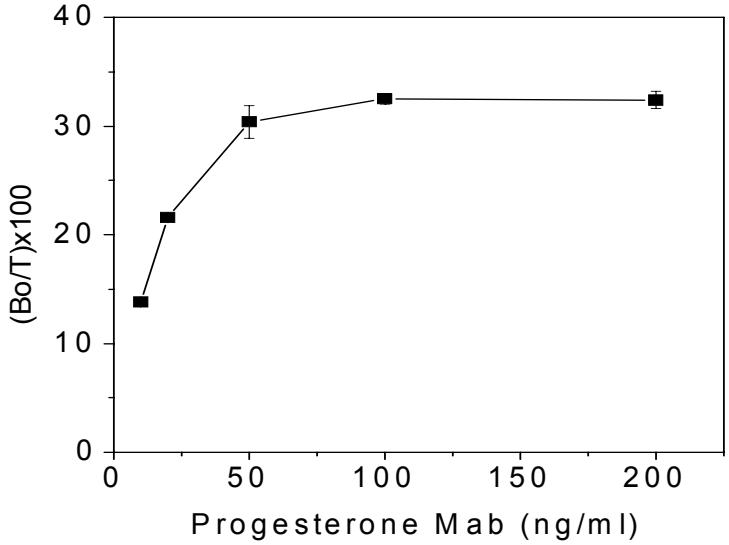

FIG. 12. Effect of mouse monoclonal anti-progesterone antibody concentration on the ^{125}I-progesterone bovine γ-globulins binding in presence of zero calibrator.

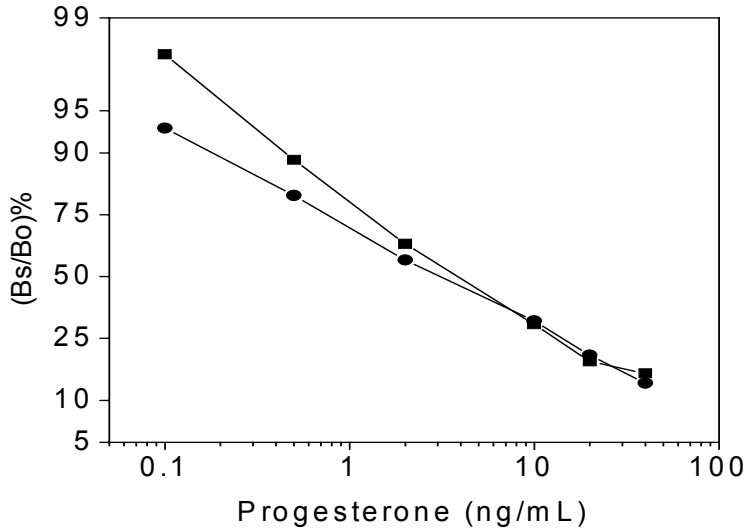

FIG. 13. Progesterone calibration curves obtained either by simultaneous incubation of the MAb, immobilized through second antibody, with the standard solution and the tracer (■) or pre-incubation of the MAb, immobilized through second antibody, with the standard solution and subsequent addition of the tracer (●).

After rigorous optimization, the final assay protocol was established and the analytical characteristics of the assay such as detection limit, dynamic range, within-run CV, between-run CV and dilution linearity were determined. The detection limit of the assay developed, defined as the analyte concentration corresponding to the mean radioactivity value of 20 replicates of zero standard -2SDs was 0.1 ng/mL. The dynamic range of the assay was 0.2–40 ng/mL. The within-run precision of the assay was determined by 12 replicate determinations of three control milk samples in a single assay and the between-run precision by duplicate determinations of the same control samples in 20 different runs. The within-run CVs were between 0.6% and 5.4%, whereas, the between-run CVs ranged between 2.3% and 7.8% over the range of concentrations covered by the calibrator solutions.

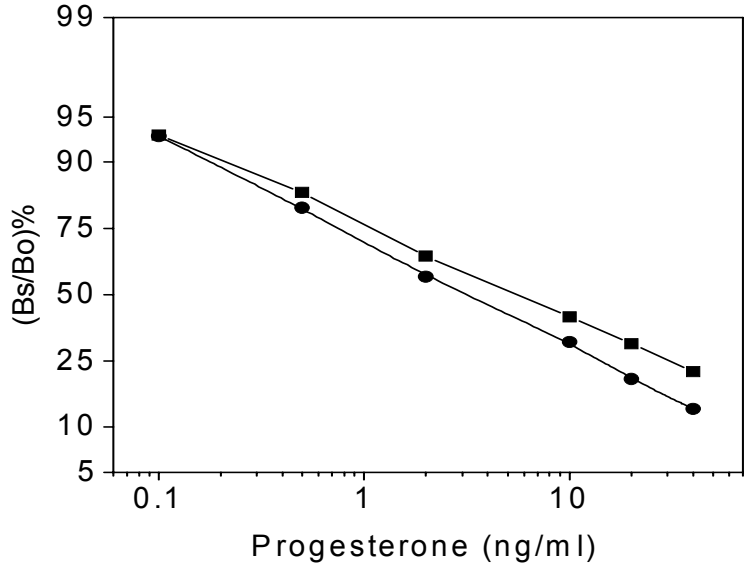

FIG.14. Progesterone calibration curves with tubes pre-coated with second antibody and simultaneous incubation with the anti-progesterone antibody, the standard solution and the tracer (■) or by tubes pre-coated with anti-progesterone antibody through second antibody which were pre-incubated with the standard solution before the addition of the tracer (●).

TABLE VI. RECOVERY OF EXOGENOUS PROGESTERONE ADDED IN MILK SAMPLES

Sample	Added amount (ng/mL)	Determined amount (ng/mL)	Recovered amount (ng/mL)	% Recovery
1	0	2.7		
	4.3	6.5	3.8	88.4
	8.6	10.5	7.8	91.0
	19.0	22.7	20.0	105
2	0	7.6		
	4.3	12.0	4.4	102
	8.6	14.8	7.2	83.7
	19.0	24.0	16.4	86.3
3	0	13.6		
	4.3	17.3	3.7	86.0
	8.6	22.6	9.0	105
	19.0	31.2	17.6	92.6

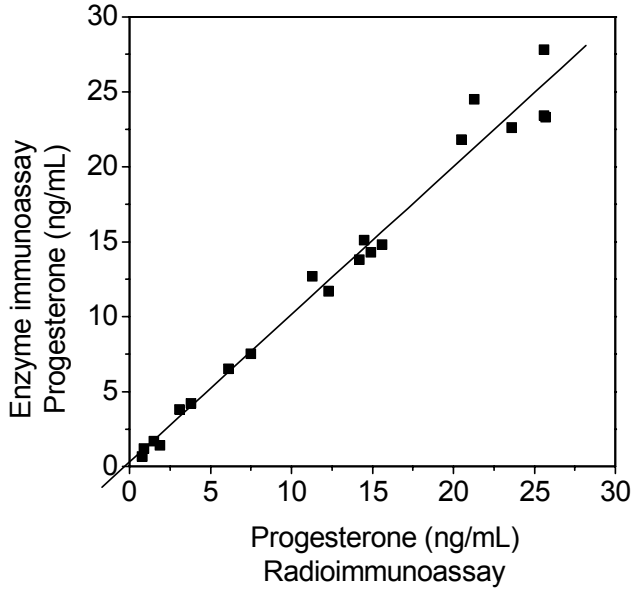

FIG. 15. Correlation of the values determined in milk samples using the developed radioimmunoassay and the enzyme immunoassay with the same assay format.

The dilution linearity of the assay was evaluated by assaying milk samples serially diluted with the zero calibrator. We found that there was a good agreement between the measured and the expected values, derived from the initial concentrations of progesterone in samples ({Determined value}=1.045x{Expected value}-0.1; r =0.975; P < 0.001; N = 22). In Table VI the results of the recovery experiment are presented. The average recovery was 93.3±8.5%. Full milk samples obtained from cows in different phases of their reproduction cycle were assayed using the developed radioimmunoassay as well as the enzyme immunoassay with the same assay format. As shown in Figure 15, there was a good correlation between the values determined with the two methods [(Enzyme assay) = 0.98654 x (RIA) + 0.27, r = 0.9892, n = 20].

5. CONCLUSION

Enzyme immunoassays for the determination of progesterone in serum and bovine milk following two different assay formats were developed. The first involved immobilization of a progesterone-protein conjugate to the solid-surface and detection through a labeled second antibody, while the second involved immobilization of second antibody and detection through a labeled progesterone-protein conjugate.

The reagents required for the development of each assay format were prepared and evaluated. A conjugate prepared using the progesterone-11αhemisuccinate derivative for introduction of progesterone moieties in bovine γ-globulins was used as solid-phase reagent in the first format and as tracer in the second format. In the latter case, the conjugate was biotinylated and used in combination with horseradish peroxidase labeled streptavidin.

The nature of the matrix for the preparation of standard solution was also determined. It was found that in order to avoid false results, it is necessary to use a matrix that resembled the sample, and it was found that full fat milk delivered by animals in estrus, which is progesterone free closely resembled the sample matrix.

Both assay formats provided calibration curves of adequate sensitivity, repeatability, and dynamic range. In addition, the assays developed were accurate as it was determined by the dilution and linearity experiments. However, the assay based on the solid-phase immobilized antibody had lower

detection limit and its application for the development of a RIA for determination of progesterone in milk samples was investigated.

In the case of the radiotracer, the use of this conjugate provided the possibility of preparing tracers with a wide range of specific activity compared to the radiolabeling of a low-molecular weight progesterone derivative. In addition, the sensitivity and dynamic range of the assay can be regulated by modification of the specific activity or the progesterone to protein ratio in the conjugate. The tracer presented adequate stability when it was lyophilized and stored at 4ºC. The lyophilization procedure did not affect the tracer reactivity whereas when the monoclonal anti-progesterone antibody was used as tracer, lyophilization resulted in complete loss of antibody activity.

The use of adsorbed second antibody is advantageous in the case of solid-phase reagent, compared with the use of directly or indirectly immobilized anti-progesterone specific antibody. Second antibody coated tubes after blocking and drying were stable for at least three months when stored at 4ºC. The fact that the immunoreaction and the immunoadsorption of the anti-progesterone antibody onto the solid-phase immobilized antibody take place at the same time simplifies significantly the procedure for the preparation of the solid-phase without affecting the performance of the assay. On the other hand, when the anti-progesterone antibody was immobilized onto the solid-phase, an additional 2-hour pre-incubation with the standard solution was necessary before the addition of the tracer in order to develop an assay of adequate sensitivity and dynamic range.

The RIA developed had a detection limit of 0.1 ng/mL and a dynamic range of 0.2-40 ng/mL. The average percent recovery was 93.3±8.5% and there was good agreement between the expected and the determined values in the linearity dilution experiment (r = 0.975). The progesterone values determined with the radioimmunoassay developed in full milk samples correlated well with those determined in the same samples by the respective enzyme immunoassay. Thus, the RIA developed can be used for the determination of progesterone in milk samples in order to define the hormonal status of the animals and have a better management of the reproduction in dairy cows.

REFERENCES

[1] POPE, G.S. AND SWINBURNE, J.K., Reviews of the progress of Dairy Science: Hormones in milk: Their physiological significance and value as diagnostic aids, Journal of Dairy Research, 47 (1980) 427-449.

[2] ALLEN, R. M. AND REDSHAW, M.R., The use of homologous and heterologous ^{125}I-Radioligants in the Radioimmunoassay of Progesterone. Steroids, **32** (1978) 467-486.

[3] SAUER, M.J., FOULKES, J.A., WORSFORD, A. AND MORRIS, B.A., Use of progesterone 11-glucuronide-alkaline conjugate in a sensitive microtitre-plate enzymeimmunoassay of progesterone in milk and its application to pregnancy testing in dairy cattle. J. Reprod. Fert. **76** (1986) 375-391.

[4] KAKABAKOS, S.E. AND KHOSRAVI, M.J., Direct Time-Resolved Fluorescence Immunoassay of Progesterone in Serum Involving the Biotin-Streptavidin System and the Immobilized-Antibody Approach. Clin. Chem. **38** (1992) 725-730.

[5] YOON, D.Y., CHO, I.M.J., CHOE, I.S., CHUNG, T. W AND BUYN, S.M., Influence of conjugation site on the specificity of monoclonal antibodies to progesterone and on the performance of direct enzyme immunoassay. Biochem. Mol. Biol. International. **33** (1993) 553-563.

[6] CLAYCOMP, R.W., CELWICHE, M.J., MUNRO, C.J. AND BON DURANT, R.H., Rapid enzyme immunoassay for measurement of bovine progesterone. Biosens. Bioelectron, **13** (1998) 1165-1171.

[7] GREENWOOD, F.C., HUNTER, W.M. AND GLOVER, I.S., The preparation of ^{131}I-labeled human growth hormone of high specific radioactivity. Anal. Biochem. **89** (1963) 114-123.

DEVELOPMENT OF RADIOIMMUNOASSAY FOR ESTIMATION OF PROGESTERONE IN BOVINE SERUM

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Abstract

The aim of the present work was to develop assays for serum and milk progesterone. Studies were carried out to evaluate the influence of the bridge as well as the site to which the iodinating species in the tracer or the protein in the immunogen is attached to progesterone on the standard curves and to optimize a suitable assay system for the estimation of progesterone in human as well as bovine serum and bovine milk. In the present work, various heterologous and homologous assay systems were evaluated to study its effect on the sensitivity, range and slope of the assay. The homologous assay system using a common hemisuccinate bridge for both tracer (progesterone-11αHS-^{125}I-histamine and progesterone-11αHS-^{125}I-TME) and immunogen (progesterone-11αHS-BSA) resulted in standard curves with poorer slopes than with a heterologous bridge assay system with the hemisuccinate linkage in immunogen (progesterone-11αHS-BSA) and the phthalate linkage in the tracer (progesterone-11αHpth-^{125}I-TME). It was also observed that a heterologous site system with hemisuccinate linkage in the immunogen (progesterone-11αHS-BSA) and carboxymethyl oxime linkage in the tracer (progesterone-3CMO-^{125}I-histamine) resulted in standard curves with better slopes. A liquid phase assay for measurement of progesterone in bovine serum has been achieved. Kit formulation for liquid phase veterinary progesterone with all in-house reagents has been carried out. The assay system uses polyclonal antibody raised against progesterone-11αHS-BSA and progesterone-3-CMO-^{125}I-histamine as the tracer. The assay has a range of 0.4-25 ng/mL and uses 50µL of sample for estimation**.** Several methods of coating antibodies on polystyrene tubes have been evaluated for its use in the development of assay for progesterone in bovine serum and milk.

1. INTRODUCTION

Progesterone (4-Pregnen-3,20-dione) is a steroid hormone produced by the adrenal glands, gonads, corpus luteum and placenta. Similar to other steroids, progesterone is also synthesized from cholesterol via a series of enzyme mediated biochemical reactions [1]. The main site of progesterone production is the corpus luteum in females during luteal phase of the menstrual cycle and by placenta during pregnancy. Thus, progesterone is an important steroid hormone and its estimation in serum is usually measured by radioimmunoassays for obtaining valuable diagnostic information. Progesterone concentration in an animal's body reflects the stage of the reproductive cycle, pregnancy and ovarian disorders. Hence, progesterone level is a good indicator for identifying luteal phase defects and to detect missed conception. Both serum progesterone levels as well as progesterone in milk have been reported to be useful in cattle management for better milk production. Consequently, assays for management of progesterone in serum or milk have been developed and used in veterinary science. Due to the non-availability of indigenous kits for progesterone, the development of a radioimmunoassay for progesterone in bovine serum and milk was taken up. All the key reagents used in the development work were prepared and characterized in-house.

2. MATERIALS

Progesterone11α-hemisuccinate, tyrosine methyl ester hydrochloride, histamine, progesterone reference standard, 11αhydroxy progesterone, phthalic anhydride, progesterone-3carboxymethyl oxime (CMO), progesterone-3CMO-BSA conjugate, progesterone-11αhemisuccinate–BSA conjugate chloramine-T, Isobutyl chloroformate, Bovine serum albumin (BSA), bovineγ globulin and sodium metabisulphite were procured from Sigma Chem. Co. USA. Carrier free ^{125}I as sodium iodide (Specific activity 15-17mCi/µg, radioactive concentration 100mCi/mL) was obtained from IZOTOP, Hungary. Whatman 3mm chromatography paper was purchased from Whatman Ltd. England. Silica gel (GF 254) and silica impregnated plastic plates were obtained from E.Merck, Darmstadt. All the other reagents were of AR grade. Preparative silica plates were prepared at the laboratory using silica gel GF254 from Acme Chemicals, India. The non-radioactive compounds were identified as UV active

zones or by exposure to iodine vapor and the radioactive zones were identified using a NaI(Tl) scintillation counter. TLC radioactive scanner, Version 1.6, was obtained from Raytest, Germany. HPLC system PU 1580 was from JASCO, Japan. The system was equipped with PU 1575 UV-Vis detector as well as a well type NaI (Tl) scintillation detector.

3. METHODS

3.1 Production of polyclonal antibodies for progesterone

Progesterone-11αhemisuccinate–bovine serum albumin conjugate was dissolved in normal saline and emulsified with Freunds complete adjuvant, in the ratio 1:2.5 to give a concentration of 1mg of conjugate per 0.5mL of emulsion. 4 New Zealand white rabbits (R2, R4, R8, R10,) were injected intradermally with 0.5mL of emulsion per rabbit. Five booster injections of emulsions at a concentration of 250μg of progesterone conjugate per 0.5mL of emulsion in the Freund's incomplete adjuvant were given intramuscularly at monthly intervals. The rabbits were bled between 10–20 days after every booster. The serum collected after every booster was evaluated for its titre, specificity and sensitivity. The chosen antiserum was dispensed into 1mL aliquots, freeze-dried and stored at 4°C until further use. Two rabbits (R14 and R18) were immunized with progesterone-3CMO-BSA conjugate in normal saline and emulsified with Freunds complete adjuvant, in the ratio of 1:2. 0.5mL of the emulsion containing ~800μg of the progesterone-3CMO-BSA conjugate was injected intradermally at multiple sites on the shaved back of the two rabbits. Booster injections were given intramuscularly at monthly intervals with one-third of the amount given initially, until a suitable antiserum was obtained. The rabbits were bled ~10 days after the boosters and the antisera was evaluated for titre, specificity and sensitivity.

3.2. Preparation of radioiodinated tracers for progesterone

3.2.1. Progesterone-11α-hemisuccinate-TME conjugate [2] (P-11αHS-TME)

Progesterone-11α-hemisuccinate (130mg) in 5mL of dry dioxane and tributyl amine (75μL) was cooled to 8-10°C. Isobutyl chloroformate (40μL) was added and the solution was incubated further for 20 minutes. Tyrosine methyl ester (TME) (69mg) in 20mL of a mixture of dioxane and water (1:1) was added to the activated progesterone hemisuccinate and the reaction mixture was stirred continuously at 4°C for 4 h. During this reaction, the pH was maintained at ~8 by the addition of 1M NaOH. The progress of reaction mixture was monitored by TLC in the solvent system benzene:acetone:methanol (5:5:2). The reaction mixture was poured into 150mL of ice-cooled distilled water and the progesterone-11α-hemisuccinate-TME conjugate precipitated. The precipitate was spun down at 6000 rpm in the centrifuge and washed sequentially with 30mL distilled water, 30mL of 1M HCl, 40mL of 1M $NaHCO_3$ and finally with 40mL of distilled water. The precipitate was dried in a vacuum dessicator. The purity of this compound was checked with TLC using the solvent system benzene: acetone:methanol (5:5:2). The yield of the conjugate was determined and the conjgate was stored at -20°C.

3.2.2. Progesterone-11αhemiphthalate-TME conjugate [3] (P-11αHpth-TME)

11αOHprogesterone (500mg) was refluxed with phthalic anhydride (1g) in 10mL of dry pyridine for 7-8 hours. The reaction mixture was cooled, acidified with 2N HCl and extracted in chloroform. The chloroform was removed using a rotary evaporator to yield 640 mg of the product. TLC in the solvent system, chloroform: methanol:water (90:10:1) was carried out to monitor the reaction. TME conjugate of the hemiphthalate derivative was prepared as in the case of P-11αHS-TME.

3.2.3. Radioiodination of TME conjugates of progesterone

P-11αHS-^{125}I-TME and P-11αHpth-^{125}I-TME

10μL (2.5μg) of ethanolic solution of the respective TME conjugates of progesterone along with 40μL of 0.5M phosphate buffer, pH 7.4 and 10-20μL (1.5mCi) of $Na^{125}I$ were taken in a culture tube. 5μL (25μg) chloramine-T dissolved in 0.05M phosphate buffer, pH 7.4 was added and the reaction was stopped after 2-3 minutes by the addition of 5μL (150μg) of sodium metabisulphite in 0.05M phosphate buffer, pH 7.4. The reaction mixture was diluted with 0.3mL of 0.05M-phosphate buffer, pH 7.4. An aliquot of 2μL was spotted on paper electrophoresis in 0.025M phosphate buffer, pH 7.4 to estimate the radioiodination yield and the specific activity.

Purification of the radioiodinated tracer was effected by solvent extraction of the radioiodinated mixture into chloroform followed by preparative TLC of the organic extract using the solvent system chloroform:methanol:water (90:10:1). The radioactivity at every one cm zone of the preparative plate was measured using NaI(Tl) scintillation counter. Silica gel from the fractions showing high radioactive counts was extracted with 2×2mL of ethanol in glass test tubes. The ethanol layer was separated by centrifugation and stored at -20°C. The tracers were evaluated for radiochemical purity, immunoreactivity and stability; and stored at 4°C.

3.2.4. Radioiodination of histamine conjugates of progesterone

P-11αHS-^{125}I-histamine and P-3CMO-^{125}I-histamine [4]

Activation: 1mg of progesterone-11α-hemisuccinate or progesterone-3CMO dissolved in 50μL of dry dioxane and 10μL of 20% tributylamine in dioxane was reacted with 10μL of 10% isobutyl chloroformate in dioxane for 30 min at 4-8°C with constant stirring. The reaction mixture was diluted to 1.4mL with dioxane.

Radiolabeling: Radioiodination of histamine was carried out as follows: To 10μL (2.2μg) of histamine in 50μL of 0.5M phosphate buffer, pH 8 and 20μL (2mCi) of ^{125}I (NaI), 10μL (50μg) of chloramine-T was added. The contents were kept stirring gently for about 2-3 minutes. The reaction was quenched by the addition of 10μL (300μg) of sodium metabisulphite. Radioiodination yield of histamine was estimated from paper electrophoresis of the reaction mixture in 0.025M PO_4^{-3} for 1h at 10 volt/cm.

Conjugation: 50μL (35.7μg) of reaction mixture of the respective derivatives prepared above was added to the radioiodinated histamine. The pH was maintained as 8.0. The reaction was carried out for 2h at 4°C with constant stirring. The reaction mixture was acidified with 0.9 mL of 0.1M HCl and thoroughly mixed with 1 mL of toluene. These two phases were separated and 0.9ml of 0.1M sodium hydroxide along with 1 mL of metabisulphite (1mg/ml) in 0.5M phosphate buffer, pH 7 was added to the aqueous fraction. The radioiodinated conjugate was extracted from the aqueous phase with 0.5ml of toluene. Equal aliquots of all the three phases viz. first toluene wash, the aqueous layer and the final toluene extract were measured for radioactivity to estimate the yield of the iodinated progesterone-11αHS-^{125}I-histamine or progesterone-3CMO-^{125}I–histamine. 200μL of the toluene extract was spotted at 4 cm from the lower end of a TLC preparative plate. The plates were developed in benzene: ethanol: acetic acid (75:24:1). The plates were dried and 1cm zones were measured for radioactivity in a NaI(Tl) scintillation counter. Silica gel from identified radioactive zones was extracted with 2×2 mL portions of ethanol. The iodinated product was stored as ethanolic fraction at 20 μCi/mL. These tracers were evaluated for radiochemical purity, immunoreactivity and stability when stored at 4°C.

3.3. Progesterone standards

Progesterone standards were prepared in methanol and calibrated using an UV spectrophotometer. The concentration of the primary stock solution was ascertained from its absorbance using the formula $OD = \varepsilon cl$ where ε is the molar extinction coefficient, c is the concentration and l is the path length. Higher concentrations were made initially in phosphate buffer, pH 7.5 and working standards were prepared in progesterone free bovine serum. Serum collected from bull was used as progesterone free

bovine serum and stored with 0.1% sodium azide as preservative. Milk collected during heat period was used as progesterone free milk and stored with potassium dichromate as preservative.

3.4. Separation system

Assays for serum samples as well as milk samples were optimized using both liquid phase as well as solid phase system. In liquid phase assay, second antibody-polyethylene glycol (PEG) precipitating agent was used for bovine serum based assay while 22% PEG along with BγG was used for assay in milk. In solid phase assays, four different solid phases were evaluated for use in assay of progesterone in both serum as well as milk.

3.5. Liquid phase assay procedure for bovine serum and milk

50 µL of progesterone serum/buffer standard or sample, 100 µL of progesterone antibody, 300 µL of progesterone tracer (~10 KBq) were incubated for 1h at room temperature. 50 µL of progesterone free milk was included in the standard tubes for the assay for milk. 100 µL of second antibody and 1 mL of 10% PEG solution in case of assay for serum and 1mL of 22% PEG and 100 µL of 2% BγG for assay in milk were added, incubated further for 15 minutes at room temperature and centrifuged at 2000×g for 20 minutes. The bound fraction was separated and measured for radioactivity. The percent bound was plotted versus standard concentration in a semi log graph paper. 10 ng/tube of danazol was necessary to be included in the whole assay in case samples to be analysed are from pregnant animals.

3.6. Solid phase assay procedure for bovine serum and milk

3.6.1. Coating of antibodies on polystyrene tubes

Physical adsorption

0.5 mL of different dilutions (1:100 to 1:50 000) of anti-progesterone antibody prepared in 0.1 M bicarbonate buffer, pH 9 were added to polystyrene tubes. The tubes were incubated for 24h at ambient temperature. The antibody solution in the tubes was aspirated and washed twice with 0.1% BSA. The tubes were saturated with 1% BSA for 30 min for saturating any unoccupied sites. After aspiration, the tubes were washed twice with 0.5 mL of assay buffer (0.1% BSA in PO_4^{-3} buffer, pH 7.5). 400 µL of ^{125}I-progesterone (40 000 cpm) and 50 µL of progesterone free milk or serum were added to the coated tubes for binding studies. The tubes were aspirated and washed once with 2mL of tris-HCl buffer containing 0.1% tween20). These tubes were stored at 4°C after coating.

Coating of antibodies through rabbit γ globulin and second antibody [5]

0.5 mL of 2 mg of rabbit γ-globulin per litre in 0.1 M bicarbonate buffer was added to polystyrene tubes and incubated for 24 h at ambient temperature. 0.5 mL of different dilutions of second antibody (1:50 to 1:2000) were added after washing the tubes and further incubated for 24 h at ambient temperature. 0.1 mL of different dilutions (1:50 to $1:10^5$) of primary antibody was added without aspirating the second antibody. After incubation with the primary antibody, the tubes were washed with 0.05 M phosphate buffer, pH 7.5. The tubes were saturated with 1% BSA.

Coating of antibodies on straptavidin coated tubes [6]

One mL of progesterone antiserum was precipitated with 18% sodium sulphate. The precipitate was collected by centrifugation and the concentration of IgG was estimated by spectrophotometer using BγG as standards. 0.3 mg of biotinyl-6-aminocaproic acid N-succinimidyl ester in a volume of 10 µL in dimethyl formamide was added to the antibody solution (20 mg/mL) in 0.05M bicarbonate buffer, pH 8.5. The mixture was stirred for 2h at 4°C. Unreacted biotin was separated by overnight dialysis in 0.01M PBS. Different dilutions of the biotinylated antibody were added to the commercially obtained

streptavidin coated tubes and incubated for 1h for coating. The tubes were washed as before and used for the assay.

Modification of local polystyrene tubes to improve coating

One mL of aniline hydrochloride and ammonium per sulphate solutions (1:1) were added to the polystyrene tubes to introduce amino groups on the polystyrene tubes. These tubes were washed thoroughly with deionised water. These tubes were later used for coating progesterone antibody, both by physical adsorption as well as through 0.1% glutaraldehyde.

4. RESULTS AND DISCUSSION

4.1. Preparation of progesterone antibodies in-house

Typical antibody titres from one of the rabbits immunized with P-11αHS-BSA is shown in Figure 1. It was observed that the titres improved with the boosters. The titre curves obtained from three rabbits (R4, R8, R10) after the fourth booster are shown in Figure 2a. Among the three rabbits, antiserum from R4 was found to be better in terms of titre as well as the slope of the standard curve (Fig. 2b). The optimum dilution of the antibody obtained with R4 antiserum is 1:12000 with progesterone-11α-hemisuccinate-^{125}I-histamine, 1:7000 with progesterone-11α-hemisuccinate-^{125}I-TME, 1:1000 with progesterone-11α-hemiphthalate-^{125}I-TME and 1:1500 with progesterone-3-CMO-^{125}I-histamine. The higher titre in case of the homologous tracers was due to the antibodies produced against the common bridge, whereas the titres were much lower with the other heterologous tracers. The cross reactivity of the antiserum with structurally related steroid is given in Table I.

Rabbits immunized with progesterone-3-CMO-BSA gave a titre of 1:2000 with progesterone-3-CMO-^{125}I-histamine after the third booster. (Fig. 3). Although the bridge in this case was same in tracer as well as in immunogen, the titre is low compared to the one with a hemisuccinate bridge probably due to the lower antigenicity of the oxime linkage compared to the hemisuccinate linkage as observed by Corrie et al [7]. No binding was observed with the progesterone-11α-hemisuccinate-^{125}I-histamine and progesterone-11α-hemiphthalate-^{125}I-TME. This indicates that the tracers modified at C-11 position failed to react with the antibodies against immunogen, which was conjugated at C-3 position. Cross reactivity of antiserum raised against progesterone-3CMO-BSA when used with progesterone-3CMO-^{125}I-histamine tracer is tabulated in Table II. High crossreactivity of 150% with pregnane-5β-3,20-dione, a metabolite of progesterone was observed with this antiserum, rendering it unsuitable for assay.

4.2. Radioiodinated progesterone tracers

The yields of TME conjugates were low (~10-20%) primarily due to the difficulty in recovering the precipitate. The recovery is higher with the use of a refrigerated centrifuge at 10000 rpm speed. The R_f values in various solvent systems are given in Table III. The reaction was monitored using TLC in benzene: acetone: methanol (5:5:2) system. In this system R_f of progesterone-11α-hemisuccinate was 0.73 and that of TME was 0.52. TLC of the precipitated TME conjugate showed a single spot at R_f 0.84.

Progesterone-11α-hemiphthalate was prepared by the reaction of progesterone with phthalic anhydride under vigorous conditions. TLC results of the reaction mixture showed three spots, one at R_f 0.5 for unreacted phthalic anhydride, the second R_f 0.65 for 11α-hydroxy-progesterone, and a third spot at R_f 0.25 for progesterone-11α-hemiphthalate derivative in the solvent system chloroform: methanol: water (90:10:1). The reaction mixture was extracted with chloroform and the chloroform was removed by a rotary evaporator to yield 640 mg of the crude product.

In the preparation of TME conjugate, TLC of the reaction mixture in the solvent system chloroform:methanol (80: 20) showed three spots viz one at R_f of 0.58 pertaining to progesterone

phthalate derivative, second at R_f of 0.3, which was due to unreacted TME and a third spot was found at 0.7. The TME conjugate was purified by precipitation in water and centrifuged at 6000 rpm. TLC of the purified conjugate after precipitation from water, using solvent system chloroform: methanol (80:20) showed a single spot at R_f of 0.72. TLC of the purified phthalate-TME conjugate using solvent system benzene: acetone: methanol (5:5:2) showed R_f of progesterone-11α-hemiphthalate-TME conjugate as 0.84 whereas R_f of progesterone-11α-hemiphthalate was 0.77 and that of TME was 0.5. Both the solvent systems could be used for characterization of the conjugate.

P-11α-HS-TME and P-11α-Hpth-TME were ascertained to be pure as they apperared as a single spot in TLC as well as a single peak in HPLC. After radiolabeling the tracers were purified by TLC. HPLC of the cold conjugates as given in Figure 4 shows a single peak for both the TME conjugates.

4.2.1 Radioiodination of TME conjugates of progesterone

Paper electrophoresis of the reaction mixture of radioiodinated progesterone-11α-hemisuccinate- TME indicated a radiolabeling yield of 85-90% with the remaining activity as free iodide (Fig 5). Specific activity of the tracer, when calculated from the iodination yield was found to be ~300 μCi/μg. The radioiodinated progesterone was extracted in chloroform. The chloroform extract was subjected to further purification on preparative TLC plate and developed in the solvent system chloroform: methanol: water (90:10:1). All the radioactive zones were eluted in ethanol and evaluated for its immunoreactivity.

The radioactive zone at R_f 0.7 showed immunoreactivity of ~75–80% with excess antibody and a non-specific binding of <10%. R_f of cold progesterone-11α-hemisuccinate–TME conjugate was 0.53 whereas progesterone-11α-hemisuccinate-^{125}I-TME was 0.7 thus separating the cold conjugate from the radioactive conjugate. Specific activity of the tracer calculated by self-displacement method was found to be ~740 μCi/μg [8]. The percent binding remained stable over a period of two months with no significant increase in the non-specific binding. Paper electrophoresis of the reaction mixture of radioiodinated progesterone-11αhemiphthalate-TME conjugate showed a radiolabeling yield of 75–78% (Fig 5). Preparative TLC of the purified radiotracer using solvent system as chloroform: methanol: water (90:10:1) showed R_f of 0.83 and that of cold TME conjugate of progesterone-11α-hemiphthalate in the same solvent system as 0.46. The radioactive zones were extracted in ethanol. The specific activity of the radiotracer when calculated from the iodination yield was found to be 450 μCi/μg whereas when calculated by RIA displacement method, it was found to be 1500 μCi/μg. The high specific activity obtained from displacement method is perhaps due to the effective separation of uniodinated and radioiodinated phthalate–TME conjugates as evident from their respective R_f values as mentioned.

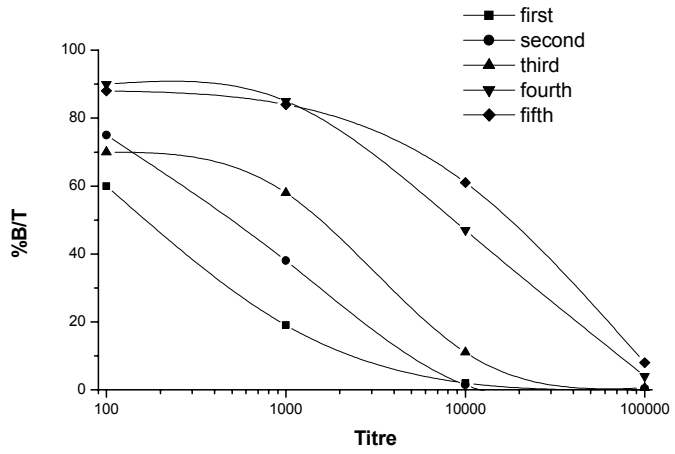


FIG.1. Titre curves of antiserum from rabbit R4 immunized against P-11αHS-BSA.

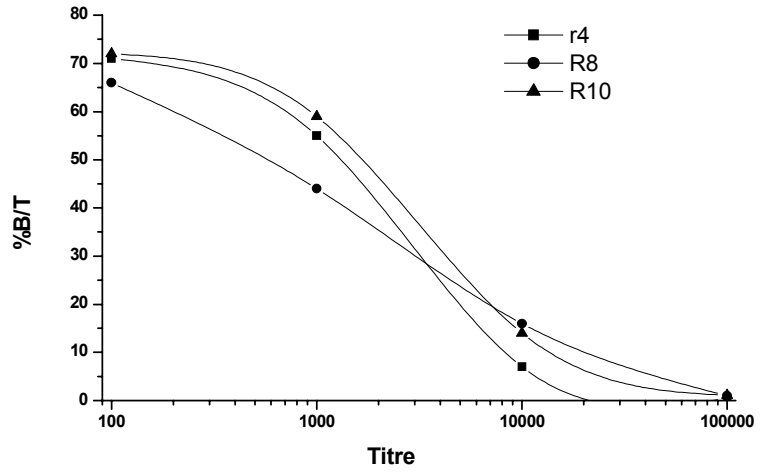


FIG. 2a. Titre curves of antibodies from three rabbits immunized against P-11αHS-BSA.

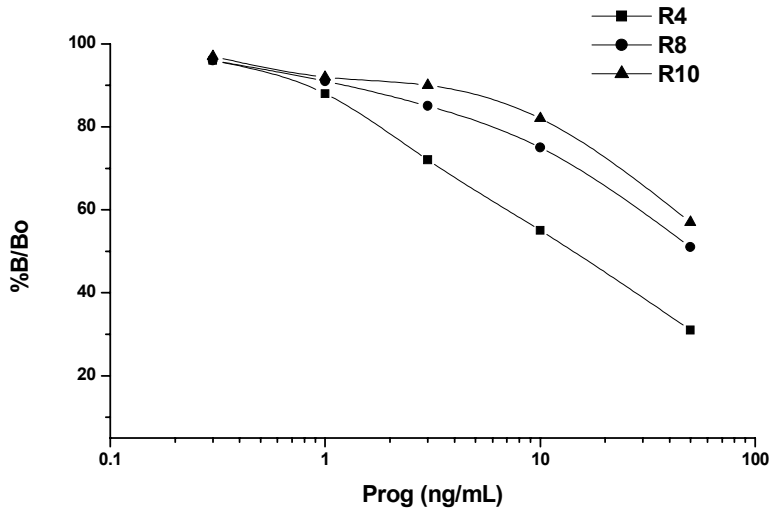


FIG. 2b. Standard curves with the antisera from three different rabbits at their optimum titre.

Immunoreactivity of the extracted fraction with excess of progesterone antibody showed a specific binding of ~ 65% and a non-specific binding of <10%. This tracer had lower stability than the other tracers, probably due to its higher specific activity.

TABLE I. CROSS REACTIVITY OF Ab AGAINST PROGESTERONE-11α-HS–BSA

	% Crossreactivity	
	Progesterone 11α-HS-^{125}I-histamine	Progesterone-3CMO–^{125}I-histamine
Progesterone	100	100
17α hydroxy progesterone	2	1.2
Hydrocorticosterone	0.1	0.01
Estriol	0.01	0.001
Corticosterone	2	0.9
Testosterone	0.64	0.09
Pregnane 5β, 3,20 dione	9	17
Danazol	0.006	0.05

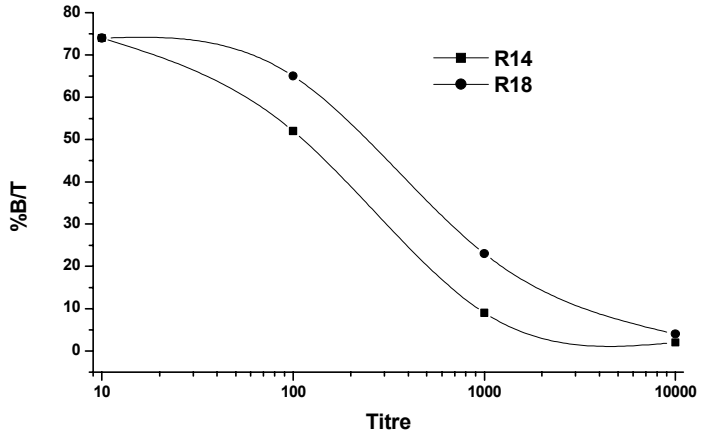


FIG. 3. Titre curves of antiserum from two rabbits immunized against P-3CMO-BSA.

TABLE II. CROSS REACTIVITY OF Ab AGAINST PROGESTERONE-3CMO -BSA

Cross reactants	% Crossreactivity
	Progesterone 3 CMO–^{125}I-histamine
Progesterone	100
11α hydroxy progesterone	1.4
17α hydroxy progesterone	4.8
Hydroxy corticosterone	0.001
Estriol	0.001
Corticosterone	0.09
Testosterone	0.2
Pregnane 5β, 3,20 dione	150
Danazol	0.001

TABLE III. R_f VALUES OF VARIOUS CONJUGATES ON TLC

Conjugates	C_6H_6:Ac: CH3OH 5:5:2	$CHCl_3$:CH_3OH 80:20	$CHCl_3$:CH_3OH :H_2O 90:10:1	C_6H_6:C_2H_5O H :AcOH 75:24:1
Tyrosine methyl ester (TME)	0.52			
Progesterone-11-HS	0.73			
Progesterone-11-HS-TME	0.84			
11α-OH-Progesterone			0.65	
Phthalic anhydride			0.5	
Progesterone-11-phthalate	0.77	0.58	0.25	
TME	0.5	0.3		
Progesterone-11-phthalate-TME	0.84	0.7		
Progesterone-11-HS-TME			0.53	
Progesterone-11-HS-^{125}ITME			0.7	
Progesterone-11-HS				0.4
Progesterone-11-HS-^{125}Ihistamine				0.56
Progesterone-11-phthalate- TME			0.46	
Progesterone-11-phthalate-^{125}ITME			0.83	
Progesterone-3-CMO				0.33
Progesterone-3-CMO-^{125}Ihistamine				0.54

4.2.2. Radioiodination of histamine conjugates of progesterone

The radioiodination yield of histamine as estimated by paper electrophoresis was about 45-65% (Fig. 6). The iodinated histamine was coupled to the activated progesterone derivative. On extraction of the reaction mixture at acidic pH, unconjugated progesterone derivative is removed in the toluene layer while the free iodide and the radioiodinated histamine not coupled to progesterone derivative along with the radioiodinated progesterone conjugate remained in the aqueous phase.

The progesterone-histamine conjugate predominantly extracted into the toluene layer at neutral pH. Radioiodination yield as indicated by extraction in the toluene layer at neutral pH was 15–25%. TLC of the toluene extract further purifies the radioactive conjugate from traces of impurities. In TLC, using solvent system benzene:ethanol:acetic acid (75:24:1) the radioiodinated progesterone-11α-hemisuccinate and progesterone-11α-hemisuccinate-^{125}I-histamine showed a R_f of 0.4 and 0.56, respectively. The R_f's being very close, separation becomes very crucial, as the presence of unconjugated progesterone hemisuccinate in the labeled compound will reduce the specific activity of the tracer. The radiochemical purity of this purified radiotracer was 98%. The specific activity of the tracer by displacement analysis was found to be only 460 μCi/μg probably due to the close R_f values of the progesterone-HS and the radiolabeled compound. However, the immunoreactivity studies showed ~85% binding with excess of progesterone antibody with a non-specific binding of <10%. The labeling studies with histamine resulted in overall low yields (15–20%) with respect to the iodine

activity used. This in turn necessitates the need for handling a larger quantity of activity for iodination to get reasonable amounts of labeled compound when it needs to be scaled up to production levels. In TLC, using solvent system as benzene: ethanol: acetic acid (75:24:1), the progesterone-3CMO and progesterone-3CMO-^{125}I-histamine showed R_f of 0.33 and 0.54, respectively. This separation is also very crucial, as the presence of unconjugated progesterone-3-CMO in the labeled compound will reduce the specific activity of the tracer. The specific activity of the tracer by RIA displacement method was found to be 522 μCi/μg. The radiochemical purity of the purified radiotracer was 98%. The immunoreactivity results showed a binding of >80% with excess of progesterone antibody where the non-specific binding was <10%.

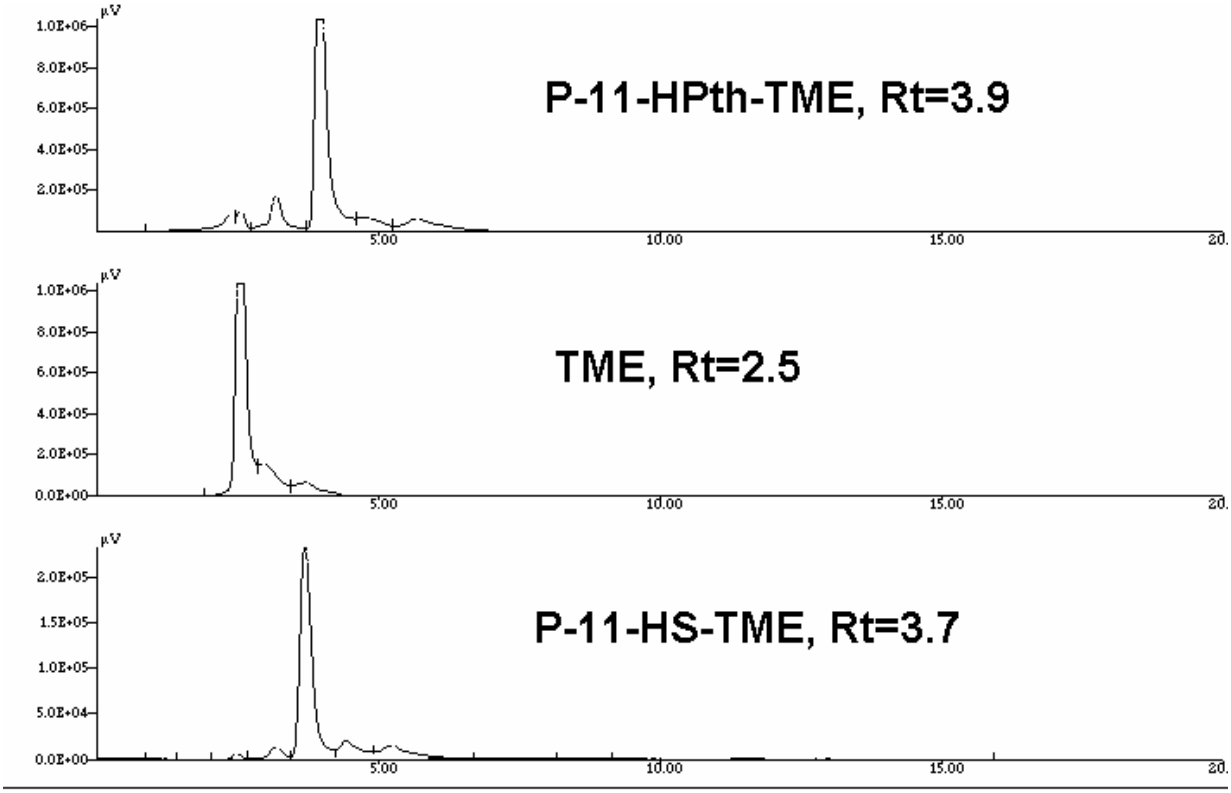


FIG. 4. HPLC of TME and progesterone-TME conjugates.

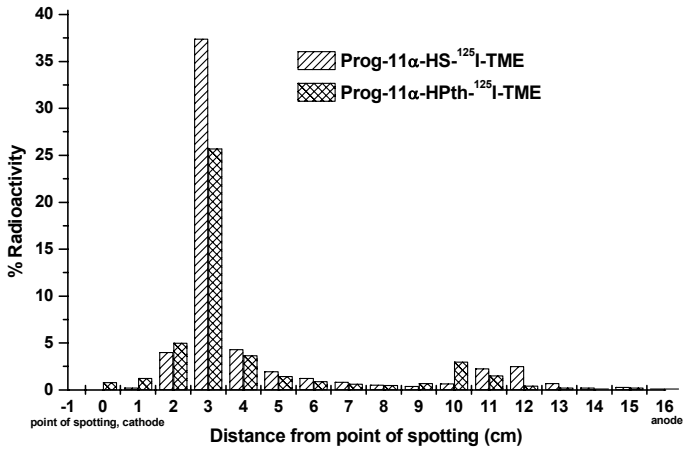


FIG. 5. Paper electrophoresis of radioiodinated progesterone-TME conjugates.

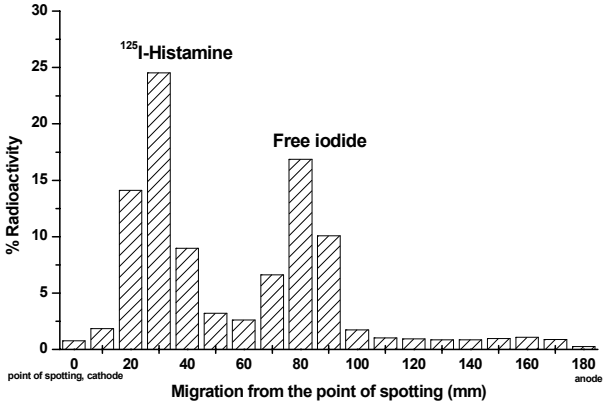


FIG. 6. Paper Electrophoresis of radioiodinated histamine.

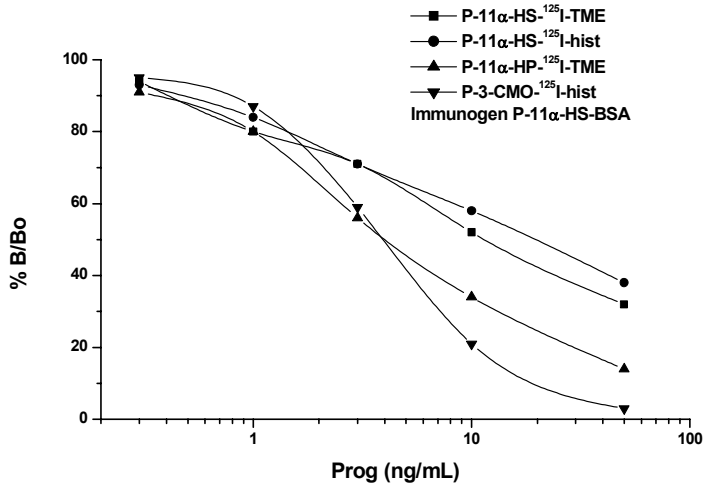


FIG. 7. Standard curves with different tracers.

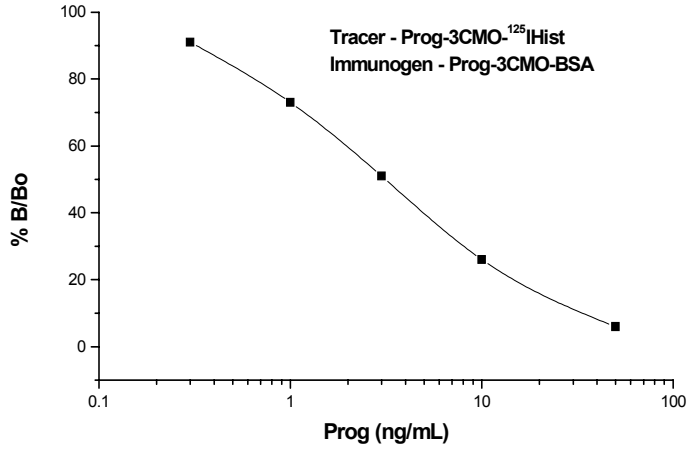
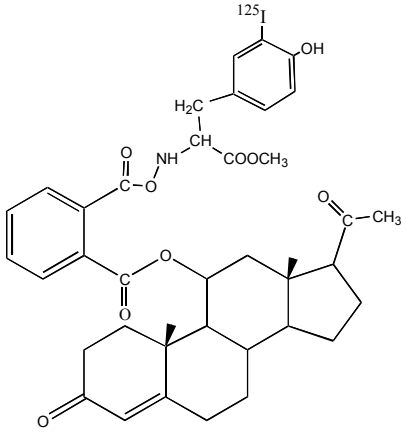
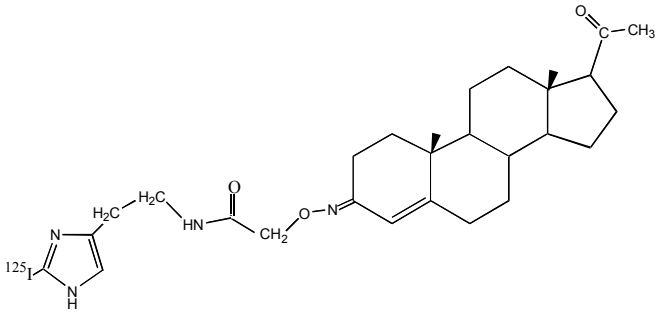


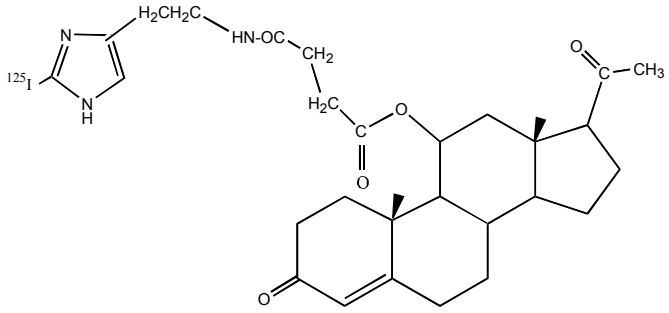
FIG. 8. Standard curve using 3CMO linkage in tracer and immunogen.



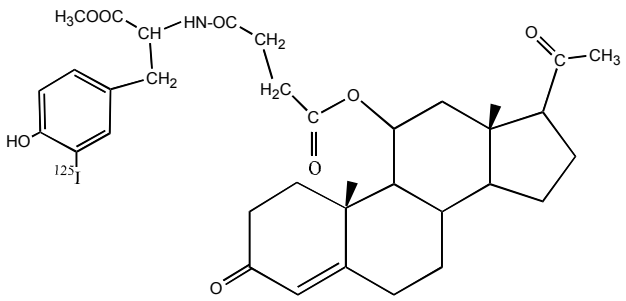
Progesterone-11α–hemiphthalate-^{125}I-TME



Progesterone-3CMO-^{125}I-histamine



Progesterone-11α-hemisuccinate-^{125}I-histamine



Progesterone-11α-hemisuccinate-^{125}I-TME

FIG. 9. Four different progesterone tracers.

TABLE IV. FEATURES OF LIQUID PHASE RIA FOR PROGESTERONE IN BOVINE SERUM

Parameters	Value
Sensitivity	0.4 ng/mL
Range	0.4 – 25 ng/mL
Intra-assay precision	3.4 – 9 %
Inter-assay precision	< 12 %
Recovery test	85 – 115 %
Parallelism	87 – 110 %

In the present studies, various heterologous and homologous assays (Fig. 7) were evaluated in a human serum system to study its influence on assay sensitivity, range and slope of the assay. The homologous assay using a common hemisuccinate bridge for both tracer (progesterone-11α-HS-^{125}I-histamine or progesterone-11α-HS-^{125}I-TME) and immunogen (progesterone-11αHS-BSA) resulted in a standard curve with poorer slope (slopes -0.5 and -0.6). The heterologous bridge assay system with the hemisuccinate linkage in immunogen (progesterone-11αHS-BSA) and the phthalate linkage in the tracer (progesterone-11α-Hpth-^{125}I-TME) and a heterologous site system such as hemisuccinate linkage at the position 11 in the immunogen (progesterone-11α-HS-BSA) and carboxymethyl oxime linkage at position 3 in the tracer (progesterone-3CMO-^{125}I-histamine) resulted in standard curves with better slopes viz -0.8 and -1.2, respectively. The homologous assay system using the CMO linkage in both immunogen and tracer resulted in a standard curve (Fig 8) with comparatively better slope (-0.97) but the antiserum showed high cross reactivity with the immediate metabolite and hence cannot be used for estimation. The different tracers are shown in Figure 9.

4.3. Liquid Phase Assay Procedure For Bovine Serum And Milk

The standard curves obtained for liquid phase assay for bovine serum samples with three tracers and antiserum against P-11α-HS-BSA are shown in Fig.10. Among these, the standard curve with tracer P-3-CMO-^{125}I-histamine gave the best slope. The imprecision profile for the system employing anti P-11α-HS-BSA and P-3-CMO-^{125}I-histamine is shown in Fig 11. The assay characteristics with the optimized system are shown in Table IV. Figure 12 gives the average of standard curves carried out by 20 different persons. This system is used for kit formulation. Fig 13 gives the liquid phase milk assay with two tracers.

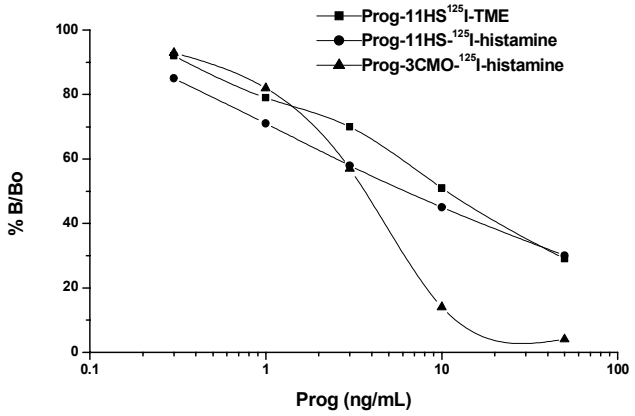


FIG. 10. Standard curve for liquid phase bovine serum.

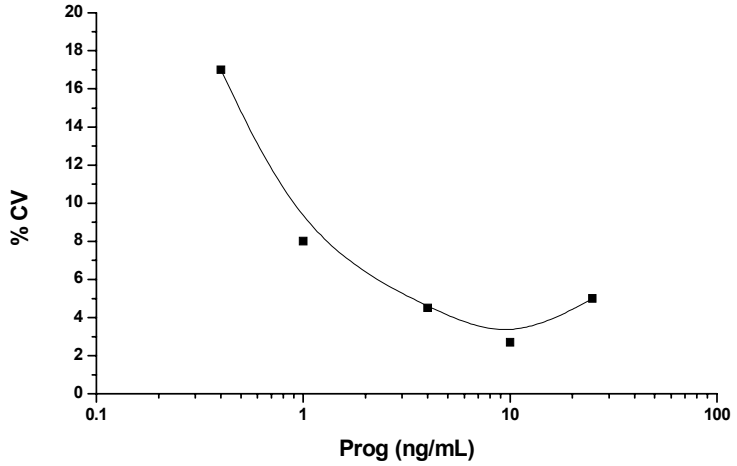


FIG. 11. Imprecision profile for liquid phase assay in bovine serum.

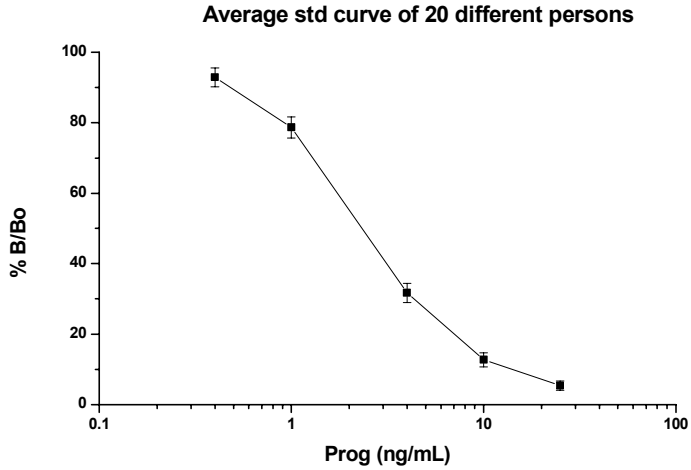


FIG. 12. Average of 20 standard curves obtained from20 different persons.

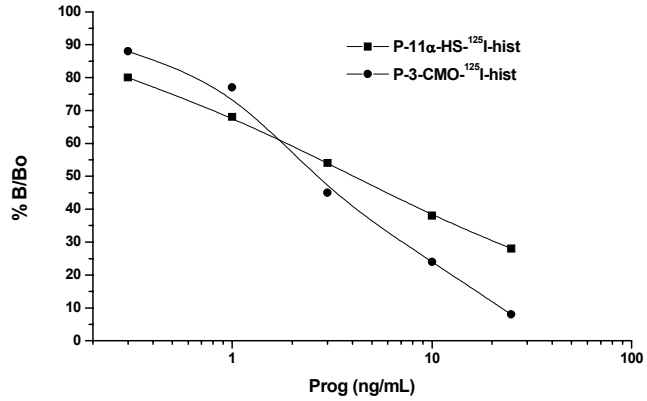


FIG. 13. Standard curve for liquid phase milk assay.

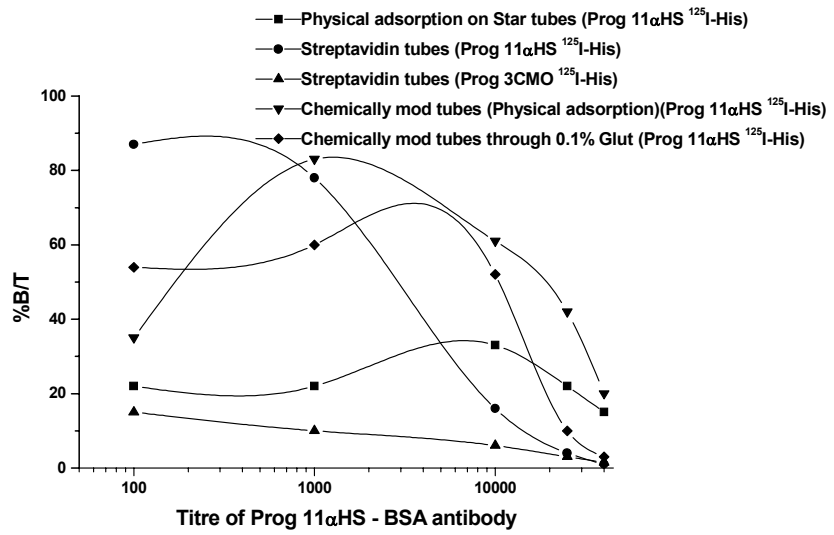


FIG. 14. Titre curve for the different solid phases for milk assay.

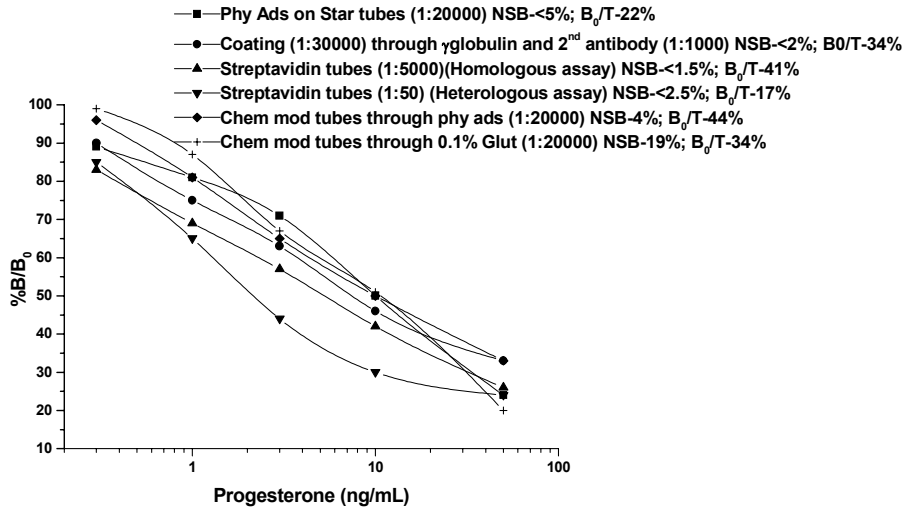


FIG. 15. Standard curves for the different solid phases for milk assay.

4.4. Solid Phase Assay for Bovine Serum and Milk

Four different solid phases were evaluated for their suitability to develop assays. Figure 14 shows the titres of the primary antibody for the different solid phases. It was observed that the titres widely differed with the solid phase used. The highest titre was obtained for the chemically modified tubes. Physical adsorption worked well only with imported star tubes. Local tubes on physical adsorption of antibody were found to give poor precision (>10% CV). Hence, efforts were made to improve the precision as well as to enhance the binding capability of the antibody by introduction of amino groups on the tubes. Aniline was used for this modification.

Figure 15 gives the standard curves for all the solid phase assays in milk. The chemically modified local tubes showed an optimum binding of ~45 % at 1:20000 dilution of antibody with 5% NSB with a homologous tracer whereas a heterologous tracer a NSB of 18-20% and a low binding of ~12% at the optimum titre. But the procedure for preparation of the coating of tubes by chemical modification was very critical. Studies with streptavidin coated tubes showed that the homologous assay system using these tubes had better characteristics in terms of sensitivity, non-specific binding and precision. The non-specific binding was ~1%. The binding was ~40-45% at an optimum titre of 1:5000. The heterologous system showed a low binding of 15-20% at a titre of 1:50 with NSB <2.5%. The coating procedure was short and the NSB was very low as compared to other system. However, the use of streptavidin-coated tubes will add to the cost of a kit.

Another system that was found suitable was the assay with the tubes coated with antibody through the rabbit gamma globulin and second antibody. In this system the non-specific binding was 2-3%, the primary antibody had a higher titre, the tubes showed high precision, the slope of the standard curve was reasonably good, but the coating procedure was long and due to the number of steps during the coating procedure, a coating machine will become mandatory. Figure 16 depicts the standard curves for assays in bovine serum. Although similar observations were made for assays in milk, the milk samples showed less reproducibility due to the fat content.

In essence, the bovine serum based liquid phase assay employing antibody against P-11αHS-BSA and P-3CMO-^{125}I-histamine as tracer is feasible for large-scale production. A solid phase assay for bovine serum and milk assay is also feasible with either streptavidin-coated tubes or gamma globulin and second antibody-coated tubes provided one has a coating machine.

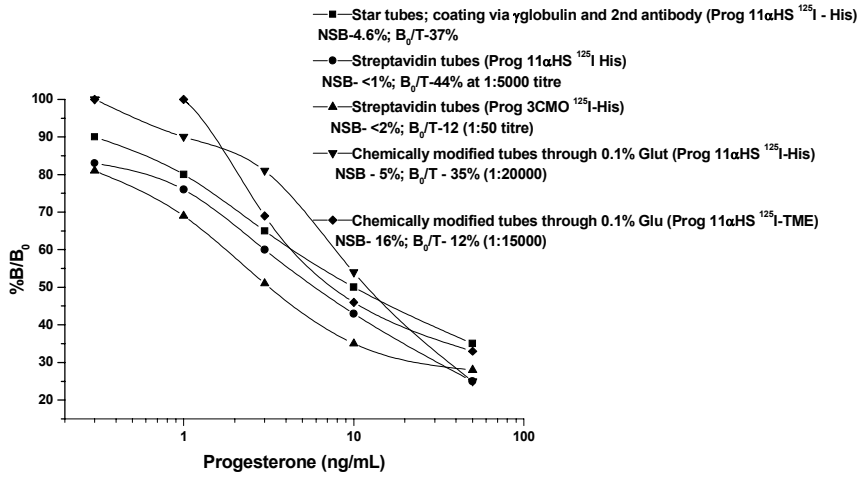


FIG. 16. Solid phase assays for progesterone in bovine serum.

5. CONCLUSION

A liquid phase assay for measurement of progesterone in bovine serum was standardized. Kit formulation for liquid phase veterinary progesterone with all in-house reagents has been carried out. The assay system uses polyclonal antibody raised against progesterone-11α-HS-BSA and progesterone-3-CMO^{125}I-histamine derivative as the tracer. The assay procedure involves addition of three components viz. standard or sample, tracer and antibody with a short incubation of 1 h at 25°C. Second antibody-polyethylene glycol precipitation is used for separation of bound and free. The assay has a range of 0.4-25 ng/mL and uses 50 µL of sample.

The assay validation that included the quality control parameters such as precision profiles, parallelism, recovery tests and quality control samples has also been carried out. Several solid phases

have been evaluated for coating of antibody. Coating of antibody through streptavidin-biotin system and through second antibody was found to be the most suitable. Scaling it to a regular production will demand a coating machine.

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REFERENCES

[1] MILLER, W.L., Molecular biology of steroid hormone synthesis. Endocrine Rev. **9** (1988) 295 318.

[2] KOTHARI, K., PILLAI, M.R.A., Preparation and characterisation of ^{125}I labeled progesterone derivatives for the development of a radioimmunoassay for progesterone, J. Radioanal. & Nucl. Chem. Articles. **177** (1994) 261-269.

[3] ALLEN, R.M. AND REDSHAW, M.R., The use of homologous ^{125}I-radioligands in the Radioimmunoassay of Progesterone, Steroids. **32** (1978) 467-487.

[4] GRACE, S., MEERA, V., BALAKRISHNAN, S.A. AND SARMA, H.D., Preparation of ^{125}I labeled testosterone derivatives for use in radioimmunoassay, J. Radioanal. & Nucl. Chem. Articles. **223** (1997) 83-87.

[5] PETROU, P.S., KAKABAKOS, S.E., KONPPARIS, M.A. AND CHRISTOFIDIS, I., Antibody coating approach involving gamma globulin from non-immunised animal and second antibody antiserum, J. Immunoassay. **19** (1998) 217-293.

[6] Biotinylation of IgG. Ed Gosling J. P., Immunoassays.: A Practical Approach, Protocol 15, (2000) p123

[7] CORRIE, J.E.T., HUNTER, W.H. AND MACPHERSON, J.S., A strategy for Radioligand, Clinical Chemistry. **27** (1981) 594-599.

[8] MORRIS, B.J., Specific Radioactivity of Radioimmunoassay Tracer determined by Self-Displacement: A Reevaluation, Clin Chim Acta. 73 (1976) 213-216.

DEVELOPMENT OF RADIOIMMUNOASSAYS KITS FOR NON CLINICAL APPLICATIONS : LOCAL PRODUCTION OF PRIMARY REAGENTS FOR MILK PROGESTERONE

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Abstract

The aim of the work was to develop a radioimmunoassay (RIA) kit using coated tubes for the detection of progesterone in cow's milk. The polyclonal anti-progesterone antibodies generated in rabbits were coated on polypropylene tubes. The radioiodinated progesterone was prepared using chloramine T method. Series of milk standards were made by dilution of bulk standard solution with progesterone-free milk. The antibody with titre at 1:12,000 was obtained after one primary injection and eight boosters. Preparation of ^{125}I-progesterone resulted in an average yield of 80% with a radiochemical purity of 97%, specific activity of 395 μCi/μg and stability of two months. Comparison of the prepared milk progesterone standard with standards from commercial kit was performed which showed a correlation coefficient of 0.95. The sensitivity of the assay was 2.3 ng/mL with a non-specific binding of 0.8% and an average B/T of 30% at zero standard.

1. INTRODUCTION

Progesterone level in cow's milk can be used to detect estrus onset in the animal in order to ensure optimum fertility and reproduction. Monitoring of cow's milk for progesterone levels is desirable both for its convenience and cost effectiveness. Studies were taken up to develop an assay for estimation of progesterone in cow's milk, due to the non-availablility of a local kit and the high cost of the imported commercial kit. The present study describes the work carried out to develop an immunoassay method to determine progesterone in milk sample with high sensitivity. Radioimmunoassay (RIA), which is an in-vitro technique, is a sensitive and specific method suitable for identification of low levels of substances such as progesterone. Reported procedures have used polyclonal anti-progesterone in RIA [2,3,5] as well as in ELISA [9]. Monoclonal antibodies have been used in RIA for the detection of buffalo milk progesterone [8,9]. In this project, the development of RIA kit for progesterone was carried out using anti-progesterone antibodies coated on Nunc tubes.

2. MATERIALS

Progesterone-11α–hemisuccinate and progesterone-11α–hemisuccinate-TME were provided by Bhabha Atomic Research Centre, India. Na^{125}I was purchased from Nordion. Progesterone (4-Pregnen-3,20-dione) and other chemicals were obtained from Sigma Chem Co., USA. Multiple Gamma Counter (DPC), vortex mixer (Thermolyne, USA), Eppendorf pipettes of varying volumes (5-1000μL) polypropylene star bottomed tubes (NUNC, Denmark), tips for the pipettes (IPHA, Indonesia) were used during the study. The data from assay was processed using IAEA immunoassay data processing program.

3. METHODS

3.1 Homologous assay

Anti-progesterone used for coating the tubes was generated using progesterone-11α–hemisuccinate-BSA, and the tracer was produced by iodination of progesterone-11αhemisuccinate-TME.

3.1.1. Production of polyclonal anti-progesterone

The procedure from Thorell *et al* [7] was adopted for the preparation of the BSA conjugate. 26 mg of progesterone-11α–hemisuccinate was mixed with 1 mL dioxane and 20 µL tri-n-butylamine (0.30 mmol). The mixture was cooled to a temperature of 11ºC and 10µL isobutylchloroformate was added. The mixture was allowed to react at 4ºC for 20 min. 4 mL of 84 mg BSA in dioxane: water (1:1) was added to the mixture in 4ºC and stirred for 4 h in 4ºC, the pH was adjusted to 8-9 using 0.1M NaOH. The mixture was dialysed for 24 h against distilled water. The pH was adjusted to 4.5 using HCl and stored for 4 days in 4ºC. The mixture was centrifuged at 2500 rpm for 15 min. The supernatant was discarded and the pellet was redissolved in 1 mL aquadest. A 200 µL of 5% $NaHCO_3$ was added and redialysed for 4 h using aquadest. The conjugate was dispensed as 40 µL aliquots into a vial and stored at -20^{o}C.

1 mL of NaCl and 1.2 mL Freund's adjuvant complete were mixed with 3 vials (300 µg/40µL/vial) of the above imunogen until an emulsion was obtained. The emulsion was injected into two rabbits. Primary injection was given subcutaneously at multiple sites on the back of the rabbits. Further boosters were given at intervals of one-month each.

Evaluation of polyclonal anti-progesterone

The blood from the rabbits was collected after every booster injection, and the serum was used for determining the titre. Serial dilutions were made and the binding with progesterone tracer was studied to determine the titre. Cross reactivity of the polyclonal anti-progesterone to several other cross reactant were also evaluated.

Preparation of antibody-coated tubes

The procedure for immobilization of anti-progesterone on polypropylene tubes was adopted from Joint FAO/IAEA Programme in Animal Production and Health [1] "Self Coating Milk Progesterone RIA Kit" with a slight modification. A 500 µL of anti-progesterone serum raised in rabbit at 1:12500 dilution in 0.05 M carbonate-bicarbonate buffer pH 9.6 was dispensed into a polypropylene star-bottomed tube (NUNC). Three hundred tubes were coated in one batch. All the tubes were incubated overnight at 4ºC. The tubes were decanted and washed with 2x500 µL of distilled water containing tween-20. The tubes after coating were stored at 4ºC.

3.1.2. Preparation of ^{125}I-Progesterone

The procedure by Kothari *et al* was adopted for the preparation of the tracer. [4]. 10 µL (2.5µg) of methanolic progesterone-11α–hemisuccinate-TME in a tube was mixed with 10 µL of 0.5 M phosphate buffer pH 7.5 and 1mCi $Na^{125}I$ (± 2.5 µL). 10 µL (10µg) of chloramineT in 0.05M phosphate buffer pH 7.5 was added to the mixture. The tube was gently mixed in a vortex mixer for 1 minute and 25 µL (25µg) of $Na_2S_2O_5$ in 0.05 M phosphate buffer was immediately added into the reaction tube. The mixture was loaded on a PD-10 column which was saturated with 1mL of 5% BSA. Fractions of 500 µL per tube were eluted with 0.05 M phosphate buffer pH 7.5. The peak fraction was stored in methanol (1:1) in a dark bottle at 4ºC. The radiochemical purity was determined by electrophoresis and the immunoreactivity of the peak fraction was evaluated by studying the binding of the tracer with antibody.

3.1.3. Preparation of standards in milk

Preparation of progesterone standard in milk was adopted from the method of FAO/IAEA (1). 0.1% sodium azide was added to the milk followed by centrifugation at 2000 rpm for 20 min. After leaving it for 15 min at room temperature, the fat layer was discarded and the milk stored at 4^{o}C. 50 mg of progesterone (4-pregnen-3,20-dione) was dissolved in 12.5 mL methanol. 0.5mL of this solution was pipetted into a 10 mL volumetric flask. The methanol was evaporated by flushing N_2 gas. Centrifuged

milk was added to the flask up to the mark. A 50 μL of this solution was pipetted and placed in a 50 mL flask. Milk was added up to the mark. Concentration of this bulk solution was 200 ng/mL. Series of standard solutions of concentration 0, 0.25, 0.5, 1, 5, 10, 20 and 50 ng/mL were made from this stock solution.

4. RESULTS AND DISCUSSION

4.1. Homologous assay

4.1.1. Production of polyclonal anti-progesterone and coated tubes

Polyclonal anti-progesterone generated in rabbits gave a titre value of 1:12000 after one primary injection and eight boosters. 50 mL of blood was collected which gave 12 mL of serum after centrifugation. Aliquots of 500 μL of the diluted antibody were stored in freezer (-20^{0}C). The anti-progesterone antiserum was incubated with progesterone, pregnenolone, corticosterone, 17α-11β-hydroxyprogesterone, cortisone, testosterone and less then 1% cross reactivity was observed. Three hundred tubes were prepared in one batch of anti-progesterone coated-tubes. All the tubes were dried and stored at 4ºC in a sealed container.

4.1.2. Production of ^{125}I-progesterone

^{125}I-progesterone tracer was prepared using chloramineT method. High radiochemical purity of more than 97% with specific activity 395 μCi/μg and an average yield of 80% were obtained. Iodinated progesterone was purified on PD-10 column and the fractions were collected and counted in gamma counter. Three peaks were obtained which were tested for their characteristics including the non-specific binding, maximum binding, radiochemical purity and yield (Table I). The third peak at tube fraction no. 30 gave higher maximum binding (68.9% Bo/T) compared to the other two peaks (13.6% and 1.5% Bo/T respectively). The radiochemical purity was 93.3%. Several preparations showed consistency in this pattern and hence this fraction was used for tracer as ^{125}I-progesterone in further study.

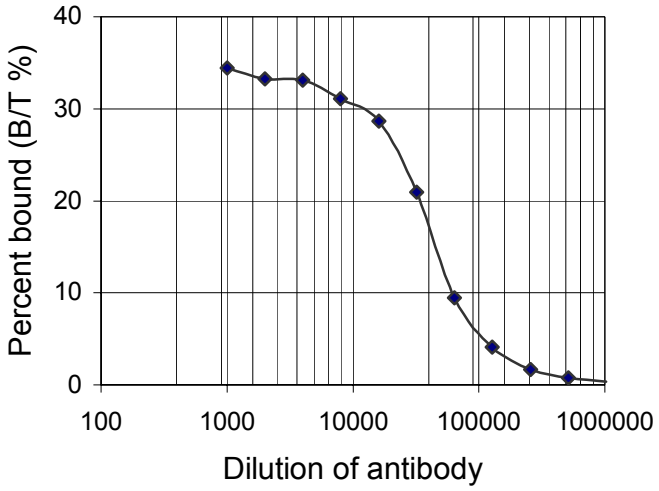


FIG 1. Titre curve of polyclonal anti-progesterone serum raised in rabbit.

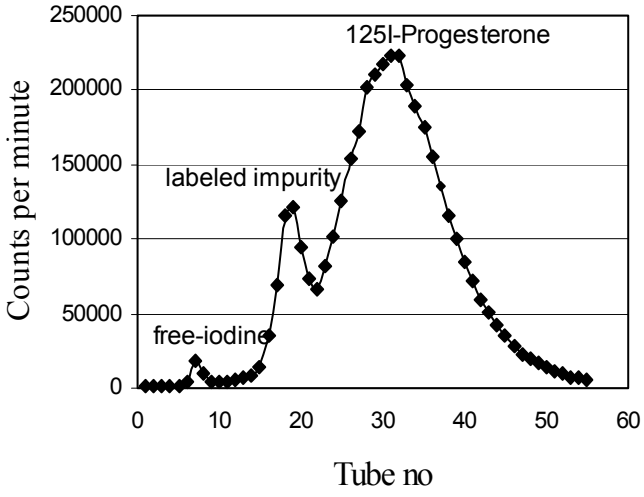


FIG 2. Purification of ^{125}I-Progesterone on a PD-10 column..

TABLE I. EVALUATION OF PROGESTERONE TRACER

Characteristics	Peak no 1 (free ^{125}I)	Peak no 2 (labeled impurity)	Peak no 3 (^{125}I- progesterone)
NSB (%)	0.4	0.2	0.4
Bo/T (%)	13.6	1.5	68.9
Radiochemical purity (%)	12.5	13.7	93.3
Yield (%)	2.5	16.2	81.3

Stability of the ^{125}I-progesterone

The quality of the tracer was monitored using parameters of radiochemical purity, non-specific binding (NSB) and zero binding (Bo/T) as shown in Figure 3. A low NSB at less than 1% with a zero binding of 30% was obtained at an antisera dilution of 1:12000. The product was found to be stable for 2 months. The tracer showed a constant radiochemical purity of more than 97% during this period.

4.1.3. Preparation of progesterone standards

Series of progesterone standards using milk matrix with concentration ranging from 0 to 80 ng/mL were prepared.

4.1.4. Assay Optimization

Optimization of the assay was carried out using varying volumes of standard, time and temperature of incubation, volume and concentration of tracer. Volumes of 25, 50, 100 and 200 µL were studied. The results showed that 200 µL standard gave a steep curve with higher zero binding compared with other volumes (Figure 4). Incubation times of 1, 2, 3 and 4 h were studied (Figure 5). Three hours incubation at room temperature gave higher binding with better standard curve compared to the incubation of 1 or 2h. 4h incubation showed higher binding with lower sensitivity. Therefore three hour incubation was chosen for further study.

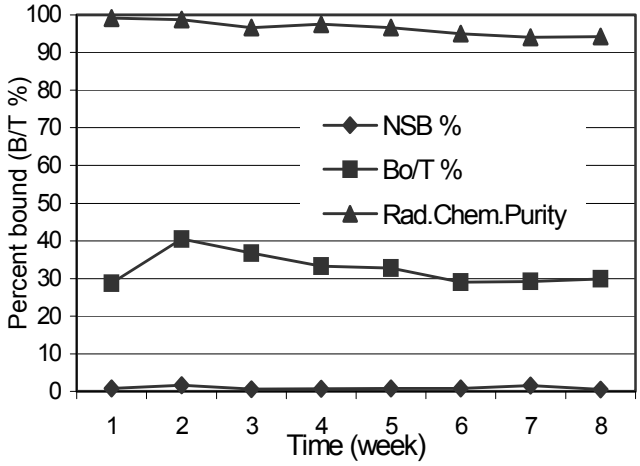


FIG 3. Stability profile of ^{125}I-progesterone.

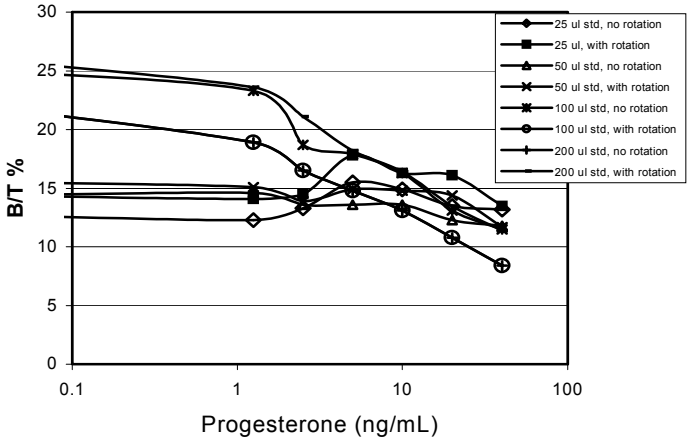


FIG 4. Effect of volume of standard on assay of progesterone.

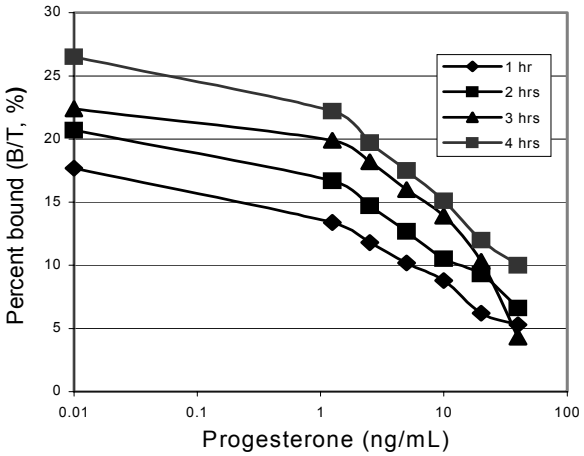
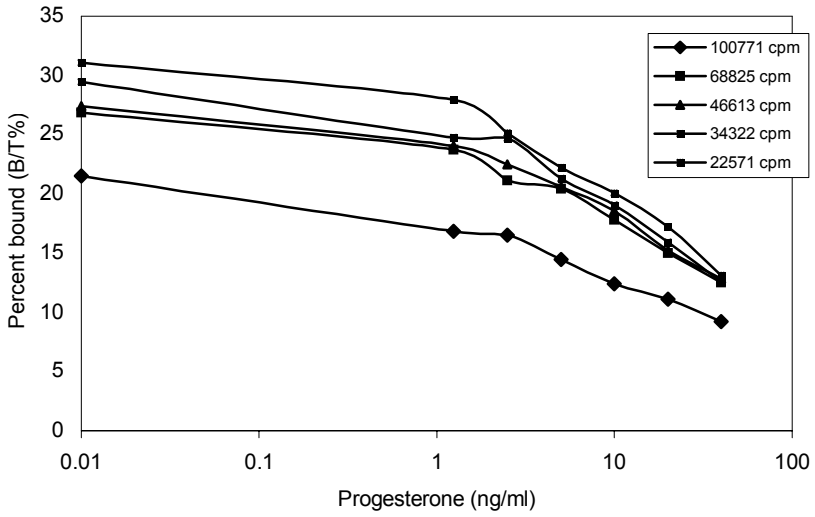


FIG 5. Effect of incubation time on the assay of progesterone.

Effect of tracer concentration on the assay was also studied using 20000 to 100000 cpm of tracer. The results gave a higher zero binding when using 23000 cpm compared to other concentration of tracer. Hence a tracer with activity of about 20000 cpm was used for further study. Effect of volume of the tracer in the assay was studied from 50µL to 400µL. The results showed very little difference in the percentage binding (Figure 6). However, 300µL was chosen for further study as it gave higher maximum binding compared to the other conditions A typical standard curve of the kit is given in Figure 7. The detection limit, which is the concentration as calculated from 2SD of 15 replicates of zero standard, was 2.3 ng/mL. Working range was determined from imprecision profile of the assay, and gave a range upto 10 ng/mL of progesterone



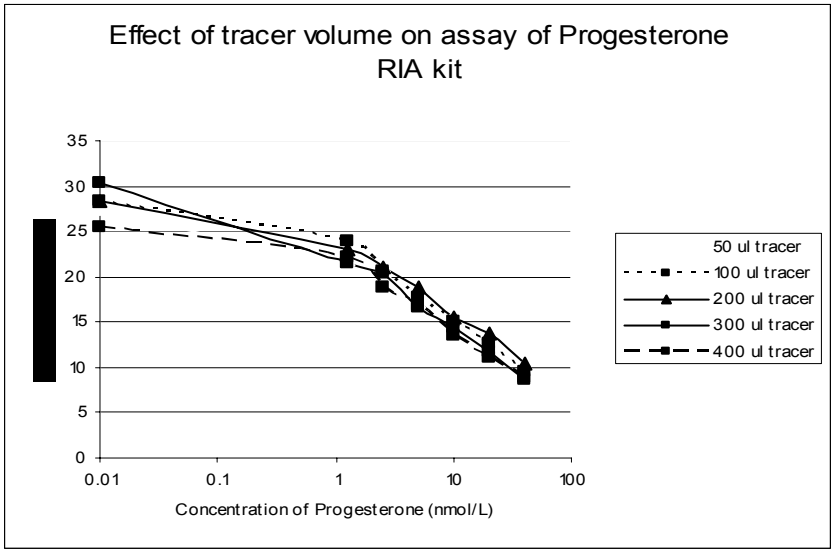


FIG 6. Effect of volume of tracer and counts on assay of progesterone.

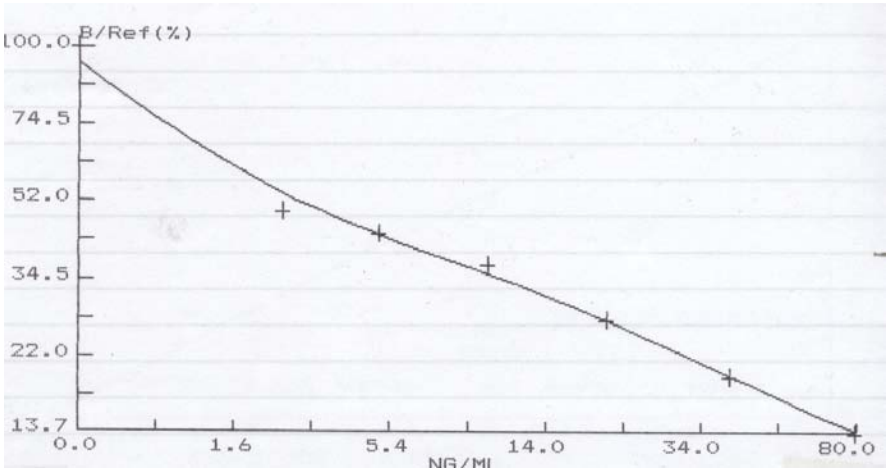


FIG. 7. Typical standard curve of the progesterone RIA kit.

TABLE III. WITHIN ASSAY VARIATION

Number	Number of replicates	Mean (ng/mL)	SD	% CV
1	5	6.3	0.22	3.6
2	5	17.0	0.27	1.6
3	5	91.4	1.48	1.6

TABLE IV. BETWEEN ASSAY VARIATION

Number	Number of replicates	Mean (ng/mL)	SD	% CV
1	15	4.2	0.18	4.3
2	15	14.6	0.56	3.9
3	15	83.2	3.12	3.8

Within assay at 5 replicates (Table III) and between assays at 15 replicates (Table IV) were performed at the optimized assay conditions. The assay used 200µL standard solution, 300µL of ^{125}I-progesterone with an incubation of 3h at RT. The within assay results showed a low CV of 1.6% when the standard concentration of progesterone was used at 17 ng/mL or 91.4 ng/mL while at a low concentration of 6.3 ng/mL, it gave a CV of 3.6%. Similar results were obtained for between assays. Stability of the kit was observed every week as shown in Figure 8. Binding percentage remained constant for a period of five weeks, but decreased slightly when the assay was performed at week 5 post-production

Sensitivity of the assay was obtained from the variation of zero standard (n=15) at 2SD. The results showed 2.3 ng/mL as the sensitivity of the assay.

4.2. Heterologous assay

The procedure was adopted from Kothari *et al* [5]. The synthesis of progesterone-11α-hemiphthalate-TME conjugate for the preparation of tracer involved two steps, first was the preparation of progesterone-11α-hemiphthalate and the second step was the conjugation of the progesterone phthalate derivative to TME.

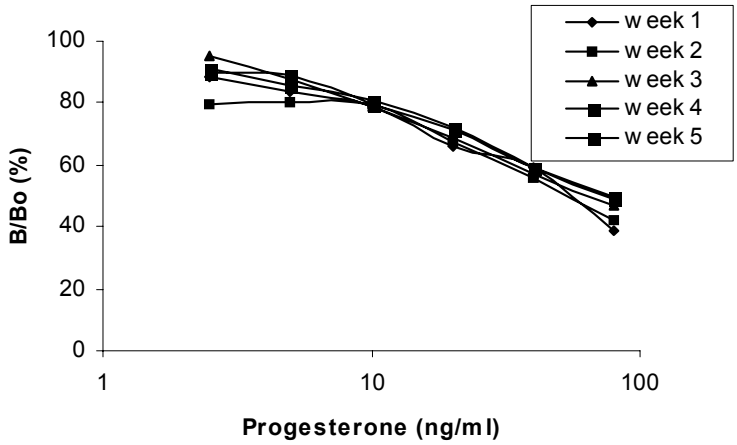


FIG. 8. Stability of progesterone RIA kit.

TABLE V. TLC USING SEPHADEX G-25 AS STATIONARY PHASE AND CHLOROFORM: METHANOL: H_2O (90:10:1) AS MOBILE PHASE

Synthesised compound	Rf	
	Reported [5]	Obtained
Progesterone-11α-hemiphthalate	0,22	0.40
Progesterone-11α-hemiphthalate-TME	-	0.70

TABLE VI. MELTING POINT

Synthesised Compound	Melting Point (^oC)	
	Reported [5]	Obtained
Progesterone-11α-hemiphthalate	239 - 241	239 – 243

The synthesised product was evaluated for purity using TLC and from its melting point data. Using TLC with sephadex G-25 as stationary phase and chloroform: methanol:H_2O (90:10:1) as mobile phase, a single spot was obtained in the synthesis of progesterone-11α-hemiphthalate (Table V). Conjugation of the derivative to TME also showed one spot at R_f 0.70. In the determination of melting point, the result obtained was close to that reported in literature (TableVI).

The NMR data was presented in Figure 9 and their chemical shifts are listed in Table VII. The data showed specific chemical shift for the reaction of hemiphthalate to the progesterone at 7.42-7.77 ppm. Based on these results, further studies were carried out. Progesterone-11α-hemiphthalate-TME was radioiodinated for use as a tracer.

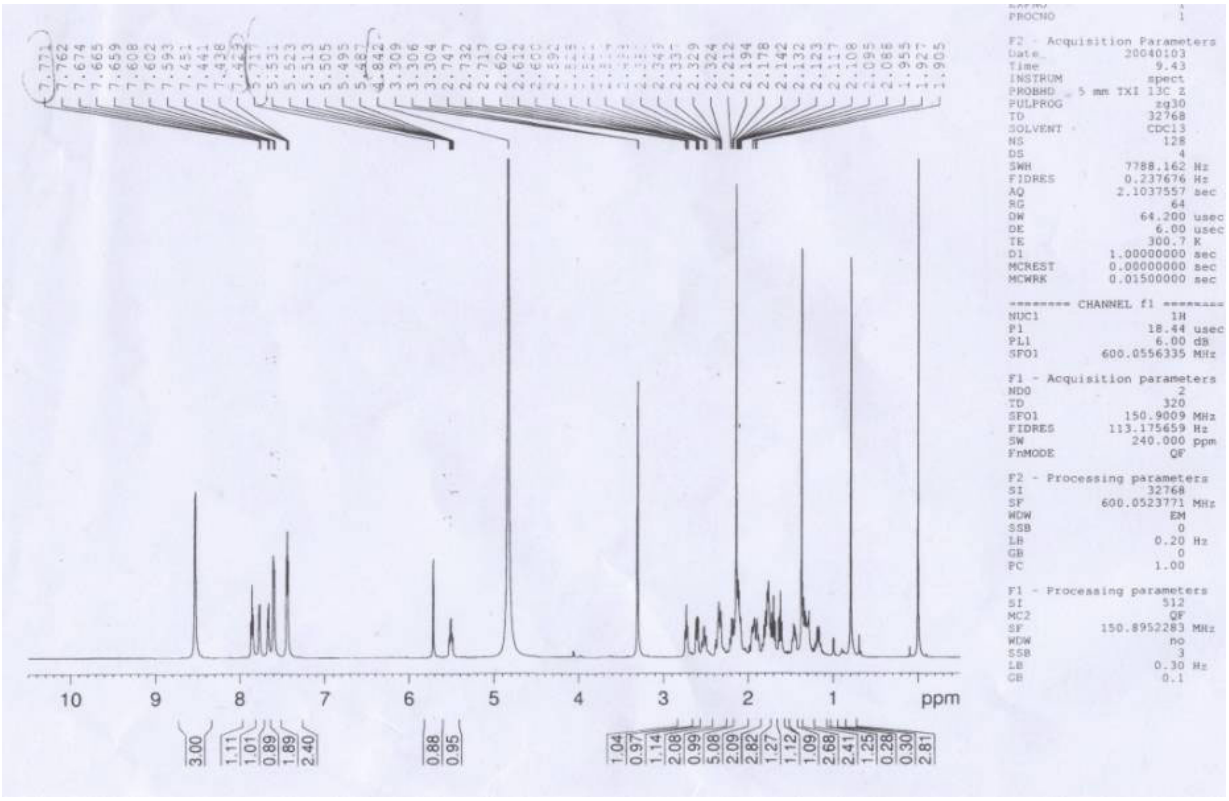


FIG 9. NMR of progesterone-11α–hemiphthalate with CD_3OD

TABLE VII. CHEMICAL SHIFT (δ PPM) FOR PROGESTERONE-11αHEMIPHTHALATE IN ^{1}H-NMR WITH CD_3OD

δ (ppm)		^{1}H
Reported [5]	Obtained	
0.81	0.79	3H, s. 18-C<u>H</u>$_3$
1.38	1.38	3H, s. 19-C<u>H</u>$_3$
2.16	2.14	3H, s. -COC<u>H</u>$_3$
5.47 – 5.56	5.50 – 5.71	1H, m, 11-<u>H</u>
7.60 – 7.81	7.71	4H, m, Ar-<u>H</u>

The progesterone-11α-hemiphthalate-^{125}I-TME was prepared using chloramineT method. The yield of iodination was obtained as ~33%. When the tracer was tested for immunoreactivity, the binding obtained was very low. The purity of the conjugate may have contributed to the result obtained.

5. CONCLUSION

A polyclonal antibody to progesterone was raised in rabbit. The immunogen used for the injection in the animal was progesterone-hemisuccinate-BSA at a dose of 200 μg. After one primary injection and 9 boosters, the antibody was harvested and the titre determined. The result showed a 1:12000 titer and the antisera was stored in a deep freezer after dilution and dispensing into smaller volumes.

A procedure for the preparation of the ^{125}I-progesterone using progesterone-hemisuccinate-TME has been standardised. The method used is chloramineT as it is simple, fast and direct (without extraction) resulting in reasonably good yield. After purification by PD-10 column, expected high radiochemical purity at an average of 97% and moderate immunoreactivity at an average of 30% were obtained accompanied with low non-specific binding value of less than 1%. The tracer was evaluated every week after production and found stable upto a period of eight weeks. These results support our expectation on the preparation of tracer. The above procedure was used as standard protocol for our tracer preparation.

The antiprogesterone-coated tubes prepared according to the FAO-IAEA method [1,6] gave about 30% binding. The tubes, which were stored in a sealed container in refrigerator, were checked for stability at weekly intervals. The kit was found to be stable for 4 weeks, which was less than the expected stability of more than two months. After the fifth week, the binding decreased. This condition was probably due to the instability of the coated tube. Efforts to increase stability of the kit was made by including pre-treatment prior to immobilization, increasing drying time and adding manitol as stabilizer, but the binding did not improve significantly. A change in the quality of antibody with time may also have caused the problem. The quality of the antiprogesterone after being stored at -20°C needed to be evaluated further.

The optimised assay was a homologous system with a common bridge in the conjugate of the tracer and the immunogen used for generating antibody, which were progesterone-hemisuccinate-^{125}I-TME as tracer and progesterone-hemisuccinate-BSA as immunogen for antibody production. A homologous bridge system was known to give lower sensitivity compared to a heterologous system [5, 10]. The present studies showed a sensitivity of 2.3 ng/mL for the homologous assay. Effort to obtain better sensitivity was made by preparing a heterologous bridge, progesterone-hemiphthalate-TME for the tracer and progesterone-hemisuccinate-BSA for the immunogen, but the synthesis of progesterone-hemiphthalate-TME was obtained at a low yield, which hampered the purification step. PD-10 column was used to purify the iodinated compound based on the results of purification of progesterone-hemisuccinate-TME. Further studies on various purification methods are underway.

A heterologous system was evaluated in which hemisuccinate bridge for immunogen for generating antibody and hemiphthtalate bridge for tracer were used. However, the synthesis of the hemiphthalate conjugate gave low yield that hampered the purification step, which in turn gave a tracer preparation with a low binding with the PD10 column purification. Hence, attempts are being made to improve the yield.

A radioimmunoassay kit for the measurement of progesterone in milk has been produced in the Center for Development of Radioisotopes and Radiopharmaceuticals (CDRR-BATAN, Indonesia) under the IAEA Research Contract No 11790/RO.

REFERENCES

[1] IAEA Regional Cooperative Agreement for Asia and The Pacific, RAS/5/035-Feed Supplementation and Reproductive Management of Cattle. Training Workshop on the Production of Iodinated Tracer for Self-Coating RIA of Progesterone, 8-12 May 2000, Office of Atomic Energy for Peace, Bangkok, Thailand.

[2] TARVEEN KARIR, P.R., CHAUDHURI, A., SAMAD, U.H., NAGVEKAR, N., SIVAPRASAD, Development of Direct Radioimmunoassay of Progesterone in Bovine Milk Book of Abstracts of the Int. Conf.on Isotope and Nucl. Anal. Tech. For Health and environment. June 2993, Vienna, IAEA-CN-103/167, p 133.

[3] KANCHAN KOTHARI, RAMJI LAL AND M.R.A. PILLAI, Development of A Direct Radioimmunoassay for Serum Progesterone, Journal of Radioanalytical and Nuclear Chemistry Articles, **196** (1995) 331-338.

[4] KANCHAN KOTHARI AND M.R.A. PILLAI., Preparation and Characterization of ^{125}I labeled Progesterone Derivatives for the Development of A Radioimmunoassay for Progesterone, Journal of Radioanalytical and Nuclear Chemistry Articles, **177**, (1994) 261-269.

[5] KANCHAN KOTHARI AND M.R.A. PILLAI., Direct Radioimmunoassay of Serum Progesterone Using Heterologous Bridge Tracer and Antibody, Journal of Radioanalytical and Nuclear Chemistry, **231** (1998) 77-82

[6] Joint FAO/IAEA Programme in Animal Production and Health, Self-coating 'Milk' Progesterone RIA Kit, Bench Protocol Version – SCRIA 3.0, January 1997

[7] J.I. THORELL AND S.M. LARSEN., Radioimmunoassay and Related Techniques, United States of America, (1978) 258.

[8] ROSANNA CAPPARELLI, DOMENICO IANNELLI AND ALDO BORDI.,, Use of monoclonal antibody for radioimmunoassay or water buffalo milk progesterone, Journal of Dairy Research **54** (1987) 471-477.

[9] WALDMAN., Enzyme immunoassay (EIA) for milk progesterone using monoclonal antibody. Animal Reproduction Science **34** (1993) 19-30.

[10] ROBERT, M., ALLEN AND MARTIN R. REDSHAW., The use of homologous and heterologous ^{125}I-radioligands in the Radioimmunoassay of Progesterone. Steroids, **32** (1978) p 467.

NEW RIA KIT FOR DETERMINATION OF PROGESTERONE IN COW MILK

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Abstract
The determination of progesterone concentration in whole and fat-free milk 19-24 days after conception enables to distinguish fertile and non-fertile insemination, which is important in cattle breeding. The aim of this work was to develop a simple and quick radioimmunoassay test for the determination of progesterone in cow milk. Two types of solid-phase tubes coated with specific polyclonal anti-progesterone antibody from ORION Diagnostica and BIOSOURCE International, two different progesterone derivatives viz. progesterone-3-carboxymethyl oxime (CMO) and progesterone-11α-hemisuccinate (HS) conjugated to ^{125}I-histamine and the HPLC system with Lichrospher RP-18 column along with 65% acetonitrile/water as eluent to purify the tracers were used to carry out this work. Progesterone-3CMO-^{125}I-histamine had a retention time of 13.2 min and progesterone-11α-hemisuccinate-^{125}I-histamine had a retention time of 7.8 min. Two kinds of kits (kit I and kit II) were prepared, first with progesterone-3CMO-^{125}I-histamine as the tracer and coated tubes from Progesterone Veterinary RIA kit of ORION Diagnostica and the second with progesterone-11αHS-^{125}I-histamine as the tracer and coated tubes from Progesterone Veterinary RIA kit from BIOSOURCE International. Progesterone from Sigma and selected fat-free cow's milk without progesterone as zero progesterone milk matrix were used for standard preparation. The optimal assay procedure was as follows: 50µL standards, controls and fat-free milk samples were pipetted into coated tubes followed by addition of 500µL of diluted tracer. The tubes were incubated for 2h incase of kit I and 3h for kit II at RT. After the incubation, the tubes were decanted and counted. The assay range was 0 to 270 nmol/L for kit I and 0 to 300 nmol/L for kit II. The sensitivity of the kit with ORION coated tubes was better (0.8 nmol/L) than that of BIOSOURCE tubes which was 1.5 nmol/L. Validation of these assays in terms of specificity, accuracy (recovery), precision (within-assay and between-assay variations) was carried out. The concentration of progesterone in milk samples from pregnant and non-pregnant cows determined using the new RIA test with ORION coated tubes and the commercial kit from ORION Diagnostica were highly consistent. One step, ready to use, simple, economical RIA tests for the determination of progesterone in cow milk were developed.

1. INTRODUCTION

Progesterone, a major hormone secreted from the corpus luteum, has two main biological functions. It transforms the estrogen-stimulated endometrium into the secretory phase, which allows implantation of the fertilized ovum. It also protects pregnancy by decreasing uterine contractility. The presence of corpus luteum is necessary for the maintenance of pregnancy in cows as in a vast majority of animals.

Progesterone has been found to be of significant clinical value in most domestic species. The cyclic behaviour of progesterone levels during the oestrous cycle and its relatively high levels during pregnancy resulted in this hormone being widely used as the pregnancy test. Also plasma progesterone, progesterone in whole milk, milk fat and fat-free milk has been estimated and a high correlation found between the values.

The development of radioimmunoassay (RIA) techniques for hormone determination in domestic species created laboratory procedures that are relatively simple to perform, inexpensive, specific and sensitive and have the potential to be used as diagnostic aids in reproductive studies.

One of the most important veterinary applications of RIA is its usage in the determination of progesterone in cow milk as an economical fertilization test in cattle. By determination of progesterone concentration in milk, insemination failures can be detected as early as 21-23 days after breeding. Cows inseminated when not in estrus can also be identified and a second insemination can be attempted. The economic advantage of this approach is obvious. Fertilization checks is considered as a routine procedure allowing better utilization of cows' reproductive potential in which an insemination failure would have been detected much later if determination of progesterone in milk was not used as an aid. To make the processing of such large number of samples economical in terms of

cost and time, progesterone determination should be attempted directly in whole milk without any prior sample extraction. However, in the laboratory, the best results have been obtained when fat-free milk was used. The different fat concentration in milk affects the progesterone concentration and separation of the fat thus substantially diminished this effect [1].

The aim of this work was to prepare a ready-to-use RIA kit for determination of progesterone in cow milk. In the developed kit, the tubes coated with specific antibodies were used as the solid phase. The assay is simple in operation, fast (single step) and acceptably accurate.

2. MATERIALS

Coated tubes were obtained from ORION Diagnostica, Finland and from BIOSOURCE International. The cross reactivity of the progesterone antiserum coated on the ORION tubes was as follows: progesterone-100%, pregnenolone-3.9%, corticosterone-0.9%, other progesterone derivatives<0.7%, other steroids <0.01% and the antiserum coated on the BIOSOURCE International tubes were progesterone - 100%, 5-alpha-pregnan-3,20-dione - 3.95%, 20-β-dihydroprogesterone - 1.83%, corticosterone - 1.07%, other progesterone derivatives and steroids - <0.7%.

Progesterone (99%), progesterone-3-O-carboxymethyl oxime (CMO), histamine dihydrochloride and ursodeoxycholic acid were procured from Sigma, USA and 4-pregnen-11α-ol-3, 20-dione hemisuccinate (HS) from Steraloids Inc. USA. Tri-n-butylamine, dioxane, isobutyl chloroformate, chloramineT, cortisol, danazol, acetonitrile were obtained from Merck, Germany and Na-^{125}I from IZOTOP, Hungary.

All milk samples (milk zero from non-pregnant cows, controls and unknown samples from pregnant cows) were received from Institute of Animal Breeding, Polish Academy of Science, Poland.

HPLC system with Lichrospher RP-18 column (250x4 mm), 5µm from Merck (Germany), with UV detectors was used for purification.

3. METHODS

3.1 Progesterone-3CMO-^{125}I-histamine and progesterone-11αHS-^{125}I-histamine

A three-step procedure for radioiodination of progesterone derivatives [2,3,4] was used.

Step 1- Activation
2 mg (~7µmol) of progesterone-3CMO or progesterone-11αHS was diluted in 50µL dry dioxane and placed in water-ice bath (+10ºC). 10µL of 1:5 diluted tri-n-butyl amine in dry dioxane and 10µL of 1:10 diluted isobutyl chloroformate in dry dioxane were sequentially added. The mixture was incubated for 45 min at +10º C.

Step 2- Histamine iodination
About 3.5 mCi (~10µL) of Na-^{125}I, 3.65 µg of histamine dihydrochloride in 10 µL of 0.1 M phosphate buffer, pH 8.3, 50 µg of chloramineT in 10 µL of 0.1M phosphate buffer, pH 8.3, 75 µL of 0.1M phosphate buffer were added and vortexed for 90 sec. 300 µg of metabisulphite in 0.1M phosphate buffer, pH 7.5 were added, vortexed and put in an ice- bath (0ºC).

Step 3 – Conjugation
2.4 mL of dry dioxane was added to the mixture after 45 min. of activation and mixed. 50µL of this solution (38.7 µg = 100 nmol of progesterone) was transfered immediately to the iodinated histamine and 10 µL of 0.1 M NaOH was added. The mixture was incubated in an ice-bath for 60 minutes (mixed 5-6 times). The conjugate was ready for purification on the chromatographic column in HPLC system.

The reaction mixture after conjugation was purified by HPLC using 65% acetonitrile and 35% water as eluent with a flow rate 0.5 or 1 mL/min and using UV detectors (at 240 nm for progesterone and 220 nm for histamine). The radioactive peaks were identified [5-10]. The collected peaks were diluted with tracer diluent buffer and their immunoreactivity was determined The tracer diluent buffer was PBS buffer, pH 7.4 with 0.05% BSA containing kathon-1ml; strawberry red -70mg; blockers: ursodeoxycholic acid - 2mL, cortisol - 0.2 mL, danazol - 0.4 mL per litre of buffer. Specific activity of the tracer was determined by displacement analysis [11]. The ready to use tracer had activity about 3.5 μCi in 11mL of tracer buffer, which was diluted to 55 mL for use in assay.

3.2. Zero progesterone milk marix

Milk without progesterone (milk zero) was obtained from cows 8–10 days after calving when the function of the corpus luteum was absent. Preservatives were added to these milk samples and lyophilized after they were defatted by centrifugation for 30 min at 1200 g at 4ºC and removal of fat layer.

3.3. Standards and Controls

Standards were prepared in PBS, pH 7.4 containing 0.5% BSA and 0.1% sodium azide. Six levels of standards were prepared viz. 0, 3–3.5, 15-20, 45–50, 135-150, 270–300 nmol/L. Standards were stored at 4ºC. Control samples were prepared from pregnant cow samples after dilution with fat-free milk with zero progesterone and lyophilized.

4. RESULTS AND DISCUSSION

4.1. Radioiodinated progesterone derivatives

The elution profile of reaction mixture (flow rate 1ml/min.) after conjugation of progesterone-3-CMO with iodinated histamine is presented in Figure 1. The first peak was identified as ^{125}I–histamine (RT = 1.9 min, 10% of radioactivity), the second (RT = 11.2 min, 41% of radioactivity) and the third (RT = 13.2 min., 49% of radioactivity) were identified as iodinated progesterone derivative. The first peak was not immunoreactive, as it did not show any binding with the antibody-coated tubes from ORION. Immunoreactivity (B_0/T) of the second peak was 8% and that of the third was 37%. The third radioactive peak was used as the tracer (^{125}I-progesterone I) by dilution in tracer diluent. Specific activity of the tracer determined by displacement analysis [11] was 112 μCi/μg. The tracer in the kit had activity of about 3.5 μCi in 11mL of tracer dilution buffer (100 tubes kit).

The elution profile of reaction mixture (flow rate 0.5 ml/min.) after conjugation of progesterone-11α-hemisuccinate with iodinated histamine is presented in Figure 2. The first peak (a, b) identified as ^{125}I-histamine (RT = 2.6 and 3.2 min., 12% of radioactivity) did not show any binding with the antibody-coated tubes from BIOSOURCE. The peaks 2,3,4 which had retention time of 5.5, 6.1, 6.9 min, (45 % of radioactivity) showed an immunoreactivity of <10%(B_0/T). The fifth peak with RT = 7.8 min, (43 % of radioactivity) which gave a binding of ~43 % was identified as iodinated progesterone derivative The fifth peak was used for preparing the tracer (^{125}I–progesterone II) by dilution in the same tracer diluent. Ready to use tracer had activity ~4 μCi in 56mL of tracer diluent buffer.

4.2. Standards

The optimal buffer for preparation of standards was PBS, pH 7.4 containing 0.5% BSA and 0.1% sodium azide. The range of the assay of progesterone in milk should be different from that of serum. The optimal range of our assay for progesterone was 0–300 nmol/L. Six levels of standards were prepared: 0, 3–3.5, 15–20, 45–50, 135–150, 270–300 nmol/L. Each standard of 0.5mL volume was pipetted into the vial and kept in solution at 4–8ºC. Progesterone (ng/mL) = progesterone (nmol/L) x 0.314.

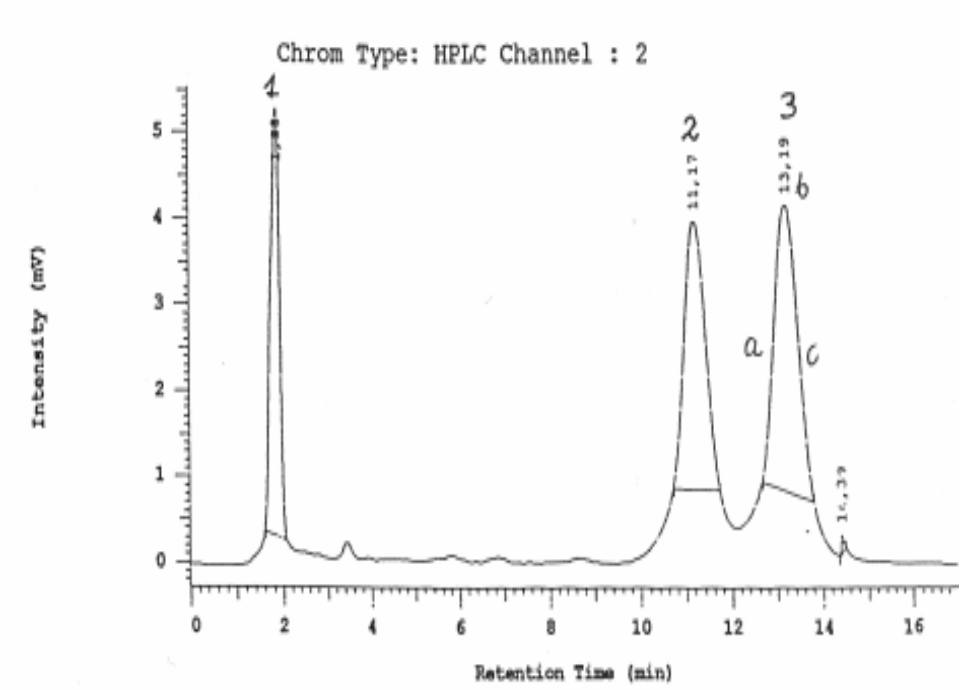

FIG. 1. Radiochromatogram of the reaction mixture of Progesterone-3-CMO with ^{125}I-histamine on HPLC after iodination.

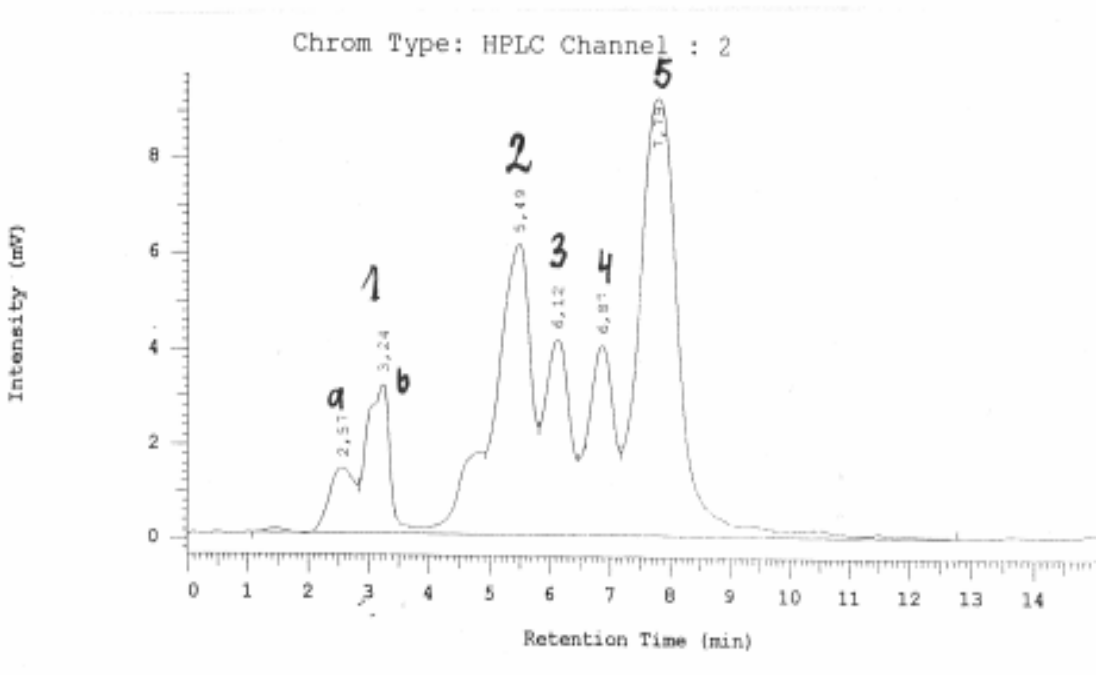

FIG. 2. Radiochromatogram of the reaction mixture of progesterone-11α-hemisuccinate with ^{125}I-histamine on HPLC.

Milk zero matrix and Control samples
The optimal matrix for the assay was fat-free milk with zero progesterone. It was lyophilised in 1mL portions and stored at –20ºC. Control samples were prepared from pregnant cow milk samples after dilution with fat-free milk zero and lyophilised in 0.5 ml portions.

4.3. Optimization of the assay

4.3.1. Assay using ORION Diagnostica coated tubes as the solid phase and ^{125}I-histamine-3-CMO progesterone as the tracer (Progesterone Veterinary RIA I test)

In order to develop a simple, short and reproducible assay with adequate sensitivity, the conditions of the assay were optimized. The total volume of the reagents pipetted into the tubes was 550 µL as the volume of coating was 500 µL. To 45 mL of tracer diluent buffer, one mL of the tracer was added and 500 µL of the prepared tracer solution was pipetted into the coated tubes. The volume was same as in Progesterone-RIA-SPECTRIA kit, ORION-OBRI. The optimal standard zero binding (B_0/T) and shape of standard curve obtained are shown in Figure 3 when activity of the pipetted tracer was 35000–50000 cpm per tube (Table I). For obtaining comparable results of progesterone concentration in unknown milk samples with commercial kits, it was necessary to use fat-free milk zero matrix for preparation of standards. The optimal proportion of milk zero matrix to the standard solution pipetted into the test tubes was 1:1 and the optimal quantity of these components was 25 µL. The volumes of controls and unknown milk samples were 50 µL. The optimal incubation conditions were 2h at room temperature without rotation (Table II and Figure 4). Under these conditions, the assay system reached equilibrium. Similarly in the assay for determination of progesterone in human serum with ORION-OBRI, POLATOM kit, the solution after incubation was decanted (or aspirated) and the tubes were not washed after incubation.

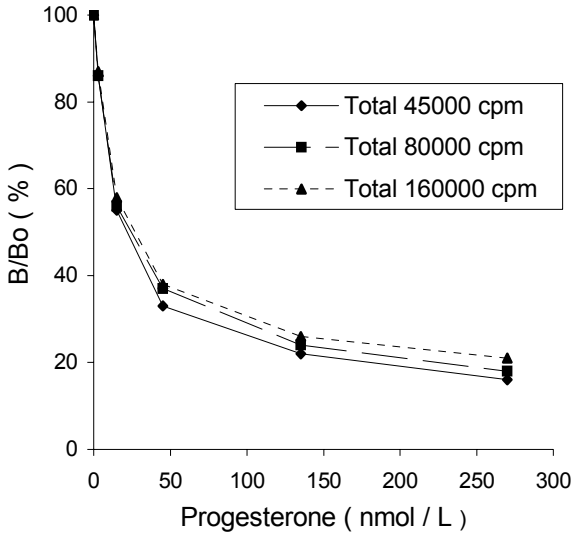

FIG. 3. Calibration curves of Progesterone Veterinary RIA I assay at different tracer concentrations.

TABLE I. EFFECT OF TRACER CONCENTRATION ON BINDING OF ZERO STANDARD (B_0/T) (PROGESTERONE VETERINARY RIA KIT I, OBRI POLATOM)

Total counts (cpm)	B_0/T (%)
21944	34.5
45200	33.3
80150	29.2
160000	26.0

TABLE II. EFFECT OF INCUBATION TIME AND ROTATION OF TUBES ON THE STANDARD CURVE OF PROGESTERONE VETERINARY RIA I ASSAY.

Progesterone (nm/L)		1h room temp.		2h room temp.		3h room temp.	
	%	With rotation	Without rotation	With rotation	Without rotation	With rotation	Without rotation
0	B/T	31.6	28.5	36.8	36.0	36.1	36.0
3	B/B_0	85	92	82	85	85	86
15	B/B_0	52	64	54	56	54	53
45	B/B_0	33	44	31	35	32	34
135	B/B_0	21.5	28	19.5	23	21	22
270	B/B_0	17.6	20.0	16.5	16	16	15.6

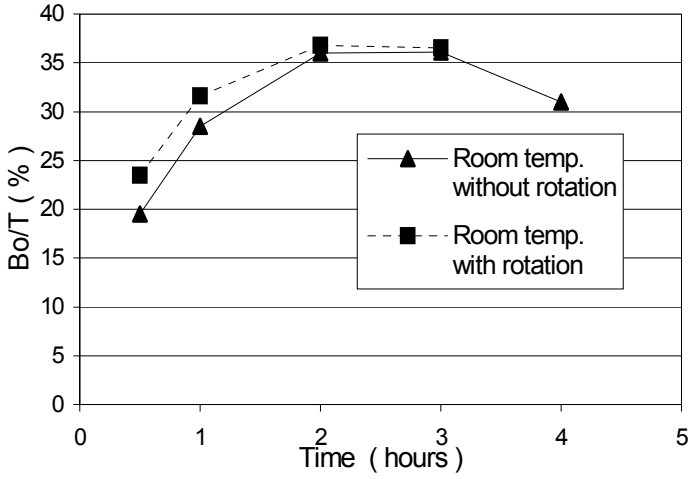

FIG. 4. Effect of incubation time and rotation on the zero standard binding in Progesterone Veterinary RIA I assay (OBRI POLATOM).

4.3.2. Assay using BIOSOURCE International coated tubes as the solid phase and progesterone-11α-hemisuccinate-^{125}I-histamine as the tracer (Progesterone Veterinary RIA II)

The tubes from Biosource have the volume of coating similar to the coated tubes from ORION and the total volume pipetted into the tubes was also similar. The optimal standard zero binding (B_0/T) and standard curve were obtained when activity of the pipetted tracer was 35000-50000 cpm per tube (Table III and Figure 5). As in Progesterone Veterinary RIA I test, for obtaining comparable results of progesterone concentration in unknown milk samples with commercial kits, it was necessary to use fat-free milk zero matrix for preparation of standards. The optimal proportion of milk zero matrix to the standard solution pipetted into the test tubes was 1:1 and the optimal quantity of these components was 25 µL. The optimum volumes of controls and unknown milk samples were 50 µL. The optimal incubation conditions were 3 h at room temperature without rotation (Table IV and Figure 6). In these conditions the assay system reached equilibrium. The solution after incubation was decanted (or aspirated).

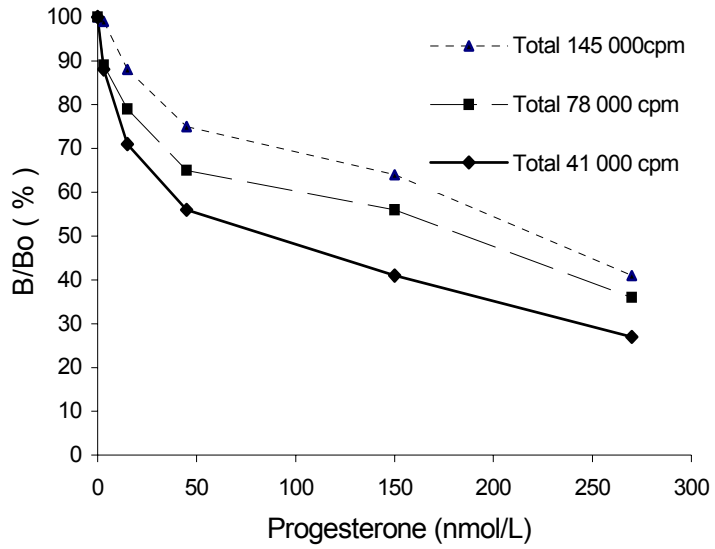

FIG. 5. Calibration curves of Progesterone Veterinary RIA II assay at different tracer concentration.

TABLE III. EFFECT OF TRACER CONCENTRATION ON BINDING OF ZERO STANDARD (B_o/T) IN PROGESTERONE VETERINARY RIA II ASSAY

Total counts in 500µL (cpm)	Bo/T (%)	
	With rotation	Without rotation
28000	46	43
45000	44	44
83000	40	40
143000	34	33

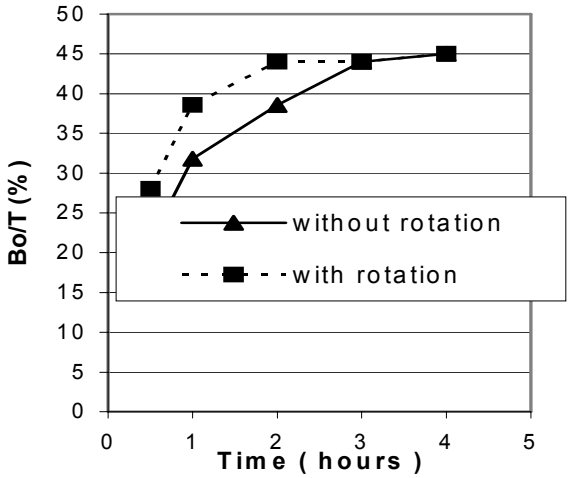

FIG. 6. Effect of incubation time and rotation on the binding of zero standard in Progesterone Veterinary RIA II assay (OBRI POLATOM).

Optimised Progesterone Veterinary RIA I assay

The final optimized assay protocol of Progesterone RIA Veterinary I test was as follows. 25 µL of standard sample alongwith 25 µL fat-free milk zero matrix were added into the coated tubes for standard curve and 50 µL of milk controls and unknown samples 500 µL of diluted tracer, mixed and incubated at room temperature for 2 h, the tubes were decanted and measured for radioactivity.

TABLE IV. EFFECT OF INCUBATION TIME AND ROTATION OF TUBES ON THE STANDARD CURVE OF PROGESTERONE VET. RIA II ASSAY. % B_0/T VALUES FOR ZERO STANDARD AND % B/B_0 FOR OTHER STANDARDS

Progesterone (nmol/L) standard (nmol /L)	1 h room temp		2 h room temp		3 h room temp	
	With rotation	Without rotation	With rotation	Without rotation	With rotation	Without rotation
0	36	29	39	36	43	43
3.5	92	92	99	94	95	89
20	75	75	79	77	75	75
50	57	60	61	63	60	57
150	48	48	54	50	48	44
300	31	33	34	35	31	27

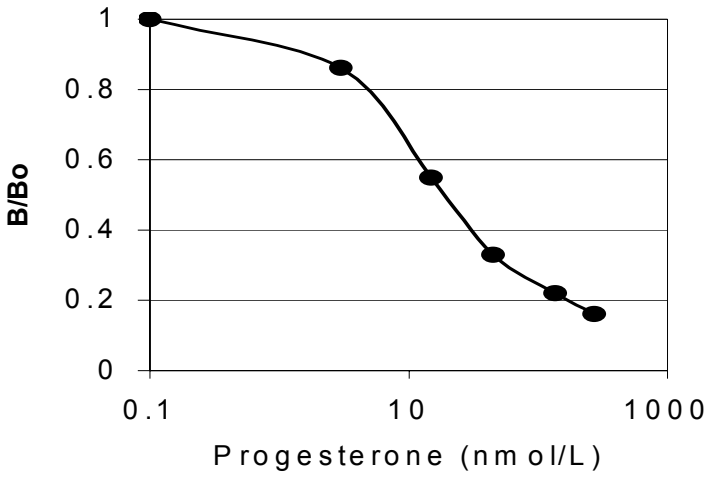

FIG. 7. Typical calibration curve of Progesterone Veterinary RIA I assay (OBRI POLATOM).

The typical calibration curve of the Progesterone Veterinary RIA I assay (OBRI POLATOM) is presented in Figure 7.

Optimised Progesterone Veterinary RIA II assay

The final optimized assay protocol of Progesterone RIA Veterinary II test is as follows: 25 µL of standard alongwith 25 µL fat-free milk zero matrix were added into the coated tubes for standard curve but 50 µL of milk for control and unknown samples, 500 µL diluted tracer, mixed and incubated at room temperature for 3 h, the tubes were decanted and measured for radioactivity. The typical calibration curve of the Progesterone Veterinary RIA II assay (OBRI POLATOM) is presented in Figure 8.

4.4. Analytical characteristics of the Progesterone Veterinary RIA assays

Sensitivity

The detection limit of the assay defined as the concentration of progesterone corresponding to a signal with 2 SD under the mean of 20 replicates of zero calibrator was 0.77 nmol/L for Progesterone Veterinary RIA I kit and 1.5 nmol/L for Progesterone Veterinary RIA II kit. The sensitivity of commercial ORION Diagnostica SPECTRIA Progesterone Veterinary RIA kit is lower then 1 nmol/l.

Recovery

Different concentrations of progesterone in fat-free milk zero were added to three fat-free milk samples (with the progesterone concentration in the range 7–30 nmol/L), analyzed for progesterone content and the recovery of added analyte were calculated (observed to expected values). The recovery was calculated for Progesterone Veterinary RIA I (Table V) and for Progesterone Veterinary RIA II (Table VI) assays. The % recoveries ranged from 92 to 118.

Parallelism testing

Parallelism was tested by dilution of four fat-free milk samples having high progesterone concentration with standard zero matrix and estimating the progesterone values in these samples using the developed kits. Values measured were between 88-104 % of expected values for Progesterone Veterinary RIA I kit (Table VII) and between 86-117 % for Progesterone Veterinary RIA II kit (Table VIII). The values of serially diluted two milk samples on plotting gave a response curves parallel to the standard curve as depicted in Figure 9 for Progesterone Veterinary RIA I kit and Figure 11 for Progesterone Veterinary RIA II kit. This suggests the identity of behavior between milk samples and the standard matrix in the Progesterone Veterinary RIA developed tests. As it is shown in Figures 10 and 12, the values determined (measured) for the diluted samples correlated well with the expected values Y = 0.9362x + 0.7813, R^2 = 0.9956 (kit) and Y=0.9521x+0.2823,R^2 =0.9973 kit I)

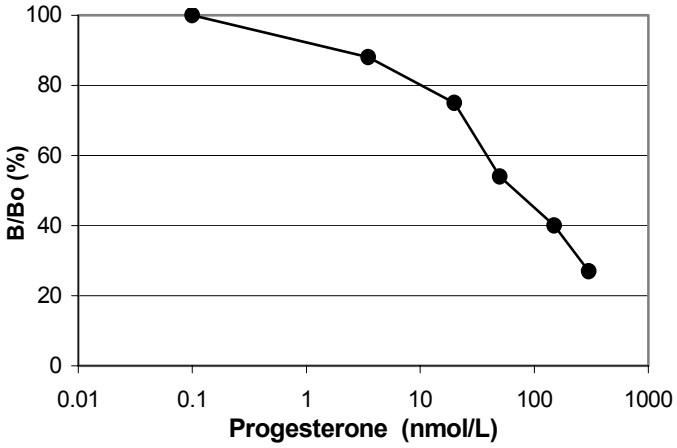

FIG. 8. Typical calibration curve of Progesterone Veterinary RIA II assay.

TABLE V. RECOVERY TEST FOR PROGESTERONE VETERINARY RIA I KIT

Progesterone in sample (nmol/L)	Progesterone added (nmol/L)	Expected Progesterone (nmol/L)	Observed Progesterone (nmol/L)	% Recovery
7.4	6.8	14.2	13.8	97
7.4	16	22.4	21	94
7.4	41	48.4	49	101
14	6.8	20.8	20	96
14	16	30	29	97
14	41	55	53	104
26.3	6.8	33.1	33	100
26.3	16	42.3	40	95
26.3	41	67.3	65	97

TABLE VI. RECOVERY TEST FOR PROGESTERONE VETERINARY RIA II KIT

Progesterone in sample (nmol/L)	Added Progesterone (nmol/L)	Expected progesterone (nmol/L)	Observed progesterone (nmol /L)	% Recovery
12	6	18	19	106
12	22	34	35	103
12	40	52	52	100
22	6	28	30	107
22	22	44	45	102
22	40	62	57	92
30	6	36	40	111
30	22	52	59	118
30	40	70	77	110

TABLE VII. PARALLELISM TEST FOR PROGESTERONE VETERINARY RIA I KIT

Sample	Dilution	Expected Progesterone value (nmol / L)	Measured Progesterone value (nmol / L)	% Recovery
1	Undiluted		79	
	2 x	39.5	38	96
	4 x	19.8	18.9	96
	8 x	9.9	9.6	97
	16 x	4.9	4.8	98
2	Undiluted		146	
	2 x	73	72	99
	4 x	36.5	37	101
	8 x	18.3	19.0	104
	16 x	9.1	9.5	104
	32 x	4.6	4.7	103
3	Undiluted		175	
	2 x	87.5	86	98
	4 x	43.8	39.5	90
	8 x	21.9	20	91
	16 x	10.9	9.5	87
	32 x	5.45	4.8	88
4	Undiluted		225	
	2 x	112.5	101	90
	4 x	56.3	55	98
	8 x	28.1	28.7	102
	16 x	14.1	14	100
	32 x	7.0	7.3	104

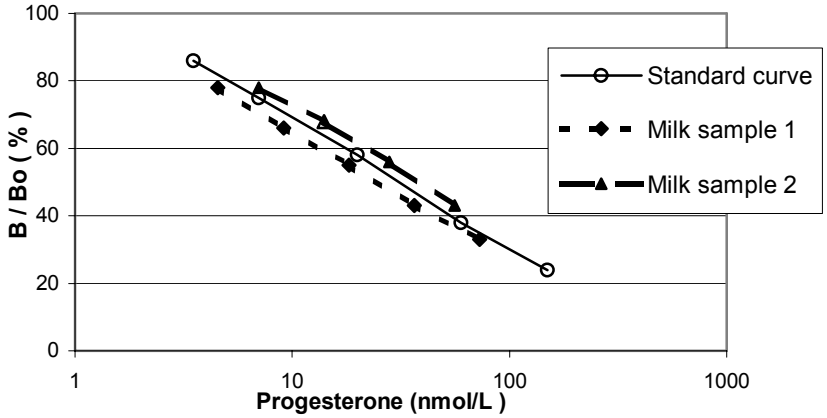

FIG. 9. Parallelism between milk samples (progesterone values of 73 and 56 nmol/L) and standards in Progesterone Veterinary RIA I assay.

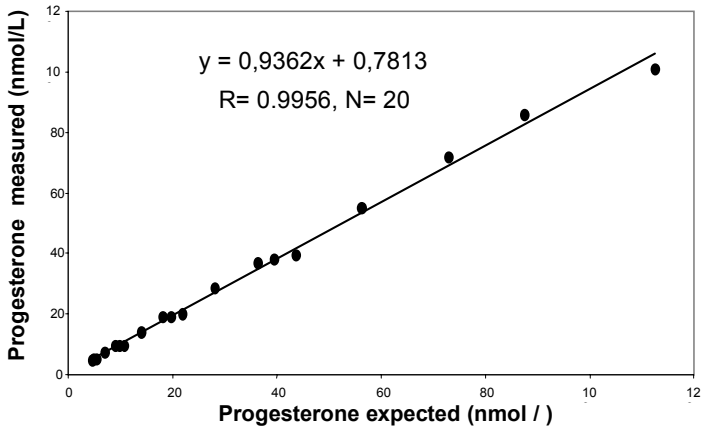

FIG. 10. Comparison between measured and expected values of four milk samples with high progesterone levels diluted with standard zero milk matrix (Progesterone Vet. RIA I kit).

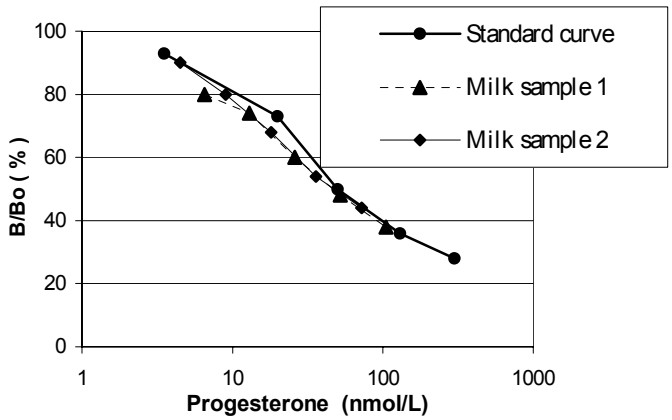

FIG. 11. Parallelism between milk samples (progesterone values of 72 and 105 nmol/L) and standards in Progesterone Veterinary RIA II assay (OBRI POLATOM).

TABLE VIII. PARALLELISM TEST FOR PROGESTERONE VETERINARY RIA II KIT

Sample	Dilution	Expected Progesterone (nmol/L)	Measured Progesterone (nmol/L)	% Recovery
1	Undiluted		82	
	2 x	41	41	100
	4 x	20.5	23	112
	8 x	10.23	11.7	114
	16 x	5.13	6.0	117
2	Undiluted		138	
	2 x	73	65	94
	4 x	36.5	32	93
	8 x	18.23	16	93
	16 x	9.1	10	116
3	Undiluted		335	
	2 x	167.5	164	98
	4 x	83.8	78	93
	8 x	41.9	36	86
	16 x	20.9	18	86
	32 x	10.5	10	95
4	Undiluted		265	
	2 x	132.5	125	94
	4 x	66.3	60	90
	8 x	33.1	32	97
	16 x	16.6	18	109
	32 x	8.3	9.2	111

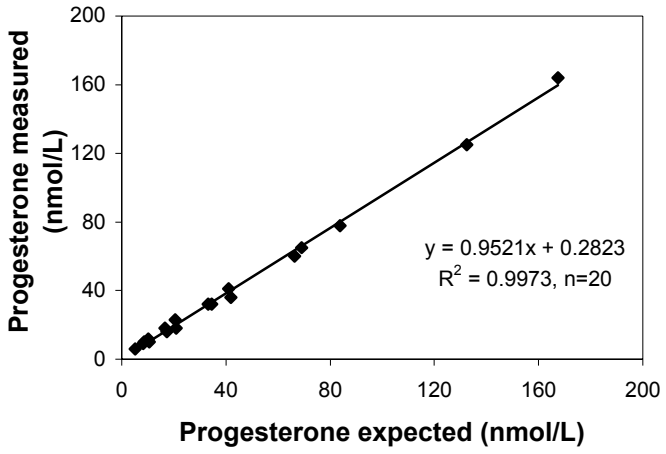

FIG. 12. Comparison between measured and expected values of four milk samples with high progesterone levels diluted with standard zero milk matrix (Progesterone Veterinary RIA II kit).

Precision

Intra-assay and inter-assay variations were estimated for the developed kits using six milk samples. These results are shown in Table IX (for Progesterone RIA kit I assay) and Table X (for Progesterone RIA kit II assay). The coefficients of variation (CV%) were between 2.4 and 8.4 %.

TABLE IX. PRECISION OF PROGESTERONE VETERINARY RIA KIT I ASSAY

INTRA-ASSAY PRECISION				
Sample number	No. of replicates	Mean (nmol/L)	SD (nmol/L)	% CV
1	20	6.46	0.23	3.6
2	20	22.3	1.06	4.8
3	20	33	1.8	5.8
4	20	42.6	1.67	3.9
5	22	104.5	5.18	4.9
6	20	181.6	9.96	5.5
INTER-ASSAY PRECISION				
Sample number	No. of duplicates	Mean (nmol/L)	SD (nmol/L)	% CV
1	5	6.7	0.34	5.2
2	5	20	1.0	5.0
3	5	35.13	1.06	2.9
4	5	61.5	1.5	2.4
5	5	101.3	2.89	2.9
6	5	220	13.2	6.0

TABLE X. PRECISION OF THE PROGESTERONE VETERINARY RIA II ASSAY

INTRA - ASSAY PRECISION				
Sample number	No. of replicates	Mean (nmol/L)	SD (nmol/L)	% CV
1	10	11.45	0.906	7.9
2	10	23.1	1.23	5.3
3	10	41.68	1.99	4.79
4	10	75	5.23	6.9
5	10	127.8	5.63	4.4
6	10	231	14.96	6.45
INTER - ASSAY PRECISION				
Sample number	No. of duplicates	Mean (nmol/L)	SD (nmol/ L)	% CV
1	5	11.0	0.84	7.7
2	5	23.3	1.51	6.5
3	5	40.9	2.45	6.0
4	5	76.9	6.47	8.4
5	5	127.2	8.3	6.5
6	5	233.4	18.1	7.8

Comparison with the commercial kit

The comparison of the typical calibration curves obtained using both the developed kits of Progesterone-Veterinary-RIA kits from I and II (OBRI POLATOM) with calibration curve in commercial Progesterone-RIA-SPECTRIA-Veterinary kit from ORION Diagnostica are presented on Figures 13 (kit I) and 14 (kit II). Progesterone levels in control fat free milk samples were determined using both the in-house (OBRI POLATOM) Progesterone Veterinary RIA kits I and II and commercial kit from ORION Diagnostica. These results are shown in Table XI (for RIA kit I) and XII (for RIA kit II).

Progesterone levels in milk samples from pregnant and non-pregnant cows were determined using SPECTRIA Veterinary Progesterone RIA (ORION Diagnostica) kit and the new Progesterone Veterinary RIA I (OBRI POLATOM) kit. Results were compared by regression analysis. The correlation coefficient reflects good agreement of these two kits (R^2=0.9871) (FIG.15)

TABLE XI. COMPARISON OF PROGESTERONE VALUES OBTAINED USING PROGESTERONE VET RIA I KIT (OBRI POLATOM) AND PROGESTERONE VETERINARY RIA SPECTRIA (ORION DIAGNOSTICA) KIT.

Sample number	Progesterone (nmol/L)	
	Progesterone Vet. RIA I (OBRI POLATOM I)	Progesterone Vet. RIA SPECTRIA (ORION Diagnostica)
1	5.8	4.4
2	17	17.3
3	35	34.5
4	66	65
5	126	114
6	227	224

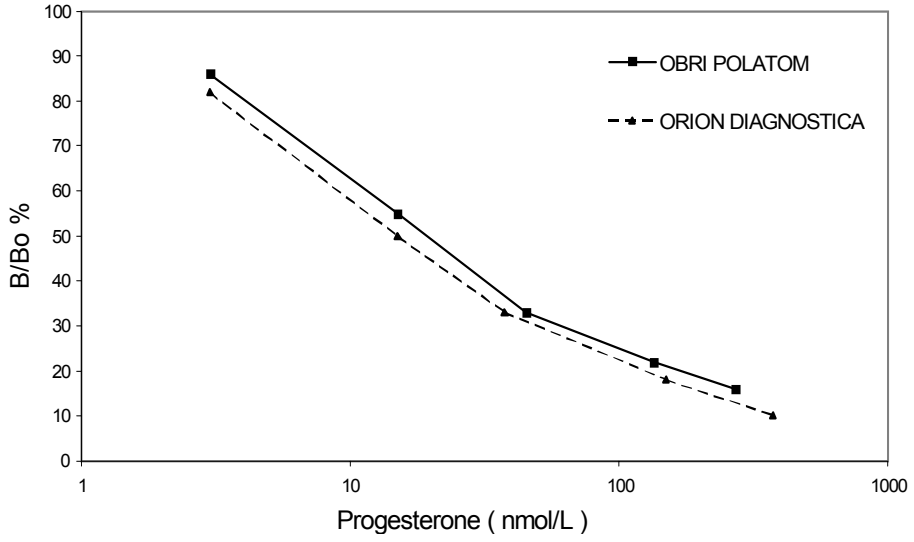

FIG. 13. Comparison of progesterone calibration curves of Progesterone Veterinary RIA-SPECTRIA (ORION Diagnostica) and Progesterone Veterinary RIA I (OBRI POLATOM).

TABLE XII. COMPARISON OF PROGESTERONE CONCENTRATION OBTAINED USING PROGESTERONE VETERINARY RIA II KIT (OBRI POLATOM) AND PROGESTERONE VETERINARY RIA SPECTRIA (ORION DIAGNOSTICA) KIT

Sample number	Progesterone (nmol/L)	
	Progesterone Vet. RIA II (OBRI POLATOM)	Progesterone Vet. RIA SPECTRIA (ORION Diagnostica)
1	9.7	8.5
2	22	19
3	36	32
4	65	63
5	115	113
6	250	220

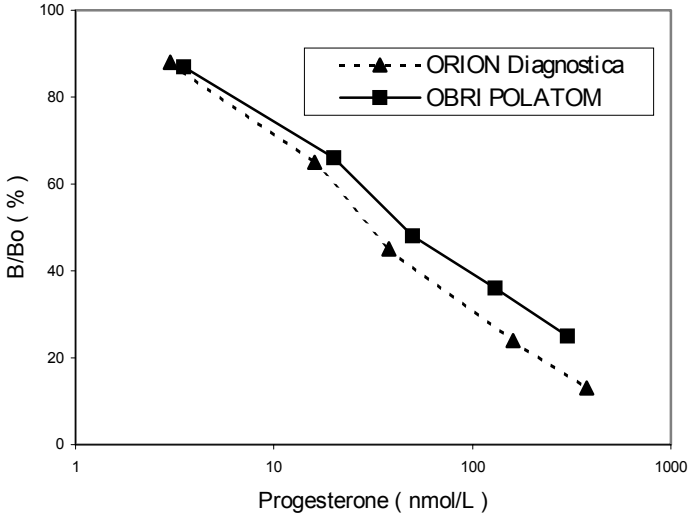

FIG. 14. Comparison of progesterone calibration curves of Progesterone Veterinary RIA-SPECTRIA (ORION Diagnostica) and Progesterone Veterinary RIA II (OBRI POLATOM).

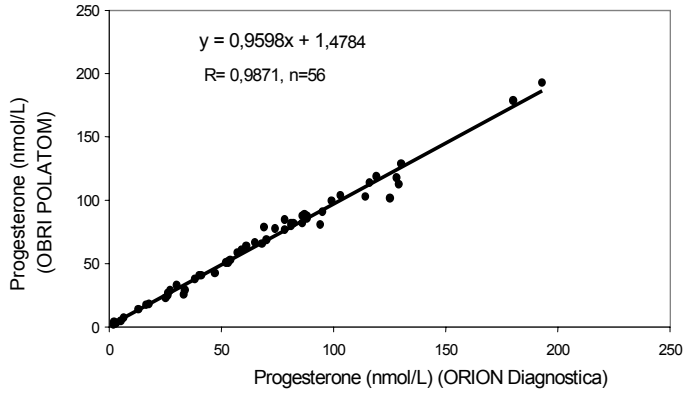

FIG. 15. Correlation between progesterone values using the Progesterone Veterinary RIA I (OBRI POLATOM) and Spectria Veterinary Progesterone RIA assay (ORION Diagnostica).

TABLE XIII. EFFECT OF STORAGE (4–8ºC) OF READY-TO-USE PROGESTERONE VETERINARY RIA I KIT (OBRI POLATOM)

Parameters	Time of storage (weeks)						
	0	2	3	4	5	7	9
B_0/T (%)*	37.6	38.1	36.5	36.2	37.0	37.6	36.5
Control 1#	6.2	6.4	6.5	6.1	5.8	5.9	5.8
Control 2#	17	18	16.2	18.5	16.8	17.5	16.1
Control 3#	29	31	29	32	30	28	32
Control 4#	62	66	63	69	61	63	62
Control 5#	107	106	96	104	97	101	97
Control 6#	196	204	200	190	205	195	210

**Binding of standard zero (B_0/T%) and #progesterone concentration in control milk samples (nmol/L)*

TABLE XIV. EFFECT OF STORAGE (4–8ºC) OF READY-TO-USE PROGESTERONE VETERINARY RIA II KIT (OBRI POLATOM)

Parameters	Time of storage (weeks)						
	0	2	3	4	5	6	8
B_0/T (%)*	42	39	39	40	42	40	41
Control 1#	9.8	9.8	10.3	9.5	10.1	9.7	10.8
Control 2#	19.7	20.5	21	20	20.3	19.8	20.5
Control 3#	38	40	38	39	40.2	42	41
Control 4#	76	79	77	85	84	80.5	77
Control 5#	117	125	111	130	113	115	120
Control 6#	255	268	247	250	289	295	270

**Binding of standard zero (B_0/T%) and #progesterone concentration in control milk samples (nmol/L)*

Stability of the new Progesterone Veterinary RIA kits (I and II) (OBRI POLATOM)

Stability of in-house ready to use kits was tested for a storage period of 9 weeks at 4–8ºC. Tables XIII (RIA I kit) and XIV (RIA II kit) show binding of standard zero and progesterone levels in control milk samples obtained using these two kits.

5. CONCLUSION

In the developed kit for determination of progesterone in cow's milk, commercial solid phase tubes coated with anti–progesterone antibody were used. OBRI produced kits for determination of progesterone in human serum (Progesterone-SPECTRIA-RIA kit in cooperation with ORION Diagnostica Finland) in which tubes coated with specific polyclonal anti-progesterone antibody were used. It was possible to purchase coated tubes alone from ORION. Using these tubes, the Progesterone Veterinary RIA I assay was developed. A second procedure for measurement of progesterone viz. Veterinary RIA II kit was worked out using coaed tubes from Biosource International.

From the studies carried out for the development of assay for progesterone determination in human serum, animal serum or animal milk, it was observed that the main problem was in preparing a tracer

suitable to bind to the antibody coated on the tubes. It was necessary to select a suitable progesterone derivative for radiolabeling [2,3,4] as the progesterone derivative used for preparing antibodies used for coating was not known in case of commercial coated tubes. Hence, we have evaluated two different conjugates for radiolabeling and selected the more suitable one to construct the standard curve using both the set of coated tubes (RIA kit I and II). The second main problem encountered was in choosing the right matrix for preparing the standards which on dilution should be parallel to that of diluted cow's milk samples [1]. In the commercial kit from ORION (Progesterone Veterinary SPECTRIA RIA), serum was used as the matrix for preparing standards. We observed the best results while using the standards prepared in 1:1 mixture of buffer containing bovine serum albumin and fat-free milk without progesterone (milk zero matrix). Optimization of the reagents should result in an assay that is simple, fast and which can measure progesterone in normal milk (with fat).

The best method for preparing the tracer was indirect labeling (three steps) using conjugation of activated progesterone derivative with iodinated histamine (^{125}I-histamine) [2,3,4] and the best method of purification was HPLC using reverse phase (RP-18) column [5–10]. Iodinated progesterone-CMO derivative (progesterone-3CMO-^{125}I-histamine) showed a binding of 36% to coated tubes from ORION and 42% to tubes from Biosource. Although the tubes from Bioource showed binding, it did not result in a standard curve. Another iodinated progesterone derivative viz. progesterone-11α-hemicuccinate-^{125}I-histamine was suitable for Biosource antibody coated tubes as it was possible to obtain a standard curve (Progesterone Veterinary RIA II) but quality of the assay with ORION coated tubes and iodinated CMO-progesterone derivative (Progesterone Veterinary RIA I) was better. Despite low maximal binding in Progesterone Veterinary RIA I assay, the standard curve in this assay was better than that in RIA kit II, B/B_o, of the first standard was 85%, and last standard 16%, with a steeper curve. Incubation in RIA I kit was shorter and washing of the tubes was not necessary. The sensitivity of the RIA I kit was 0.77 nmol/L and in RIA II kit, it was 1.5 nmol/L. The quality of the developed Progesterone Veterinary RIA I kit was better than RIA II kit in terms of quality control parameters such as recovery which was 94–104% (RIA kit II 92-118%), recovery in parallelism test was 88-104% (in RIA kit II 86–117%), CV% was 2.4-6.0% (RIA kit II 4.4–8.4%).

The only commercial kit available for assay of progesterone in cow's milk is from ORION Diagnostica (Finland). The results of progesterone concentration in control and pregnant cow's milk samples obtained using RIA I kit OBRI POLATOM and commercial ORION kit were similar. In general, the progesterone concentrations in fat free milk samples compared with normal milk samples were similar. The optimized kits were found to be stable up to 9 weeks at 4–8ºC. During this period, the maximal binding (B_0/T) and progesterone concentration in the control milk samples remained constant. The work carried out has resulted in a good quality Progesterone Veterinary RIA I test which is simple, quick and specific for the assay of progesterone in cow`s milk.

REFERENCES

[1] HRUSKA, K., FRANEK, M., BURSA, J., KALA, B.P., Radioimmunoassay of progesterone in cow milk. Endocrinologia Experimentalis. 17 **(**1983) 213-218.

[2] BOLTON, A. E., Radioiodination techniques. Review 18, Amersham International plc, UK (1985) 65-66.

[3] DEWANJEE, M.K., Radioiodination: Theory, Practice and Biomedical Application. Kluwer Academic Publishers, Boston Dordrecht, London, (1992) 538-539.

[4] KOTHARI, K., PILLAI, M.R.A., Direct radioimmunoassay of serum progesterone using heterologous bridge tracer and antibody. J.Radioanal. and Nucl. Chem. 231 (1998) 77-82.

[5] HANAI, T., Liquid chromatography in biomedical analysis. J. Chromatography Library Elsevier. 50 **(**1991) 255-282.

[6] MESMER, M.Z., SATZGER, R.D., Determination of anabolic steroids by HPLC with UV-Vis- Particle Beam Mass Spectrometry. J.Chrom.Science. **35 (**1997) 38-42.

[7] ALLENMARK, S., SON BERG, A.A., HAMMAR, M., LINDSTROM, E., Combined paper and reversed-phase high performance liquid chromatography method for the study of pregnenolone and progesterone metabolites. J. Chromatography. 224 **(**1981) 399-405.

[8] KESSLER, M.J., High performance liquid chromatography of steroids metabolites in the pregnenolone and progesterone pathways. Steroids. 39 (1982) 21-33.
[9] WAISMAN.Y., et al., Rapid diagnosis of congenital adrenal hyperplasia by high performance liquid chromatography. Clin. Chim. Acta. 138 **(**1984) 1-8.
[10] VON STETTEN, O., SCHLETT, R., Purification of ^{125}I–labeled compounds by high performance liquid chromatography with on line detection. J. Chromatography. 254 (1983) 229-235.
[11] BHUPAL, V., MANI, R.S., A simple method for the determination of the specific activity of ^{125}-I- tracer used in radioimmunoassay. 107 (1986) 377-383.

PRODUCTION OF ANTIBODIES AGAINST AFLATOXIN B_1 AND DEVELOPMENT OF AFLATOXIN B_1 ELISA

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Abstract
Rabbits were immunized with aflatoxin B_1-BSA conjugate and antibodies against aflatoxin B_1 were obtained after seven months. The titers of the antibodies obtained from two rabbits were 1:50000 and 1:20000 respectively as characterized by ELISA. Assay of aflatoxin B_1 ELISA was developed and the sensitivity of the assay was 0.5 ng/mL.

1. INTRODUCTION

Aflatoxins are a group of toxic metabolites produced by strains of Aspergillus flavus and Aspergillus parasiticus. They contaminate food and agricultural commodities, especially wheat, corn, soya and groundnut products. Among them, aflatoxin B_1 is considered to be one of the most potent carcinogens and is linked closely with human liver cancer. Aflatoxin B_1 at low concentration (usually ppb) can cause very damaging effects. In Southern China, the weather being very hot and humid, contamination due to mould formation is a widespread occurrence. This is harmful to humans. Accordingly, to protect human health, it was necessary to detect the level of mycotoxin such as aflatoxin B_1 in food and food products. Conventionally, the level of aflatoxin B_1 is detected by thin layer chromatography (TLC) or high-pressure liquid chromatography (HPLC) or mass/liquid chromatography. These methods are quite useful and accurate but time consuming as it involves processing of a large number of samples. In recent years, immunoassay has become very attractive because of its specificity, sensitivity and simplicity. Several agencies now supply immunoassay kits for detection of aflatoxin B_1 including RIA and ELISA kits. We have produced antibodies against aflatoxin B_1 [1] and developed an ELISA for aflatoxin B_1 using in-house developed antibodies.

2. MATERIALS

Aflatoxin B_1-BSA conjugates, Aflatoxin B_1 and Horseradish peroxidase (HRP), tetramethyl benzidine (TMB) were purchased from Sigma Chemical Co. USA.

3. METHODS

3.1. Immunization schedule

Four rabbits were administered with aflatoxin B_1-BSA conjugate at a concentration of 300 μg per rabbit in complete Freund's adjuvant for the primary injection. The rabbits were boosted with aflatoxin B_1-BSA at a concentration of 150 μg per rabbit in incomplete Freund's adjuvant after two weeks and the boosters were repeated every four weeks. After seven months, the blood was collected from rabbits' jugular vein and the serum separated to obtain the antiserum.

3.2. Preparation of anti-rabbit IgG-HRP

2 mg horseradish peroxidase (HRP) was dissolved in 1 mL of distilled water. 100 μL of 0.1M $NaIO_4$ and 400 μL distilled water were added slowly to the HRP solution while stirring on a magnetic stirrer. The mixture was kept in dark under stirring at room temperature for 20 min. The mixture was

dialyzed overnight against 0.1M, HAC-NaAC buffer, pH 4.4 at 4°C. The pH of activated HRP solution was adjusted to 9.5 with 0.2M carbonate buffer, pH 9.5. 2 mg of donkey anti-rabbit IgG (1mg/mL in 0.01M, pH 9.5, carbonate buffer) was added dropwise to the activated HRP solution. The reaction mixture was stirred on a magnetic stirrer for 2h in dark at room temperature. 50 µL $NaBH_4$ (4 mg/mL) was added to the above solution and kept at 4°C in dark for another 2 h. Dialysis against 0.01M, pH 7.4, phosphate buffer for 2 days at 4°C was carried out.

3.3. Characterization of antibody against Aflatoxin B_1 by ELISA

Each well of the 96-well plates was coated with 150 µL of 2 µg/mL of aflatoxin B_1-BSA conjugate in 0.1 M, carbonate buffer, pH 9.5. The wells were blocked with 1% BSA in 0.05 M, pH 7.4, phosphate buffer. 100 µL standard (0, 0.5, 1, 5, 10, 50, 100 ng/mL) and 50 µL of antibody against aflatoxin B_1 were added to the coated wells and incubated at 37°C for 1 h. The plates were washed three times with wash buffer (0.05M, pH 7.4 phosphate buffer containing 0.02% Tween 20). 150 µL of anti-rabbit IgG-HRP was added and incubated at 37°C for 30 min. The plates were washed four times with wash buffer and 150 µL of 0.4 mM wth H_2O_2 (0.04% w/v) in acetate buffer, pH 6, TMB solution was added. The reaction was stopped after 15 min by the addition of H_2SO_4. The absorbance at 450 nm was measured using an ELISA reader.

4. RESULTS AND DISCUSSION

Four rabbits were immunized with aflatoxin B_1-BSA, but two of them died, one in the first and the other in the fifth month. Antiserum against aflatoxin B_1 were obtained only from two rabbits. The titre curve of anti-aflatoxin B_1 antibodies is shown in Figure 1. The titres of the antibodies are 1:50000 and 1:20000 respectively. A standard curve of ELISA for aflatoxin B_1 is shown in Figure 2. The assay sensitivity was 0.5 ng/mL.

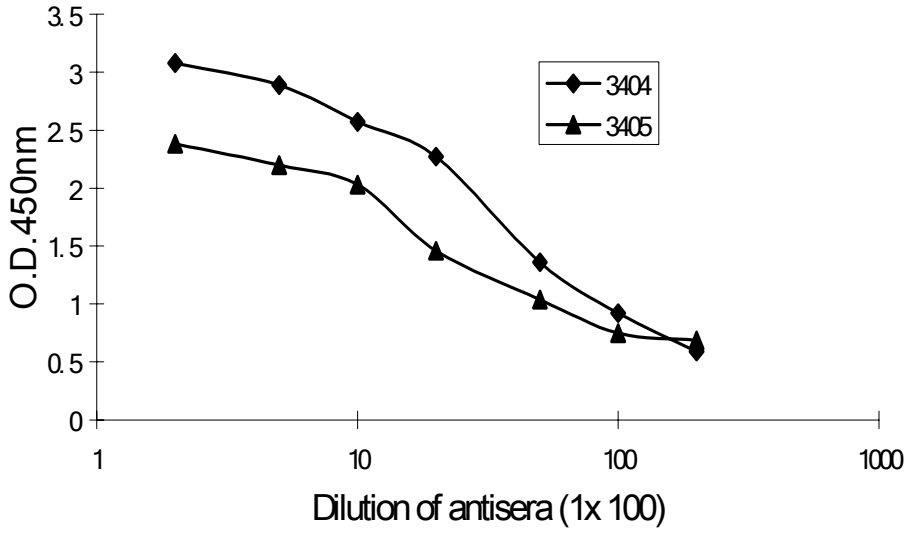

FIG. 1. Titer curve of Aflatoxin B_1.

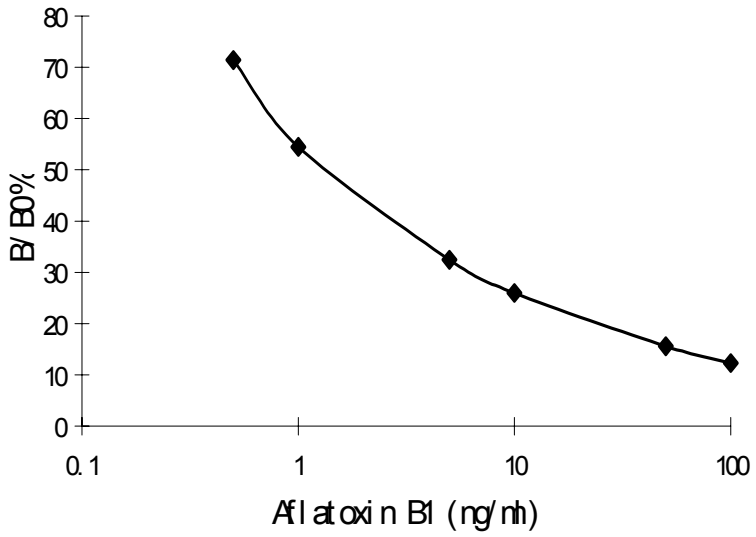

FIG. 2. Aflatoxin B_1 ELISA standard curve.

5. CONCLUSION

1. The antibodies against aflatoxin B_1 were obtained and characterized by ELISA. The titres of the antibodies are 1:50000 and 1:20000, respectively.
2. Assay of aflatoxin B_1 ELISA was developed and the sensitivity was 0.5ng/mL

REFERENCE

[1] CHU, F.S. AND UENO, I., Production of Antibody Against Aflatoxin B_1 Appl. Environ. Micobiol. **33** (1977) 1125-1128.

DEVELOPMENT OF A SOLID PHASE ASSAY SYSTEM FOR AFLATOXIN B_1

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Abstract
Antibody raised against aflatoxin B_1-BSA was coated on polystyrene tubes through the second antibody and rabbit gamma globulin as well as by physical adsorption An optimum dilution of 1:8000 for primary antibody gave a binding of 65% with a non-specific binding of <1%. A solid phase assay using these tubes with an incubation of 3 h at room temperature and a standard range of 0.5–10 ng/mL was optimized. A liquid phase radioimmunoassay standardized earlier was used for analysis of aflatoxin B_1 in samples such as groundnut, wheat, maize and soyabean obtained from different places. A correlation of 0.987 was obtained in the aflatoxin B_1 values estimated by the present method as well as by TLC and UV detection and a gross positive correlation was observed between aflatoxin B_1 estimated by the present system and the total aflatoxins as estimated by TLC. A direct ELISA system for aflatoxin B_1 with antibody coated on microtitre wells was standardized for two cut off values viz. 10 ng/mL and 25 ng/mL. An indirect ELISA was also optimized wherein BSA-aflatoxin B_1 was coated on the microtitre wells. The free aflatoxin B_1 in the sample competes with aflatoxin B_1 on the solid phase for a limited amount of the antibody-HRP. A system with cut off values at 10 ng/mL (%B/Bo–60%) and 25 ng/mL (B/Bo–25%) was developed.

1. INTRODUCTION

Aflatoxins are a group of toxic secondary metabolites of fungi, most commonly produced by certain strains of Aspergillus flavus and all strains of Aspergillus parasiticus [1]. Crops that contain high levels of starch and lipids such as peanut, corn, cottonseed, dry fruits etc. are found to be contaminated by aflatoxins in field during harvest and upon storage. The most important factor in aflatoxins production is the moisture or relative humidity surrounding the substrate. There are four closely related groups of aflatoxins viz. aflatoxin B_1, aflatoxin B_2, aflatoxin G_1 and aflatoxin G_2. Aflatoxin M_1 and M_2 are the metaboilites of B_1 and B_2, respectively. Aflatoxins exhibit both acute and chronic toxicity. Prolonged exposure to subacute levels of aflatoxins is of serious concern to human [2]. Health effects of aflatoxins include hepatotoxicity and liver cancer. It has also been found to be teratogenic in animals. Aflatoxin B_1 has been classified as the groupI carcinogen. The contamination of aflatoxin B_1 in agricultural commodities has been the subject of national and international levels. In India, under the prevention of Food Adulteration Act 1954, presence of aflatoxin B_1 in food and feed should not exceed 30 ppb. Conditions for the growth of moulds are quite conducive in the humid and hot tropical climate of India. Exportable agricultural commodities from India face rejection due to aflatoxin B_1 contamination. Thus, there is a need to have a simple, rapid and sensitive technique for the estimation of aflatoxin B_1. Currently, most agencies use the conservative TLC method for quantitation of aflatoxin B_1, which is neither very sensitive, nor specific to B_1. HPLC and mass spectrometry have also been developed and used. But these techniques are expensive and involve extensive clean-up to overcome interference due to the complex matrix. Immunoassays being specific and sensitive require simple clean-up procedure and are better suited for routine analysis of large number of samples [3]. Herein we describe the work carried out to develop a sensitive and specific solid phase immunoassay for aflatoxin B_1 using in-house prepared radiotracer and aflatoxin B_1 antibodies. Work was also carried out to develop an ELISA for aflatoxin B_1. ELISA has the advantage of being modified to a system, which can be visually analysed and could be used at any place.

2. MATERIALS

Aflatoxin B_1 and Aflatoxin B_1-BSA conjugate were obtained from Sigma Chemicals, USA. Carrier free ^{125}I as Sodium Iodide (Specific activity 15–17mCi/μg, radioactive concentration 100mCi/mL) was obtained from IZOTOP, Hungary. Whatman 3mm chromatography paper was purchased from Whatman Ltd. England. Silica gel (GF 254) and silica impregnated plastic plates were obtained from

E.Merck, Darmstadt. Preparative silica plates were prepared at the laboratory using Silica gel GF254 from Acme Chemicals. The non-radioactive compounds were identified as UV active zones or by exposure to iodine vapor and the radioactive zones were identified using a NaI (Tl) scintillation counter. All the other reagents were of AR grade.

Horseradish peroxidase, 2,2' azino-biz (3ethylbenzylthiazoline-6-sulfonic acid) (ABTS), anti-aflatoxin B_1 peroxidase conjugate were obtained from Sigma Chemical Company, USA. AflatoxinB_1 was obtained through IAEA. Immunolon Strips (flat bottom wells) were purchased from Dynex Technologies, Inc. ELX800 Universal microplate reader from Bio-Tek Instruments, Inc was used for measurement of absorbance

The oxime derivative of AfB_1 was prepared as per procedure described by Chu *et al* [4]. ^{125}I-AfB_1 was prepared by conjugating AfB_1 oxime with ^{125}I-histamine by mixed anhydride method as reported earlier [5]. The conjugated product was purified by solvent extraction followed by TLC. >99% radiochemically pure iodinated aflatoxin was obtained. Antibodies against aflatoxin B_1 were raised in rabbits by immunization with aflatoxin B_1-BSA conjugate.

3. METHODS

3.1. Liquid Phase Assay for measurement of Aflatoxin B_1

The assay uses aflatoxin B_1 polyclonal antiserum produced and characterized in-house and aflatoxin B_1-CMO-^{125}I-histamine as the tracer. The standardized procedure for the synthesis of the oxime derivative and the radioiodinated aflatoxin B_1 has been described earlier [5]. The synthetic scheme used is shown in Figure 1.

The optimized assay involves the incubation of 100 µL of standard (0.5–10 ng/mL) or extracted sample, 100 µL of antisera (1: 50000) and 100 µL of radioiodinated tracer (~40000 cpm) for 3 h at room temperature (25°C). After the assay, 100 µL of second antibody and 12% PEG solution were added to separated the bound and free.

3.2. Direct ELISA

3.2.1. Antibody coating on microtitre well plates

Different dilutions of antibody ($1:10^2$ to $1:10^5$) were made in 0.1M bicarbonate buffer, pH 9 for determining the optimum titre for the assay. 0.2 ml of these dilutions were added to the wells and incubated for 24 h at ambient temperature or 3h at 37°C. The wells were washed three times with 0.05M PO_4^{3-} buffer, blocking with 1% BSA for 30 min, and washed again twice with 0.3 mL of assay buffer (0.1% BSA in PO_4^{-3} buffer, pH 7.4). The wells were air dried and stored at 4°C.

3.2.2. Preparation of Aflatoxin B_1 -HRP Conjugate

Aflatoxin B_1 oxime synthesized and characterized in our laboratory as per earlier reported procedure was used for preparation of aflatoxin B_1-enzyme conjugate. 600 µg of aflatoxin B_1 oxime dissolved in 1:1 ethanol: water mixture was allowed to react with 5 mg of horseradish peroxidase enzyme in presence of 200 mg of 1-ethyl-3,3-diethyl amino-propyl carbodiimide (EDC). The contents were slowly mixed at ambient temperature in dark for 1 hr. An additional 100 mg of EDC was added and the reaction was continued at 4º C for 24 h. The HRP-aflatoxin B_1 was purified from free aflatoxin by gel filtration on sephadex G-75. 3 mL fractions were collected on elution with 0.05 M PO_4^{3-} buffer, pH 7.4. Fractions 3–5 were pooled, stored in 0.05 M PO_4^{3-} buffer containing 0.1% BSA and used as the tracer. The protein concentration of pooled tracer was 0.5 mg/mL.

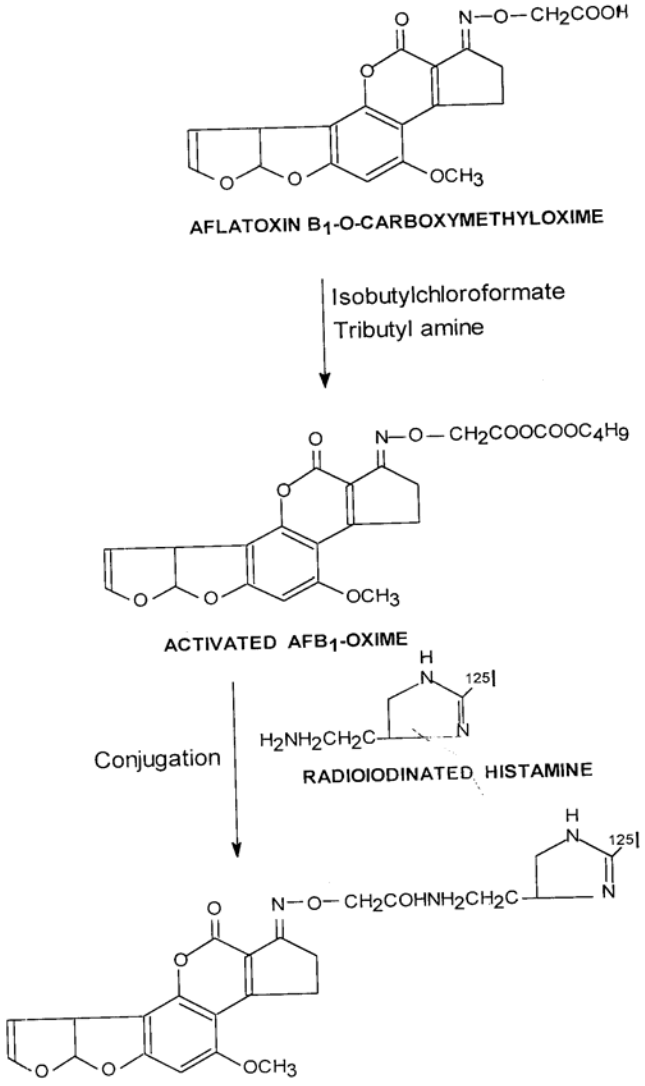

FIG. 1. Synthetic scheme of aflatoxin B_1 oxime and radioiodinated aflatoxin B_1.

Substrate system

Solution A: 10 mg of 2,2' azino-biz (3ethylbenzylthiaazoline-6-sulfonic acid (ABTS) was dissolved in 20 mL of in 0.05 M Citrate buffer, pH 5 and stored at 4°C.

Solution B: 40 µL of hydrogen peroxide was added to 20 mL of in 0.05 M Citrate buffer, pH 5. This solution is to be freshly prepared before use.

The substrate solution is prepared by mixing a 1:1 dilution of solution A and B. 200 µL of the mixed solution is added to the wells. The absorbance must be measured at 20 minutes. Since no stopping solution is added, the absorbance measurement at 630 nm after 20 min is critical.

3.2.3. Optimization of ELISA

The titre for antibody coated on the wells and amount of aflatoxin B_1-HRP conjugate was optimized. Antibody coated wells at titre of 10000 was used for assay optimization. 1:30 dilution of aflatoxinB_1-HRP tracer was found to give optimum OD and was used for the assay development.

100 μL of aflatoxin B_1-HRP along with 100 μL of standard aflatoxin was added to these antibody-coated wells and incubated for 2h at ambient temperature. The wells were aspirated and washed 5 times with 0.05 M phosphate buffer. 200 μL of substrate solution was added (ABTS- 0.5 mg/mL, H_2O_2 – 2 μL/mL). The absorbance was measured at 630 nm after 20 min.

3.3. Indirect ELISA

Indirect competitive ELISA uses aflatoxin B_1-BSA conjugate coated on microtitre plate. The coated plate is incubated with the anti-aflatoxin B_1 labeled with enzyme and standard/sample containing aflatoxin B_1. The aflatoxin B_1 in the standard/sample competes with aflatoxin B_1 on the solid phase for the enzyme-labeled antibody. The amount of antibody bound to the plate is ultimately determined by enzyme-substrate reaction.

3.3.1. Optimisation of the assay

The amount of aflatoxin B_1-BSA for coating on microtitre wells, the amount of anti-aflatoxin B_1-HRP to be used in the assay system, assay parameters such as time, temp, pH of the reaction and substrate concentration were optimized to arrive at a suitable system. In the initial studies carried out for optimization of the assay system, 20 μg of aflatoxin B_1-BSA conjugate per well was used for coating. Coating was carried out in 0.1M bicarbonate buffer, pH 9, overnight at ambient temperature. 1:100 and 1:200 dilution anti-aflatoxin B_1 antibody enzyme conjugate and aflatoxin B_1 standard (10 and 25 ng/mL) were incubated in coated microtitre wells for 30 min or 2 h. At the end of incubation absorbance was measured after substrate addition similar to the above method used for direct ELISA. Absorbance was around 1 and 0.9 for 1:100 and 1:200 dilutions of tracer, respectively.

3.4. Solid Phase assay for measurement of Aflatoxin B_1

3.4.1. Coating of antibodies by physical adsorption

Different dilutions of the aflatoxin B_1 antibody ($1:10^2$ to $1: 1x10^5$) were made in 0.1M bicarbonate buffer, pH 9. 0.5 mL of these dilutions were added to the polystyrene tubes for physical adsorption. The tubes were kept for coating for 24 h at ambient temperature. The tubes were aspirated and washed once with 0.1% BSA. The tubes were saturated with 1% BSA for 30 min for saturating any unoccupied sites. The tubes were again aspirated and washed twice with 0.6 mL of assay buffer (0.1% BSA in PO_4^{-3} buffer, pH 7.4). The tubes were air dried and stored at 4°C. Antibody dilution of 1 to 50000, which gave 35 % binding, was chosen as the titre

3.4.2 Coating of antibodies through second antibody [6]

0.5 mL of 2 mg per litre of rabbit γ globulin in 0.1 M bicarbonate buffer, pH 9 was added to the polystyrene star tubes and incubated for 24 h at ambient temperature. 0.5 mL of different dilutions of second antibody (1:50 to 1:500) in 0.1% BSA in 0.05M PO_4^- buffer, pH 7.5 were added after washing the tubes and further incubated for 24 h at ambient temperature. 0.1 mL of different dilutions (1: 1000 to 1: 20 000) of primary antibody was added without aspirating the second antibody. After the incubation with the primary antibody, the tubes were washed with 0.05 M phosphate buffer, pH 7.5. The tubes were saturated with 1% BSA for 30 min and the tubes were washed again with 0.1% BSA in 0.05 M PO_4^- buffer, pH 7.5.

The optimized assay involves the incubation of 100 μL of standard (0.2-10 ng/mL) or extracted sample, and 400 μL of radioiodinated tracer (~40 000 cpm) in antibody coated tubes for 3 h at room temperature (25°C). After the assay, 2x1 mL of wash buffer of 0.05M phosphate with 0.1% tween 20 was added and the tubes were decanted. The bound activity versus aflatoxin B_1 concentration was plotted in a logit-log paper. A solid phase assay standard curve is shown in Figure 4

3.5. Extraction of samples

5 g of finely ground feed sample was taken in a 100 ml capacity glass stoppered conical flask and 25 ml of AR grade methanol-water (55:45) was added. The flask was vigorously shaken using a shaker for a minimum of 30 min. The extract was gravity filtered into a 25 ml conical flask using Whatman no 1 or equivalent. The flasks were tightly stoppered to avoid leakage and evaporation during storage. The extracted samples were carefully stored at 2–4ºC and used for analysis after appropriate dilution. Every tenth sample was spiked with the known aflatoxin B_1 standard solution to ascertain the accuracy.

Samples such as groundnut, wheat, maize and soyabean obtained from different places were analysed for aflatoxin B_1. Sampling is very important. Usually from 2 to 40 Kg lot mixed sub samples of 25 to 50 g are selected. Few spice extracts samples were analysed for aflatoxin B_1. The analysed values are given in Table I.

4. RESULTS AND DISCUSSION

Figure 2 shows the inhibition at two different aflatoxin B_1 standard concentrations (10 and 25 ng/mL) at two antiserum dilutions (1:10 000 and 1:50 000) for the Direct ELISA. Figure 3 shows % inhibition in binding after addition of 10 and 25 ng/mL of standard aflatoxin B_1 for both 30 min and 2h incubation for the Indirect ELISA

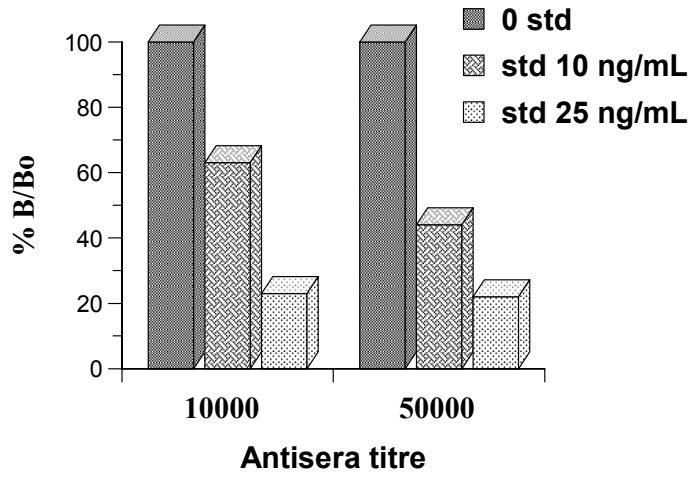

FIG. 2. Direct ELISA.

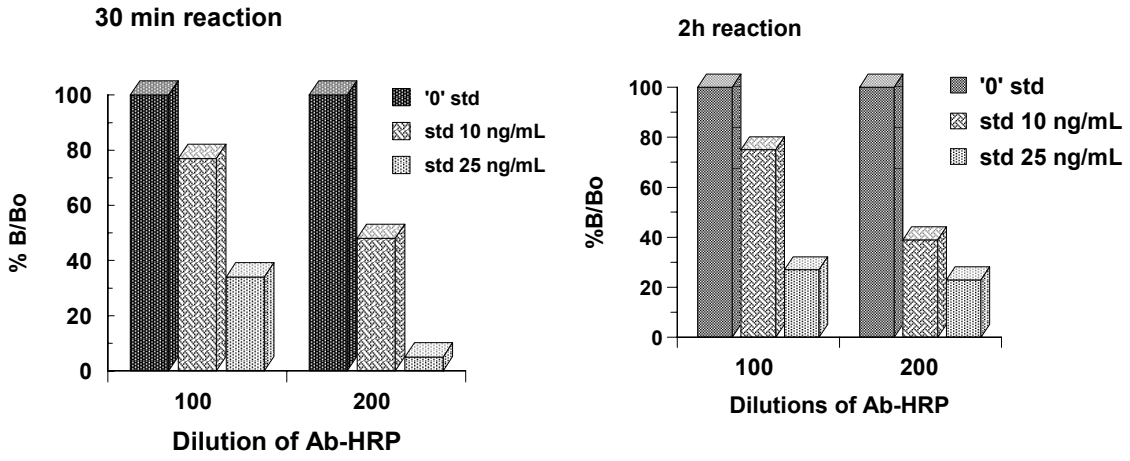

FIG. 3. Indirect ELISA.

Although the responses were good at both dilutions, the OD measured was higher at 1:10000 and hence used for further optimization.

The standard curve using coated tubes is given in Figure 4. An optimum dilution of 1:8000 for primary antibody gives a binding of 65% with a non-specific binding of < 1%. The assay has a range of 0.2–10 ng/mL. The actual value of the sample in ppb is calculated taking into account the dilution factor and volume of extraction solvent. Figure 5 gives the correlation of samples analysed by the earlier established liquid phase assay system and the developed solid phase system. Parallelism studies were also carried out with samples that exhibited higher aflatoxin B_1 values (Table I). Precision profile is given in Figure 6.

Table II gives the results of analysis of 6 groundnut samples for aflatoxin B_1 by TLC as well as by the liquid phase assay. A comparison of total aflatoxins by UV detection and aflatoxin B_1 is depicted in Table II

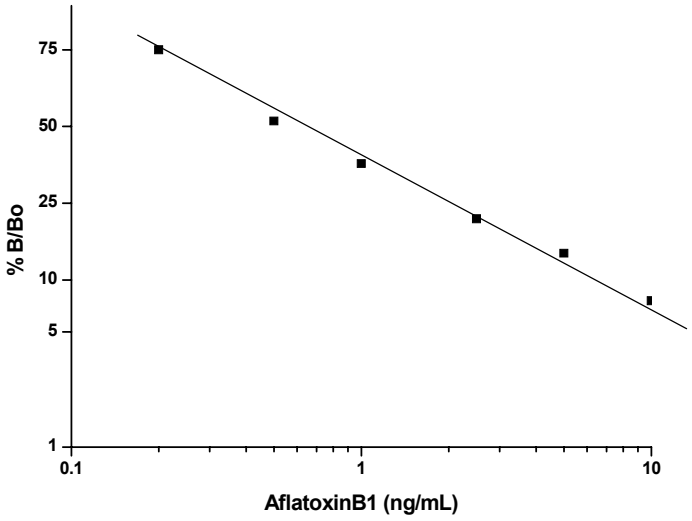

FIG. 4. Solid phase RIA for aflatoxin B_1.

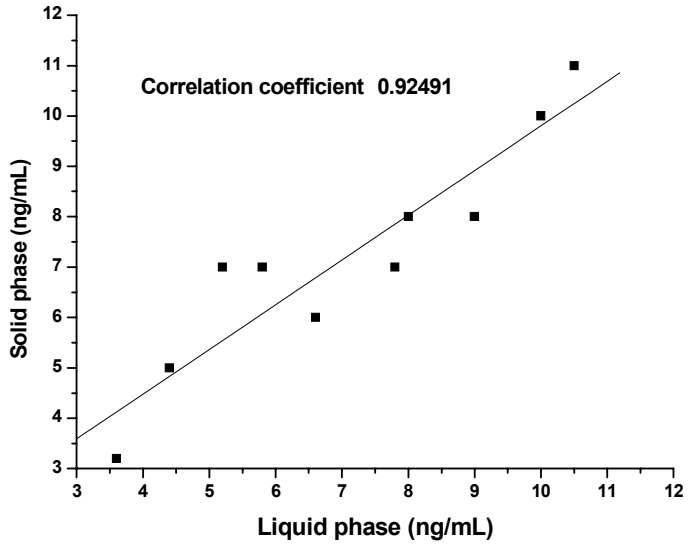

FIG. 5. Correlation between solid phase and liquid phase RIA for aflatoxin B_1.

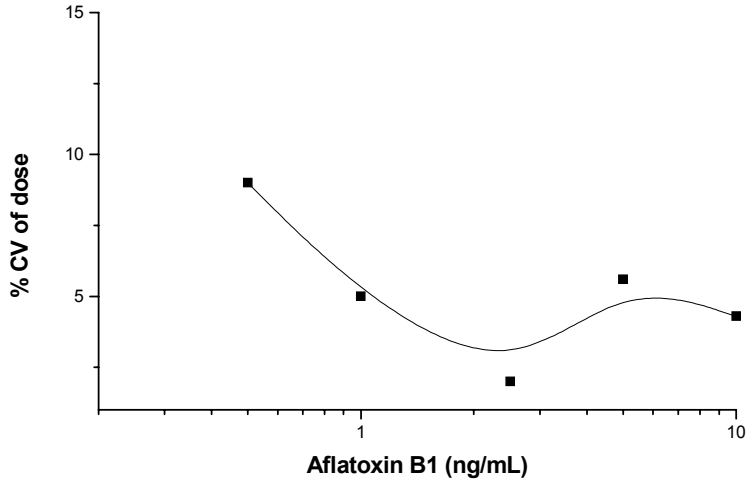

FIG. 6. Precision profile for coated tube assay of aflatoxin B_1.

TABLE I. PARALLELISM STUDIES

	Assayed values (ng/mL)	% Recovery
Sample1	5	
1:2	2.5	100
1:4	1.4	112
Sample2	12	
1:2	5.2	86
1:4	3.4	113
Sample3	12	
1:2	5.8	97
1:4	3.4	113
Sample4	8	
1:2	4.3	107
1:4	2.5	125

TABLE II. COMPARISON OF AFLATOXIN B_1 VALUES IN SPICE SAMPLES

Sample	Aflatoxin B_1 (ppb) Developed RIA	Aflatoxin B_1 (ppb) Detection by UV on TLC
1	963	1000
2	843	850
3	680	850
4	380	250
5	70	100
6	723	850

TABLE III. SAMPLE ANALYSIS OF AFLATOXIN B_1 VS TOTAL AFLATOXINS

Sample	Aflatoxin B_1 (ppb) Developed RIA	Total Aflatoxins (ppb) UV on TLC (Central Poultry Development Organisation)
1	25	110.5
2	50	166
3	52.5	140.7
4	40	188.2
5	29	166.8
6	39	166.2
7	33	165.6
8	45	218.2
9	18	51.2
10	26	77.1

The comparison of the sample values indicated in Table III are between two different methods, as this method has been the conventional way of detecting aflatoxin (total). A strikingly positive correlation is observed between the two methods in samples with high levels of aflatoxins

5. CONCLUSION

A solid phase assay for aflatoxin B_1 using antibody-coated tubes was optimized. The assay uses in-house polyclonal antibody raised against aflatoxin B_1-BSA and aflatoxin B_1-CMO-^{125}I-histamine as the tracer. The assay involves addition of standard or sample and tracer to the tubes followed by an incubation of 3h at 25°C. Decanting of the tubes after addition of wash buffer separated bound and free fraction. Samples from different feed were analysed and compared with different methods. Work was also carried out to optimize an ELISA system for aflatoxin B_1. ELISA has the advantage of being modified into a system, which can be visually analysed and could be used at any place.

ACKNOWLEDGEMENTS

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REFERENCES

[1] SINHA, K.K., BHATNAGAR, D.D., (Eds) Mycotoxins in Agriculture and Food Safety, Marcel Dekker, Inc. New York, 1998.

[2] GROOPMAN, J.D., CAIN, L.G., KENSLER, T.W., Aflatoxin exposure in Human Population: Measurements and relationship to cancer CRC Crit. Rev. Toxicol **19** (1988) 113-145.

[3] CHU, F.S., UENO, I. Production of antibody against aflatoxin B_1. Appl. Environ. Microbiol. **33 (**1977) 1125-1128.

[4] KORDE, A., BANERJEE, S., PILLAI, M.R.A., VENKATESH, M., Preparation and evaluation of ^{125}I-aflatoxin B_1 J. Radioanal. Nucl. Chem **250** (2001) 231-237.

[5] ARUNA, K., USHA, P., MEERA, V., SHARMILA, B., SARMA, H.D., HAZARE, S., SHARMA, A.K., PILLAI, M.R.A., Development of RIA procedure for aflatoxin B_1 measurement. J. Agric. Food Chem. (Articles) **51** (2003) 843-846.

[6] PETROU, P.S., KAKABAKOS, S.E., KONPPARIS, M.A., CHRISTOFIDIS, I., Antibody coating approach involving gamma globulin from non-immunized animal and second antibody antiserum, J. Immunoassay. **19** (1998) 217-293.

DEVELOPMENT OF IMMUNOMETRIC ASSAYS FOR AFLATOXIN B_1

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Abstract
Derivatives and conjugates were prepared for aflatoxin B_1. TLC alongwith UV light detection was used for monitoring the reactions. Antibodies were produced by immunization in rabbits. Commercially available immunogen was administered by subcutaneous injection into the back of two rabbits. The aflatoxin B_1-CMO-derivative was prepared by reaction with aminoxy acetic acid. Purification was carried out using silica column in selected solvents. Histamine as well as tyramine labeled with ^{125}I was conjugated to the activated CMO derivative of aflatoxin B_1. Purification was done by selective solvent extraction. TLC on samples of aflatoxin B_1 by detection under UV lamp was selected as the conventional method of quantification.

1. INTRODUCTION

Aflatoxin B_1 is the most potent carcinogen of the family of mycotoxins from Aspergillus flavus. It is present in rice, wheat, peanuts, as well as other dry vegetable products. It is highly fluorescent allowing the determination by silica-gel chromatography with high sensitivity. The furocoumarin structure is adequate for the development of specific and precise immunometric assays allowing the synthesis of derivatives for conjugation to high molecular weight proteins and cyclic compounds for iodination. Since the last decade, aflatoxin B_1 has been assayed using radioimmunoassay with a double antibody liquid homologous system. Presently, solid phase assays with specific IgG's adsorbed on plastic tubes with isotopic as well as non-isotopic tracers are being developed.

2. MATERIALS

All reagents were procured from Sigma Chemicals Company, USA. For the synthesis of conjugates, aminoxy acetic acid, pyridine, methanol, H_2O, dicyclohexyl carbodiimide, n-hydroxy succinimide, bovine serum albumin, dimethyl sulfoxide, dimethylformamide and tributylamine, iso-butyl chloroformate were used. For radiolabeling, chloramineT, sodium metabisulphite and potassium iodide were used.

TLC-Silicagel 60 with stain F254, electrophoresis and silica column separation was used. Tris buffer 0.05M pH 7.5, phosphate buffer 0.05M pH 7.4, tetrahydrofuran: ethyl acetate: n-hexane (2:13:35), chloroform: methanol (97:3), n-Butanol: NH_4OH 2N: ethanol (4:1:1) were prepared as solvents for different experiments. Reagents for cytotoxicity studies were cells in culture, Buffer Dulbecho modified medium and Buffer Hank's: acetate.

3. METHODS

3.1. Antibodies

Aflatoxin B_1-BSA conjugate obtained from Sigma was used for antibody generation in rabbits following reported procedure [1,2]. Rabbits were injected, each with 105 µg of aflatoxin B_1-BSA conjugate that was equivalent to 6 µg of antigen in the immunogen. After three weeks, rabbits were bled without any further booster. The sera were separated by centrifugation. After four months, another booster was given and rabbits were bled three weeks later. These sera have now been tested in India.

3.2. ^{125}I-Aflatoxin B_1 tracer

Aflatoxin B_1 carboxymethyl oxime was prepared following the technique described by Chu and Ueno [1]. The CMO-derivative of aflatoxin B_1 was prepared by refluxing aflatoxin B_1 with aminoxy acetic acid at 55°C in a mixture of methanol: pyridine: water (4:1:1) for two hours and overnight at 4-10°C. Purification was carried out using silica column with three solvent systems. First, the derivative was dried and recovered in $CHCl_3$ and loaded on top of the column. Secondly, the solvents were passed through the column in the following order, 6 mL $CHCl_3$, 30 mL of a mixture of $CHCl_3$ and methanol (97:3) and lastly 12 mL methanol 100%. In every case, one mL fractions were collected in numbered glass vials and a drop of each fraction was deposited in a numbered paper strip and viewed under UV lamp. Two regions were detected and chromatography on TLC with solvent $CHCl_3$: methanol (97:3) was developed to identify the derivative and free AfB_1. The second pool at R_f 0.3-0.5 was selected for further processing, as reported in the literature [3].
Histamine as well as tyramine labeled with ^{125}I was conjugated to the activated CMO derivative of AfB_1 by reaction with isobutyl chloroformate and tributyl amine alongwith dimethylformamide in dioxane [4,5,6]. These reactions were allowed to proceed overnight. Purification was carried out at alkaline pH by ethyl acetate extraction.

4. RESULTS AND DISCUSSION

The synthesized derivative showed a single species at R_f 0.3-0.5 after silica column purification in TLC as detected under UV lamp. The radioiodination yield was very low and was difficult to purify the pure product. Figure 1 shows the titre curves of antisera as evaluated by the group from India. Titres were determined by its binding with ^{125}I-histamine-aflatoxin B_1. High titres were obtained for both the antisera. Although tracers were prepared as per the standard well-described techniques, they were not suitable for developing an assay for aflatoxin B_1.

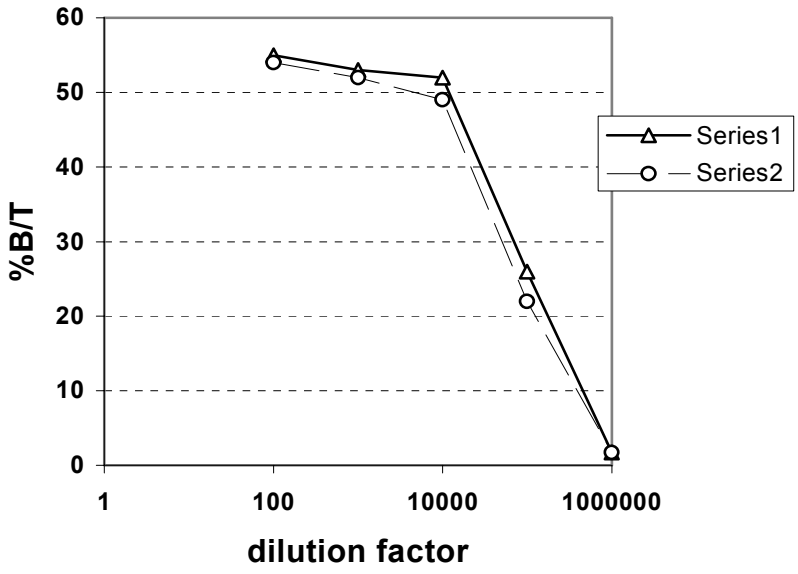

FIG. 1. Titration curve for aflatoxin B_1 antisera from two rabbits.

5. CONCLUSION

Synthesis of derivatives was performed with good reproducibility among several batches. Separation of free aflatoxin B_1 was done under controlled conditions and the follow up of the purification by TLC was simple and sensitive. Good quality antisera have been produced. Although the conjugates were prepared following the well-described techniques and with good laboratory conditions, we could not succeed in preparing a suitable tracer, probably due to the crucial steps in the procedure.

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REFERENCES

[1] LANGONE, J.J. AND VAN VUNAKIS, J.L., Aflatoxin B_1 specific antibodies and their use in Radioimmunoassays J.Nat. Cancer. Invest. **56** (1976) 591-596.

[2] MASSAGLIA, A., BARBIERI, U., SIRI-UPATHUM, CHYAGRIT AND VITALI, R. Preparation of ^{125}I-Labeled Steroid Derivatives for Radioimmunoassay. Int. J. Appl. Radiat.Isot. **24** (1973) 455-462.

[3] CHU, F.S. AND UENO, I. Production of antibody against aflatoxin B_1. Applied and Environmental Microbiology **33** (1977) 1125-1128.

[4] VAITUKAITIS, J., ROBBINS, J.B., NIESCHLAG, E. AND ROSS, G.T., A method for producing specific antisera with small doses of immunogen. J. Clin. End. **33** (1971) 988-991.

[5] KAKABAKOS, S.E., LIVANIOU, E., EVANGELATOS, S.A., EVANGELATOS, G.P. AND ITHAKISSIOS, D.S., Isolation of mono- and di-iodine 125 tyramines for conjugation labeling. Eur. J. Nucl. Med. **18** (1991) 952-954.

[6] KORDE, A., BANERJEE, S., PILLAI MRA, VENKATESH, M., Preparation and evaluation of ^{125}I-aflatoxin B_1. J. Radioanal. Nucl. Chem. **250** (2001) 1231-1237.

PRODUCTION AND CHARACTERIZATION OF ANTIBODIES AGAINST ATRAZINE

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Abstract
Atrazine-BSA conjugate was synthesized and six rabbits were immunized with this conjugate. After 6 months, antiserum were obtained and characterized by RIA. The result showed that the titres of antibodies were 1:35000 to 1:70000 and the antibodies obtained were more specific to ametryn. Atrazine-tyramine was synthesized and radioiodinated with $Na^{125}I$ by chloramineT method. A radioimmunoassay was developed for atrazine using the prepared reagents.

1. INTRODUCTION

Atrazine (2-chloro-4ethylamino-6-isopropylamino-s-trazine) is one of the most commonly used herbicides in agriculture. It is efficiently employed for control of grassy and broadleaf weeds in maize, sugarcane and other crops as well as in non-crop land situations. The use of this herbicide over the past many years has resulted in contamination of the soil, surface water, ground and drinking water. The residue analysis of atrazine is a substantial part of environmental control programs and is usually carried out by TLC, HPLC or GC/MS. A cumbersome cleanup procedure and complex equipment are often needed when these techniques are used. They are not convenient for analyzing a large number of samples. In such situations, immunoassays have proved to be useful as screening methods.

2. MATERIALS

Atrazine was donated by Shandong Vicome Greenland Chem. Co. Triazine derivatives were provided by National Institute for Control of Pesticide. Bovine serum albumin was obtained from Sigma Chemicals Company, USA. The other reagents were supplied by Beijing Chemical Co.

3. METHODS

3.1. Synthesis of Atrazine-thiopropanoic Acid [1, 2]

The method of M.H. Goodrow was followed [1]. The mixture of 1.08 g atrazine (technical grade) and 574 mg of 3-mercaptopropanoic acid in 100 mL of absolute ethanol was added to 10 mL of 85% KOH in absolute ethanol and heated under reflux for 5 h. The solvent was distilled to leave a white solid. The residue was dissolved in 25 mL of 5% $NaHCO_3$. The solution was washed with $CHCl_3$ (3 x 10 ml) and acidified to pH 2 with 6N HCl. The resultant white solid was collected, washed with water and dried to obtain 1.1 g of atrazine-thiopropanoic acid.

3.2. Preparation of Atrazine-BSA Conjugate [1, 2]

33 mg of atrazine-thiopropanoic acid was dissolved in 0.65 mL of dry DMF with equimolar concentration of N-hydroxysuccinimide. 50 mg of dicyclohexyl carbodiimide was added to the above solution and stirred for 3 h. The reaction mixture was centrifuged at 3000 rpm for 10 min and half of the supernatant was added to BSA solution (67 mg in 13.5 mL of H_2O and 3.5 mL DMF) and stirred for 22 h. The supernatant was dialyzed against 0.05 M phosphate buffer, pH 7.4 for 48 h.

3.3. Immunization

Atrazine-BSA conjugate was used as the immunogen. Primary injection of 500 μg per rabbit was administered into six rabbits in the complete Freund's adjuvant and booster doses of 250 μg per rabbit in incomplete Freund's adjuvant were given every four weeks. After six months, the blood was collected from rabbits' jugular vein and centrifuged to obtain the antiserum.

3.4. Preparation of Atrazine-tyramine

28.5 mg (0.1mmol) of atrazine-thiopropanoic acid and 13.7 mg (0.1mmol) tyramine were dissolved in 10 mL of dry DMF containing 30 mg DEPBT{-(diethyloxyphosphoryloxy)-1,2,3-benzotriazin-4(3*H*)-one} and 40 µL triethyl amine and stirred over night. 30 mL of saturated NaCl was added to the mixture and extracted with ether. The compound was purified by HPLC.

3.5. Iodination of Atrazine-tyramine

Radioiodination was carried out using chloramineT as the oxidizing agent. 100 µL of 0.1M, pH 7.4 phosphate buffer, 5 µg (5 µL) of atrazine-tyramine and 1.5 mCi of $Na^{125}I$ was taken in a test-tube. 12.5 µL (0.5 mg/mL) of chloramineT was added and mixed for 3 min. 12.5 µL (1 mg/mL) of sodium metabisulphite was added to stop the reaction. The iodination mixture was purified by HPLC. The HPLC purification pattern is shown in Figure 1.

3.6. Characterization of antibodies against atrazine

The titre, affinity and cross-reactivities of antisera from rabbits were determined using RIA. 100 µL of standard or sample, 100 µL of ^{125}I atrazine-tyramine and 100 µL of antibody were incubated for 3 h at 37°C. 500 µL of precipitating reagent was added. The tubes were centrifuged at 3500 rpm for 20 min, supernatant was discarded and the precipitate counted in a γ-counter.

4. RESULTS AND DISCUSSION

The HPLC pattern is shown in Figure 1. The titre curve and competitive inhibition curves of antiserum are shown in Figures 2, 3, 4 and 5 respectively, and the affinity and cross-reactivities shown in Table I.

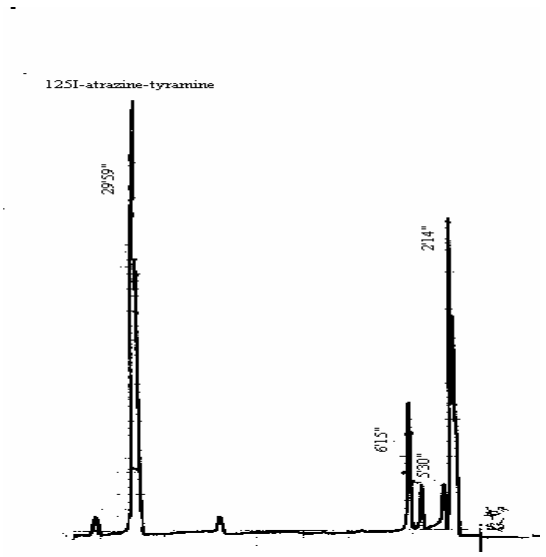

FIG. 1. HPLC pattern of purification of ^{125}I-atrazine-tyramine.

Haptens can be synthesized by two routes as shown in Figure 6. In Goodrow's experience, hapten synthesized from A results in antibodies, which are more specific for atrazine and simazine, while the hapten synthesized following the second route resulted in antibodies that recognized the S-methyl *s*-trazines such as ametryne, prometryn and simetryne. We had to choose the second route as we couldn't obtain 4,6-dichloro-N-(1-methylethyl)-1,3,5-triazine-2-amine. The results obtained confirmed that the antibodies were more specific for ametryn, prometryn and simetryne especially ametryn as shown in Table I.

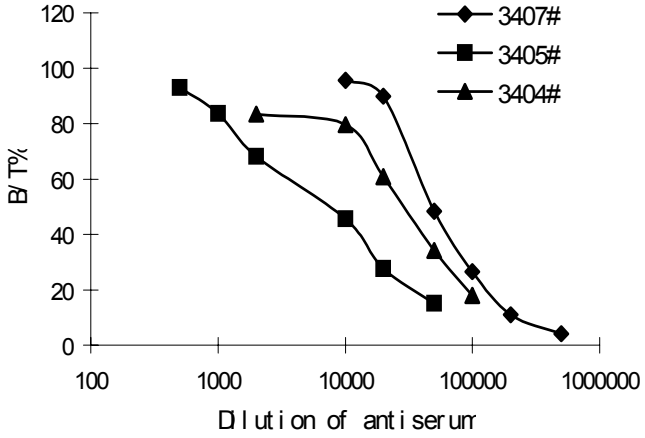

FIG. 2. Titre Curve of Antiserum.

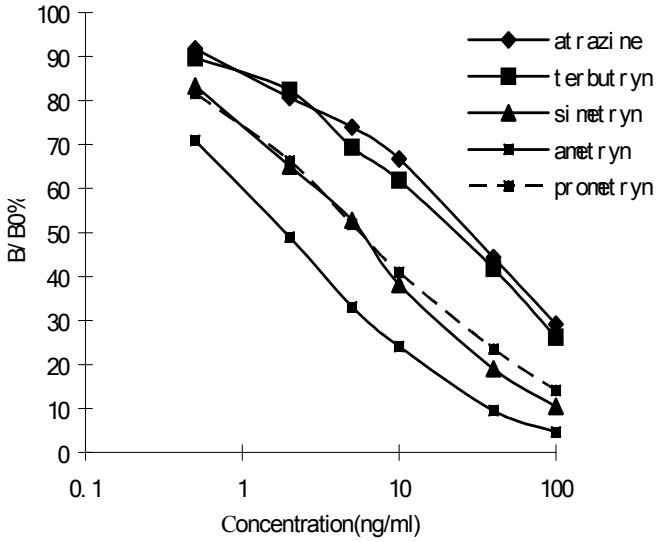

FIG. 3. Inhibition curve of Ab3404.

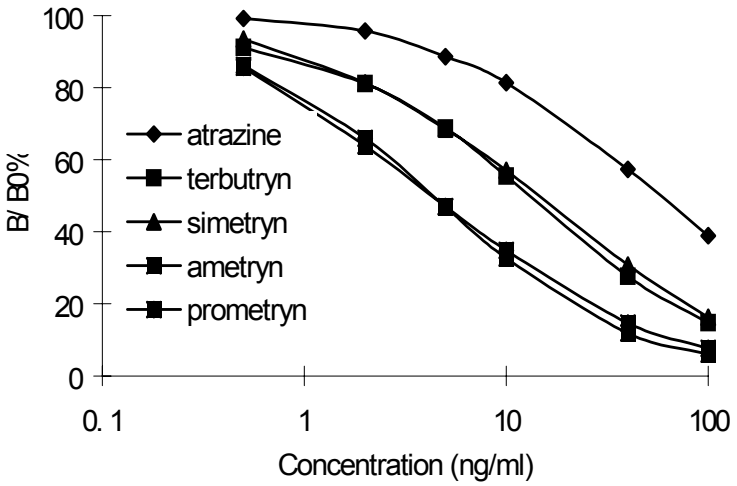

FIG. 4. Inhibition curve of Ab 3407.

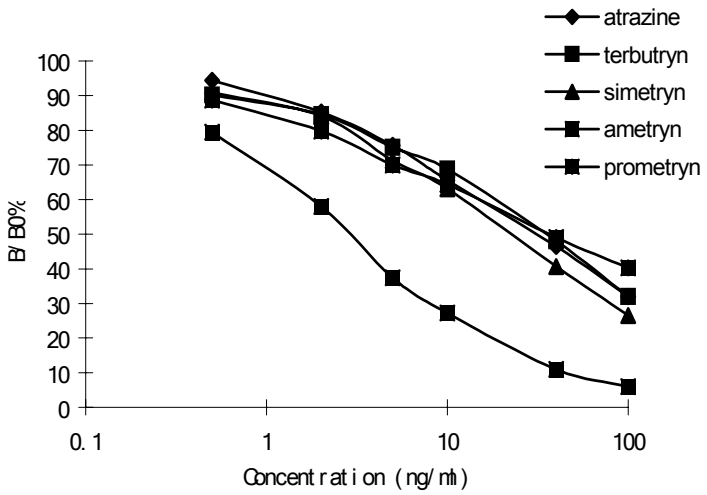

FIG. 5. Inhibition curve of Ab3405.

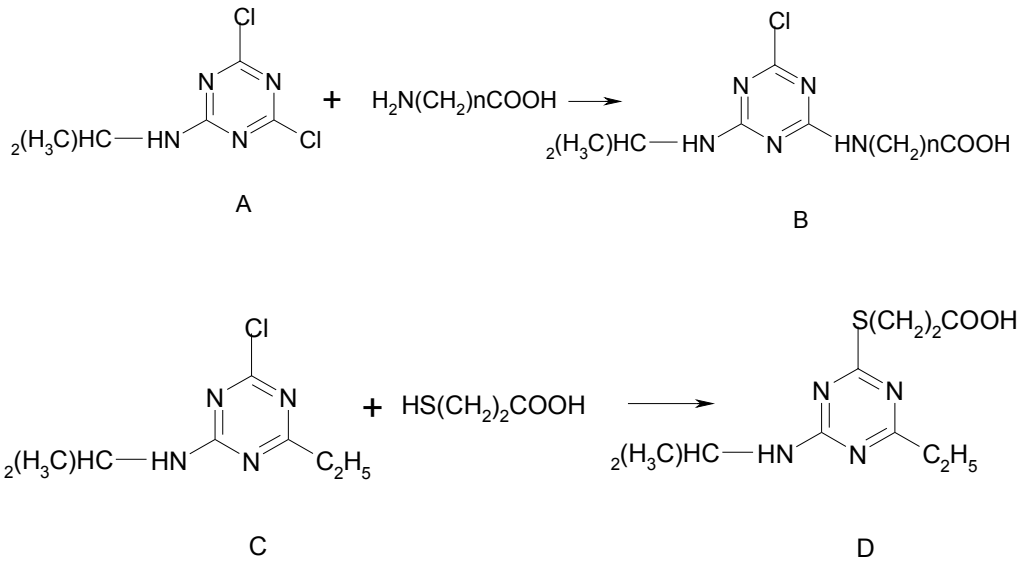

FIG. 6. Synthesis route for conjugation of atrazine derivatives to carrier protein and tyramine.

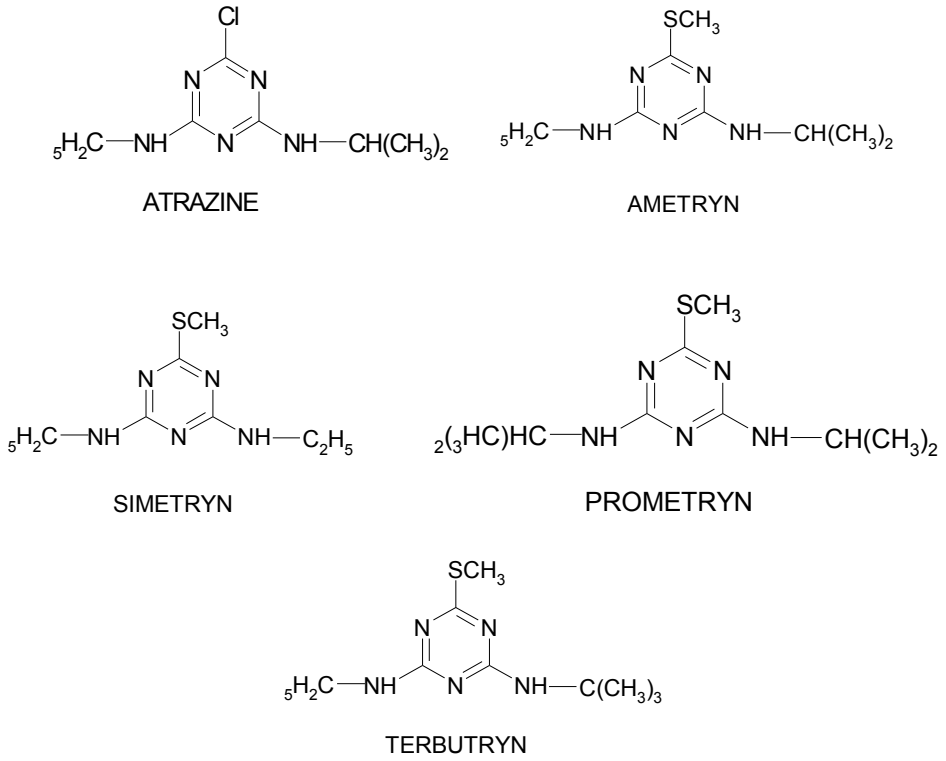

FIG. 7. Atrazine and its analogues .

TABLE I. CHARACTERIZATION OF ANTIBODIES

	No. of Ab	**3404**	**3405**	**3407**
Titer of Ab		1:35000	1:5000	1:70000
Affinity of Ab ($L.mol^{-1}$)		$2.0x10^7$	$1.7x10^7$	$1.9x10^7$
Cross-reactivity of Ab (%) based onatrazine	Ametryn	1400	1034	1289
	Simetryn	509	136.4	387
	Prometryn	509	81.1	1289
	Terbutryn	127	85.7	483
Cross-reactivity of Ab (%) based on ametryn	Atrazine	7.1	9.7	7.8
	Simetryn	36	13.2	30
	Prometryn	36	7.8	100
	Terbutryn	9.1	8.3	37.5

5. CONCLUSION

The following conclusions are drawn from our experience:

(i) Atrazine-BSA conjugate was synthesized successfully by M. H. Goodrow's method.
(ii) Rabbits were immunized with the conjugate and anti-atrazine antisera were obtained.
(iii) Atrazine-tryamine was also synthesized and iodinated with $Na^{125}I$ by ChloramineT.
(iv) The methodology of atrazine RIA was developed.
(v) The characterization of antibodies by RIA showed that titres of antibodies were 1:35000-70000, and antibodies were much more specific to ametryn.

REFERENCES

[1] GOODROW, M.H., HARRISON, R.O., HAMMOCK, B.D., Hapten Synthesis, Antibody Development, and Competitive Inhibition Enzyme Immunoassay for s-Triazine Herbicides J. Agric Food Chem. **38** (1990) 990-996.

[2] THOMAS, G., BERTOLD, H., Production of Monoclonal Antibodies for the Determination of s-Triazines with Enzyme Immunoassay Food Agric. Immunol. **2** (1990) 85-97.

DEVELOPMENT OF RIA KITS FOR THE DETERMINATION OF ATRAZINE IN WATER

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Abstract
The objective of this study was to develop a rapid and reliable solid-phase radioimmunoassay for the determination of atrazine in water. The tubes were first coated with IgG precipitated from anti-atrazine serum raised in sheep. The reaction was performed by incubating a mixture of 50 μL of standards in water and 200 μL of atrazine tracer for 45 minutes. After washing, the tubes were counted in a gamma counter. The minimum detectable concentration was 0.02 ppb, which was much below the limits permitted by the USEPA guidelines for drinking water (3 ppb). The assay allows accurate determination of atrazine in water with good specificity, precision and accuracy, and is suitable for the rapid screening of numerous surface and subsurface water samples as well as for a variety of other analytical applications.

1. INTRODUCTION

For many decades, herbicides have been used for plant protection in agriculture. However, a part of it does not reach the weeds but instead volatilizes or stays in the soil. Herbicides can be washed out of the air and can reach the rivers or lakes through water. From the soil, they also can leach into the ground water processing plant. One of the most frequently used pesticides in Thailand is atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine). It belongs to the group of triazine pesticides. To help in protecting the natural resources from unwanted load of pesticides, it is highly desirable to have a means of checking the herbicidal content in drinking water. Although there was no report on environmental impact of Atrazine in Thailand, the USEPA had conducted the survey during 1988-1990 and found that it is environmentally significant and was the second most commonly detected pesticide residue in drinking water wells in Thailand.

Immunoassay methods began as early as 1959, initially in the medical field, and were recommended for use in the environmental monitoring in 1971. Test kits have been commercially available since 1990, but widespread environmental field application of immunoassay technology has occurred only in the mid-1990. Presently, about 12 commercial manufacturers provide these kits. Environmental Protection Agency (EPA) promulgated the immunoassay method for atrazine analysis in 1999, as EPA Method 4670. Several papers during mid-1990 have compared atrazine analysis results by immunoassay with laboratory GC/MS methods, most notably in the three American Chemical Society Symposium Series publications. These papers agree that immunoassay and lab GC/MS results are comparable, but require verification analysis for a portion of the samples.

The estimation of pesticide by immunoassay reduces the cost of laboratory analysis, which allows for increased sample coverage for a reliable and more efficient verification programme. Immunoassay is generally useful for the detection of triazine herbicides, especially atrazine. The immunoassay method for atrazine has proven to be useful for monitoring vulnerable areas of contamination. Although many commercial immunoassay kits for atrazine are available worldwide, and have performed with promising results, the routine test for environmental samples could not be done in Thailand because budgets and resources were limited. Development of an assay for atrazine measurement and formulation into kits based on locally developed reagents would make routine service a practical approach in solving the problems.

2. MATERIALS

3-mercaptopropanoic acid was purchased from Merck, Germany. Atrazine was supplied from Zuellig Agro. TLC was performed on 0.25 mm precoated silica gel 60 F254 plastic sheets (Merck). Compounds were detected first by viewing under ultraviolet light (254 mm) in appropriate solvent systems as described in the experiment. Immunochemicals were obtained from Sigma Chemical Co. and ICN Immuno Biologicals. Analytical standards of atrazine (purity 98%) were purchased from Chem Service, USA. Polystyrene tube from NUNC "Star" tubes (immuno quality, 12x75 mm) were used for coating. Antisheep atrazine was obtained from Guildhay, U.K. Melting points were determined with a Thomas Hoover apparatus and infrared spectra (IR) were recorded on Perkin Elmer 1600 series FTIR. ^{1}H-NMR spectra were measured with a Varian EM-360 L 60-MHz spectrometer. Ultraviolet-visible spectra (UV) were obtained on a Varian UV Spectrophotometer. Radioactivity was measured with gamma counter from Packard Instrument. HPLC analysis was performed using a JASCO HPLC system equipped with a quarternary gradient pump (Po-980), loop (Rheodyne), and UV detector. Acetonitrile and methanol (HPLC grade) were purchased from Sigma-Aldrich and Merck, Germany.

3. METHODS

3.1. Synthesis of atrazine haptens

Haptens were synthesized by the routes shown in Figures 1 and 2. TLC confirmed the purity of compounds. The final products were further characterized by IR and ^{1}H-NMR analysis as well as high-resolution mass spectroscopy.

3.1.1. Preparation of atrazine-thiopropanoic acid (1 a)

To a stirred, heterogeneous mixture of 1.08g (5.01mmol) technical grade atrazine and 100 mL absolute ethanol was added a solution of 0.574 g (5.40 mmol) 3-mercaptopropanoic acid and 0.714 g (10.8mmol) of 85% KOH in 10 mL of absolute ethanol under N_2. The mixture became homogeneous on refluxing and a precipitate (KCl) appeared. The refluxing was continued for 5 h until atrazine was no longer detectable by TLC. The solvent was distilled to leave a white solid. The residue was dissolved in 25mL of 5% $NaHCO_3$. The basic solution was washed with $CHCl_3$ (3x10 mL) and acidified to pH 2 with 6N HCl. The resultant white solid was collected, washed with water, and dried to obtain 0.43g (30%) of product.

3.1.2. Preparation of atrazine-histamine(1b)

Atrazine-thiopropanoic acid *(1a)* was coupled covalently to histamine by the N-hydroxysuccinimide ester method of Langone and Van Vunakis (1975). The atrazine-thiopropanoic acid (1a) 0.20 mmol (57.0 mg) was dissolved in 1.0 mL of dry DMF with equimolar N-hydroxysuccinimide (23.0 mg) and a 10% molar excess of dicyclohexyl carbodiimide (45.4 mg). After 3.5 h of stirring at 25ºC, the precipitated dicyclohexylurea was removed by centrifugation, and the DMF supernatant was added to histamine solution. Histamine 0.2 mmol (22.2 mg) was dissolved in 5 mL of H_2O, and 1.05 mL DMF was added slowly to the vial with vigorous stirring. The DMF supernatant was added to histamine solution. The reaction mixtures were stirred gently at 4ºC for 22 h to complete the conjugation and extracted with dichloromethane (3x5 mL). The dichloromethane was removed under reduced pressure, leaving a white solid.

3.2. Radioimmunoassay of atrazine

3.2.1. Preparation of ^{125}I-atrazine tracer

500 μCi $Na^{125}I$ was added to 5 μL (5 μg) atrazine histamine (*1b),* 5 μL of 0.5 M phosphate buffer, 5 μL of chloramineT (1mg/ml in 0.05M phosphate buffer, pH 7.4). The contents were mixed on a

vortex mixer for 20 sec. 5 μL of sodium metabisulphite (5 μg) was added to the reaction tube. The mixture was applied to the HPLC column and eluted under the following conditions.

Column	:	Reverse phase, C18-ODS
Gradient	:	0–5 min, an isocratic mixture of 20% CH_3CN and 80% CH_3COONa 5–80 min, a 20-60% gradient of CH_3CN
Flow rate	:	1 ml/min

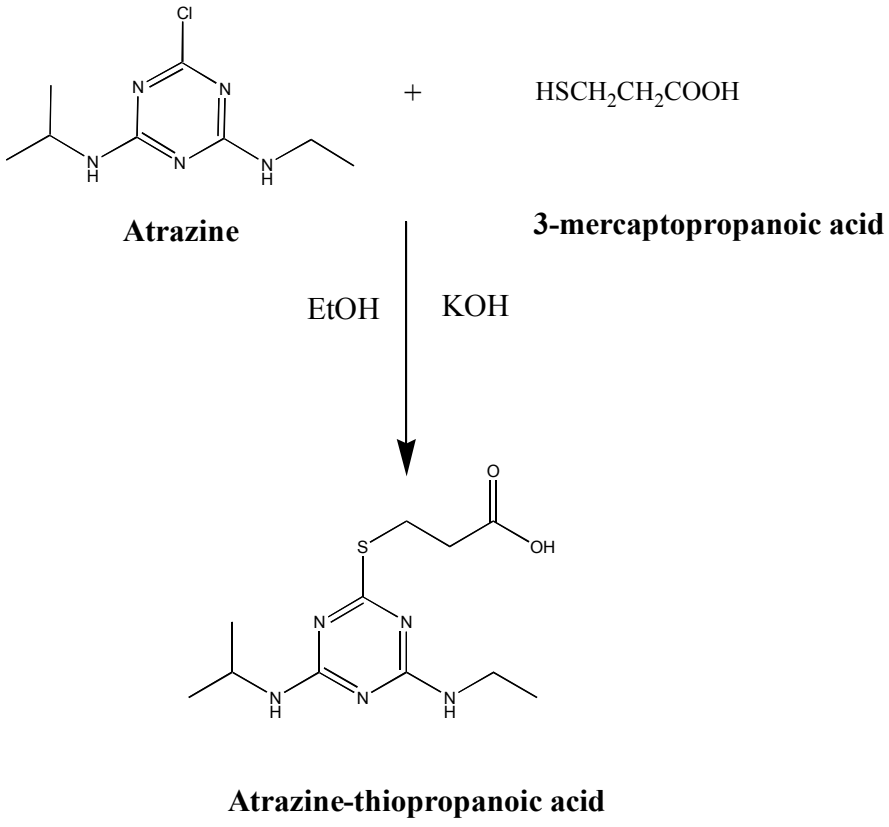
60 fractions (1 min per fraction) were collected and monitored for the activity using a gamma counter. The fractions corresponding to atrazine peak were pooled and stored at 4ºC until use.

3.2.2. Preparation of atrazine standards

Concentrated stock solution of the analyzed atrazine (1000 ppm) was prepared by dissolving 10 mg of the atrazine in 10 mL methanol. Stock solution was subsequently used to prepare standards of different atrazine concentrations. These standards were prepared in distilled water to obtain the following working concentrations 0, 1, 5, 10, 50 and 100 ppb. These working standards were used in the assay.

3.2.3. Preparation of antibody coated tubes

The IgG fraction prepared from the sheep anti-atrazine serum was coated on the polystyrene tubes as follows. 300 μL of anti-atrazine solution (1:8000 of antibody in 0.05 M carbonate buffer, pH 9.6) were dispensed into polystyrene tube (NUNC "Star" tubes immuno quality, 12x75 mm).



Atrazine-thiopropanoic acid

FIG. 1. Synthesis and structure of atrazine-thiopropanoic acid (1a).

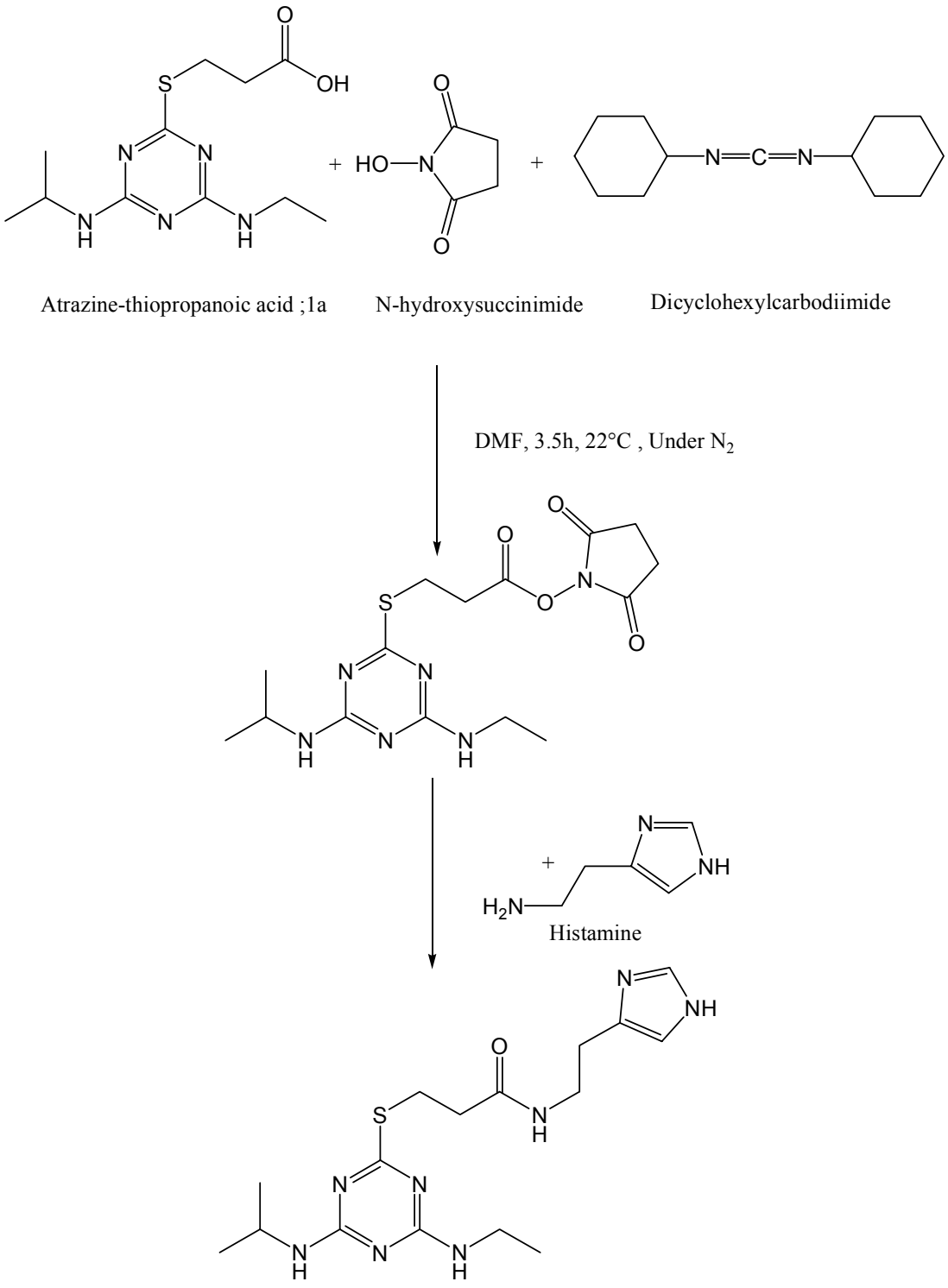


FIG. 2. Synthesis and structure of atrazine histamine (1b).

After overnight incubation at 4ºC (16–18 h), the solution was discarded and 0.5 mL of washing solution (0.2% tween-20) was added to each tube. The washing step was repeated and the tubes were stored at 4^{o}C until use.

3.2.4. Assay optimization

Effect of various factors such as incubation time and temperature, amount of antibody, amount of standard or sample, activity of tracer etc. on the standard curve and on the levels of the control serum was evaluated in order to optimize the assay. The general assay protocol was as follows: 50 µL of each standard or sample was dispensed into a corresponding coated tube, followed by 200 µL of atrazine tracer. After gently mixing on a vortex mixer, the tubes were incubated for appropriate time at room temperature. The tubes were washed with 0.5 mL wash buffer and the radioactivity of the bound fraction was measured in a gamma counter.

3.3 Assay validation

A developed assay protocol required validation to establish suitability before proceeding to practical application. Validation was carried out by evaluating the following features:

Sensitivity
The sensitivity of the method was determined as the minimum detectable concentration at two standard deviation from the zero binding value.

Recovery test
Known quantities of atrazine were added into different sample pools. The percentage of recovery of added atrazine was calculated after the assay.

Precision
3 samples of different concentration were evaluated in the same assay (intra-assay precision) as well as in different assays (inter-assay precision).

3.4. Determination of atrazine using HPLC

Atrazine stock solution having concentration of 100 mg/L was prepared in methanol in glass-stoppered volumetric flask and stored in a freezer at –20^{o}C. Six standard solutions with atrazine concentrations of 0.5, 1.0, 2.0, 3.0, 5.0 and 10.0 ppm in methanol/water (50/50 v/v) were prepared for external calibration. All standards were stored at 4ºC. Prior to analysis, the samples were allowed to attain room temperature.

Analyses of atrazine using HPLC have been studied in order to determine the optimum condition for use as reference method for atrazine RIA. HPLC analyses of the atrazine were performed on a JASCO HPLC. The sample volume injected into HPLC system was 20 µL. The operation conditions were as follows :

- Reverse phase C18 column (Crestpak C18S, 4.6x150 mm, JASCO)
- Mobile phase : isocratic 70% acetonitrile, 30% water
- 1.0 ml/min flow rate
- Detection : UV at 240 nm

4. RESULTS AND DISCUSSION

4.1. Synthesis of haptens

3-{{4-(Ethylamino)-6-[(1-methylethyl)amino]-1,3,5-triazine-2-yl}thio}propanoic acid; (1a) was obtained as a white crystalline substance (m.p 165^{o}C) with ~ 30% yield. The product showed a single

spot in TLC with R_f of 0.6 (ethyl acetate: methanol, 9:1) and 0.5 (ethylacetate: dichloromethane, 9:1). The peaks in the IR, MS, ^{1}H-NMR and UV spectrum were consistent with the expected product.

3-{{4-(Ethylamino)-6-[(1-methylethyl)amino]-1,3,5-triazine-2-yl}thio}propanoiodohistamine (1b) was obtained as a white crystalline substance (m.p. 170^{o}C) with ~20% yield. The product showed a spot in TLC with R_f of 0.1 in both systems viz. ethyl acetate: methanol, 9:1 and ethylacetate: dichloromethane, 9:1 and a small spot at the same R_f of starting material

4.2. Radioimmunoassay of atrazine

The typical assay protocol for determination of atrazine in water was as follows: 50 μL of each standards or samples was dispensed into the antibody-coated tubes. 200 μL of the atrazine tracer was added to each tube. Each tube was gently mixed on a vortex mixer and incubated for 45 min at room temperature (18-25^{o}C). After the incubation the contents of the tubes were decanted completely. 0.5 mL of washing solution was added to each tube and decanted again. The operation was repeated twice. The radioactivity in the bound fraction was measured using a gamma counter.

Pipetting scheme (volumes in μL)

	Anti atrazine coated tubes							
Specimen	S1	S2	S3	S4	S5	S6	Samples	Total
Standard 0	50	-	-	-	-	-	-	-
Standard 1	-	50	-	-	-	-	-	-
Standard 5	-	-	50	-	-	-	-	-
Standard 10	-	-	-	50	-	-	-	-
Standard 50	-	-	-	-	50	-	-	-
Standard 100		-	-	-	-	50	-	-
Water Samples	-	-	-	-	-	-	50	-
Atrazine Tracer	200	200	200	200	200	200	200	200

Incubate the tubes for 45 min at room temperature.
Decant/wash twice with 0.5 mL washing solution.
Count 1 min in gamma counter.

4.2.1. Assay optimization

Incubation time

50 μL standard or control and 200 μL atrazine tracer were incubated in the coated tubes for 0.5, 0.75, 1, 2 and 18 h at room temperature (25^{o}C) followed by separation of bound and free fractions. The tubes were counted and the count rates are shown in Table I and standard curves are shown in Figure 3. It was observed that the bound radioactivity increases with increasing incubation time, which is mainly due to the diffusion controlled antibody-antigen interaction. The standard curves obtained at different incubation times were parallel. 45 minutes was chosen as the incubation time because it was sufficiently long enough for the assay to be complete and be comparable.

Incubation temperature

Several assays were set up at room temperature, 4ºC and 37ºC and no significant difference was observed. The results are shown in Table II and Figure 4.

Sample size

This was investigated by setting up assay using 25, 50 and 100 μL of standard with 45 minutes incubation at room temperature. The count rate of the tubes and standard curves are presented in Table III and Figure 5, respectively. As expected, the bound radioactivity increases with increasing amount of standard volume. With 25 μL standard, the 0-1 ppb standards are almost indistinguishable. The optimum amount of standard proved to be 100 μL.

Activity of atrazine tracer

Atrazine tracer with varying amount of activity (20000, 30000, 40000 cpm/tube) were incubated with sample in coated tubes. After incubation, bound fraction was counted and %B/T was calculated and listed in Table IV and Figure 6. The results illustrated that activity of tracer at 20,000 cpm/tube gave good binding and equivalent to 40,000 cpm/tube. The tracer of 20,000 cpm/tube is chosen as optimal activity for the assay for it not only can save the amount of tracer used, but also reduce the exposure to the operator.

TABLE I. BOUND COUNTS OF ATRAZINE STDS WITH INCUBATION TIME

Atrazine	Binding (CPM)				
(ppb)	30 min	***45 min***	1 h	2 hr	18 hr
0	11251	***11772***	12765	13018	11567
1	9628	***10444***	10999	10501	10232
5	6582	***7222***	7236	7189	7526
10	4416	***4619***	5832	6616	6116
50	1171	***1236***	1551	1746	1957
100	670	***823***	953	1000	1217

TABLE II. BOUND COUNTS OF ATRAZINE STDS WITH INCUBATION TEMPERATURE

Atrazine	Binding (CPM		
(ppb)	4^oC	***25^oC***	37^oC
0	12247	***13214***	11918
1	7213	***7038***	7774
5	3831	***3345***	4705
10	3213	***2334***	3891
50	903	***639***	1169
100	568	***435***	689

TABLE III. BOUND COUNTS OF ATRAZINE STDS AS A FUNCTION OF SAMPLE SIZE

Atrazine	Binding (CPM)		
(ppb)	25 μL	50 μL	***100 μL***
0	11213	11992	***12388***
1	11715	10363	***7873***
5	10557	7796	***4985***
10	8838	6028	***4109***
50	2998	1921	***1226***
100	1899	1115	***713***

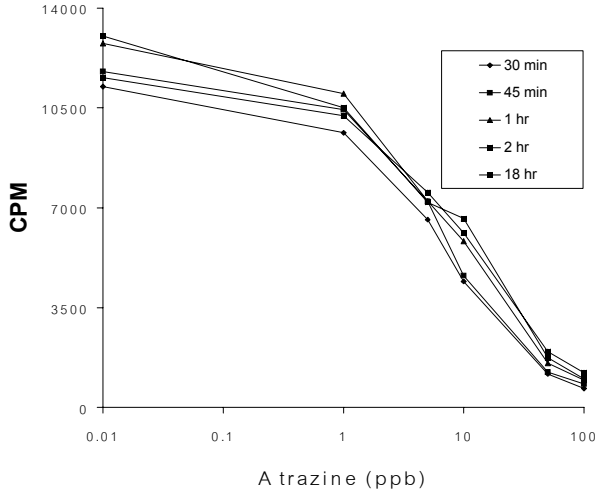


FIG.3. Standard curves for atrazine with different incubation time.

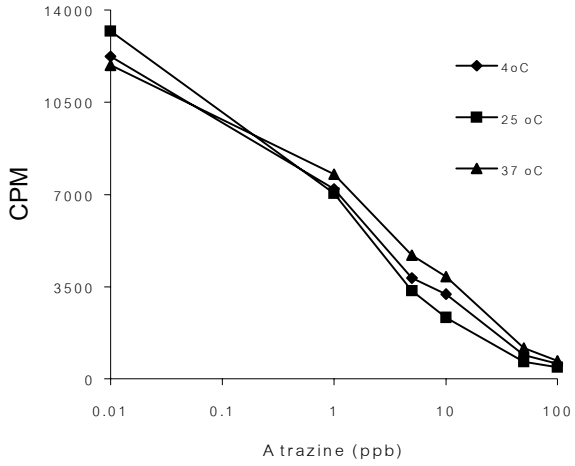


FIG. 4. Standard curves for atrazine at different incubation temperature

TABLE IV. % B/T OF ATRAZINE STANDARDS AS A FUNCTION OF TRACER ACTIVITY

Atrazine	Binding (%B/T)		
(ppb)	***20,000 cpm***	30,000 cpm	40,000 cpm
0	***42.06***	40.17	43.33
1	***39.04***	33.69	36.18
5	***28.01***	27.64	26.13
10	***22.34***	21.90	20.76
50	***6.93***	6.99	6.09
100	***3.89***	4.01	3.74

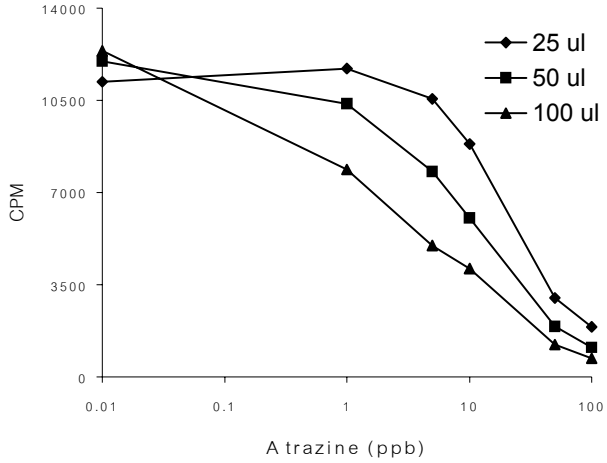


FIG. 5. Standard curves for atrazine using different sample volume.

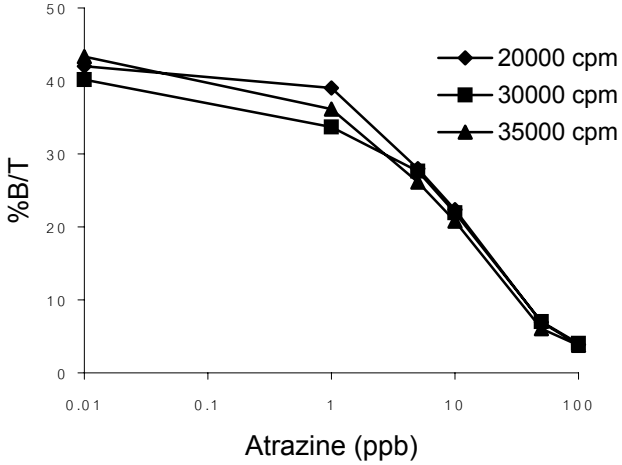


FIG. 6. Standard curves for atrazine using tracers with different activity.

TABLE V. PRECISION OF ATRAZINE RIA BASED ON COATED TUBES

	n	Mean (ppb)	S.D (ppb)	C.V (%)
Within-batch				
Sample L	20	1.25	0.06	5.18
Sample M	20	8.15	0.32	3.88
Sample H	20	27.97	1.82	6.52
Between-batch				
Sample L	10	1.30	0.12	7.52
Sample M	10	8.23	0.45	5.36
Sample H	10	28.20	1.62	5.50

TABLE VI. RECOVERY OF ATRAZINE RIA BASED ON COATED TUBES USING DISTILLED WATER AND TAP WATER

Distilled water			Tap water		
Spiked	Assayed	% Recovery	Spiked	Assayed	% Recovery
0	< 0.02	-	0	< 0.02	-
10	12.29	122.9	10	11.29	112.8
20	22.83	114.1	20	22.95	114.7
50	52.92	105.8	50	52.73	105.5

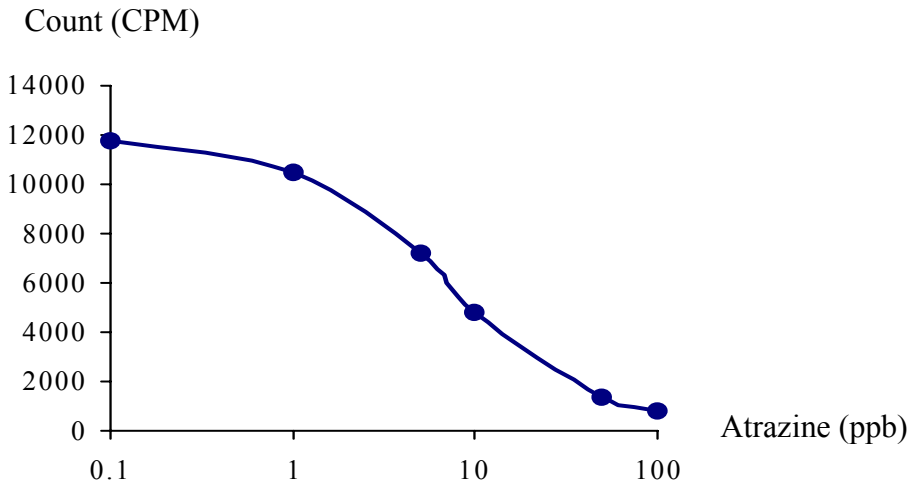


FIG. 7. Typical standard curve for atrazine by Radioimmunoassay.

4.3. Assay Validation

Sensitivity
The detection limit of atrazine by RIA was 0.02 ppb (n = 20). Figure 7 shows a typical standard curve of the atrazine assay.

Precision
The assay precision was evaluated by assaying three water samples, 20 replicates each in one assay and three water samples for 10 consecutive days. The within-assay and between-assay coefficients of variation were 3.9-6.5 % and 5.4-7.5 %, respectively. Precision data obtained over the range of 1-30 ppb atrazine is given in Table V.

Recovery
The assays were run with unspiked and atrazine-spiked water samples from different sources. None of the water samples showed any matrix effects (Table VI).

Determination of atrazine using HPLC
The standards were analyzed by HPLC methods. Several HPLC columns were tested to find the one, which gave the best separation. It was found that the column Model Crestpak C18S, 4.6x150 mm, (JASCO) gave the best results for the analysis of the standard solutions. Several isocratic and gradient elutions, with two mobile phases viz methanol/water and acetonitrile/water, were used for the separation of the compounds of interest.

The best separation with the most symmetrical peak was obtained using the mobile phase acetonitrile/water (70/30 v/v) under isocratic conditions; hence this mobile phase was used for further

investigations. Several flow rates (0.5-1.5 mL/min) were evaluated. The best separation was achieved using a flow rate of 1.0 mL/min. The chromatogram of the separation under the above mentioned HPLC conditions is shown in Figure 8. The retention time for atrazine is 2.97 minute. The peaks were quantified at a wavelength of 240 nm, where the compounds have maximum absorption.

The calibration was carried out by injecting various standard solutions into the HPLC column. The r^2 value obtained from the respective calibration curves was 0.9991 (Figure 9).

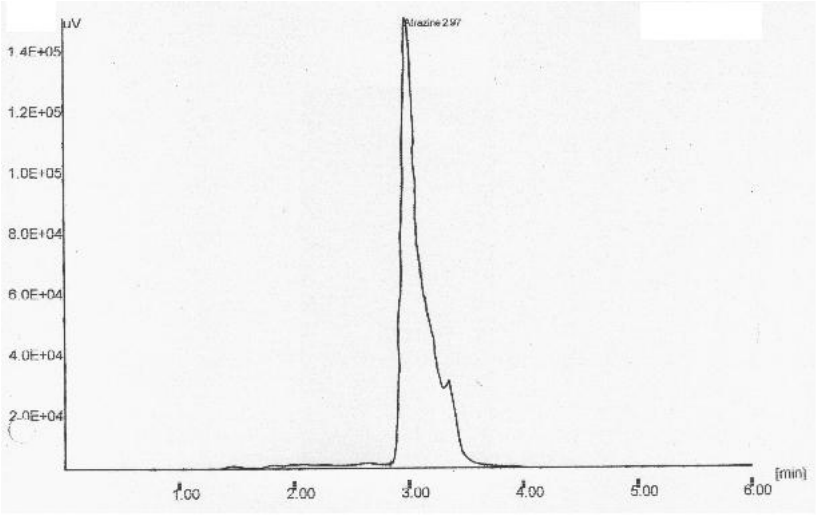


FIG. 8. Chromatogram for atrazine standard using HPLC.

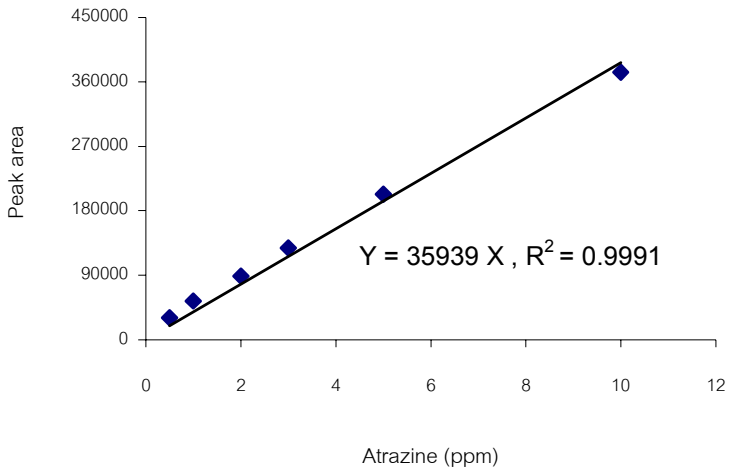


FIG. 9. Six atrazine standards by HPLC showing peak area versus concentration.

5. CONCLUSION

The radioimmunoassay of atrazine in water samples using coated tubes method has been described. The formats described here represent optimized conditions yielding a rapid, highly sensitive and reproducible assay that appears to be rugged and adaptable for field use. The assay, involving the use of "limited antibody", allows reactions to come to equilibrium after one step incubation period (45

min) at ambient temperature. The optimization and validation of the atrazine RIA produced a kit that allows the measurement of atrazine in 100 μL samples at concentrations ranging from 0-100 ppb with a sensitivity of 0.02 ppb. The assay exhibited good precision and reasonable recovery. We have shown the potential usefulness of these assays for the detection and measurement of atrazine herbicide at levels commonly found in environmental samples.

The reference method for analysis of atrazine by High Performance Liquid Chromatography (HPLC) is the technique used in this laboratory exercise. Using HPLC does not require extracting the organic analyte in non-aqueous form, as the HPLC can accept aqueous and non-aqueous injection. However, one major drawback of HPLC is its poor sensitivity to low concentration of atrazine. A pre-concentration step is therefore required. The solid phase extraction (SPE) may be used to extract atrazine, which is a non-polar organic compound, into an organic solvent. Furthermore, the solid-phase extraction and HPLC may be used in conjunction with RIA method as a confirmation technique. The combination of RIA and HPLC provides a low-cost screening analysis for atrazine herbicides in surface and groundwater and is suitable for large water-quality surveys. However, field assessment with samples from several contaminated area is the next step needed in our study before the routine screening is established. This is required to further validate the results obtained from RIA technique developed with the reference HPLC method.

BIBLIOGRAPHY

JUNGBLUTH, F., Analysis of Crop Protection Policy in Thailand. TDRI Quarterly Review. **12**, (1997) 16-23.

[MUSICK, S., CHEREPON, A., PETERS, J., Immunoassay Analysis for the Determination of Pesticides in Groundwater Samples: The Texas Experience. Texas Natural Resource Conservation Commission. http://www.nwqmc.org/2000proceeding/papers/pap_musick.pdf

HAYES, M.C., JOURDAN, S.W. AND HERZOG, D.P., Determination of Atrazine in Water by Magnetic Particle Immunoassay : Collaborative Study. Journal of AOAC International. **79** (1996) 529-537.

STEARMAN, G.K., Enzyme Immunoassay Determination of Atrazine Degradation in Soil : Moisture, Sterilization, and Storage Effects. Journal of Soil Contamination. **2** (1993) 131-140.

HARRISON, R.O., GOODROW, M.H. AND HAMMOCK, B.D., Competitive Inhibition ELISA for the s-triazine herbicides : Assay Optimization and Antibody Characterization. J. Agric Food Chem. **39** (1991) 122-128.

STEINHEIMER, T.R., HPLC Determination of Atrazine and Principal Degradations in Agricultural Soils and Associated Surface and Ground Water. J. Agric Food Chem. **41** (1993) 588-595.

http://www.epa.gov. EPA Method : Triazine Herbicides as Atrazine in Water by Quantitative Immunoassay. CD-ROM 4670, Revision 0 January 1998.

SCHLAEPPI, J.M. WERNER, F. AND RAMSTEINER, K., Hydroxyatrazine and Atrazine Determination in Soil and Water by Enzyme- Linked Immunosorbent assay Using Specific Monoclonal Antibodies. J.Agric. Food Chem. **37** (1989) 1532-1538.

HUBER, S. J., Improved Solid-Phase Enzyme Immunoassay System in the ppt Range for Atrazine in Fresh Water. Chemosphere. **14** (1985) 1795-1803.

LANGONE, J.J. AND VAN VUNAKIS, H., Radioimmunoassay for Dieldrin and Aldrin.

Res. Common.Chem. Pathol. Pharmacol. **10** (1975) 163-171.

http://www.abraxiskits.com/Products/Product%20Atrazine/Fast Screen Atrazine.htm.

DEVELOPMENT OF RADIOIMMUNOASSAY FOR THE MEASUREMENT OF LEPTIN IN HUMAN SERUM

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Abstract

Leptin is a 16 KDa polypeptide hormone encoded by the obese gene (*ob*) and secreted by adipose tissue. This hormone plays a major role in energy homeostasis and regulation of food intake and body weight. It also affects the metabolic, neuroendocrine, reproductive and haematopoietic systems. In this study, we report the development and validation of a radioimmunoassay for the quantitative measurement of human leptin in serum. Labeling of recombinant human leptin with ^{125}I was best performed by the chloramineT method, specific activities of 2.8–3.3 MBq/μg were obtained. New Zealand white rabbits were immunized with recombinant human leptin and a highly specific polyclonal antibody was obtained without measurable cross-reaction as analysed using 10 different antigens. Concentrations of human leptin from serum samples were measured by a single overnight incubation assay with a sensitivity of 0.5 ng/mL and a measuring range of 0.5–100 ng/mL. Separation of bound and free fractions was performed with a magnetizable immunosorbent suspension and in the liquid phase assay separation was performed by using a 1mL mixture of anti-rabbit IgG (second antibody raised in sheep) and 8% PEG-solution after 20 minutes incubation at room temperature. Intra-assay and inter-assay imprecision (coefficient of variation) was < 6% and 8%, respectively. Recoveries of leptin in four human serum specimens serially diluted ranged from 88% to 106%. It was concluded that serum human leptin concentrations can be accurately and precisely measured by this novel radioimmunoassay procedure. Preliminary results obtained from the measurement of serum leptin in lean, overweight and obese patients are presented. Serum leptin concentrations correlated with Body Mass Index (BMI) and were significantly higher in women than in men. Such good correlation helped in the follow-up of the treated obese patients.

1. INTRODUCTION

Zhang and co-workers reported the discovery of leptin (1994), a hormone secreted primarily by adipose tissue [1]. Leptin is encoded by the obese gene (*ob*) that has been identified and characterized in human as well as in a number of animal species. After intracellular processing of the 167 amino acid pro-hormone to cleave a 21 amino acid signal peptide segment, the mature form of leptin is secreted into the bloodstream where it circulates as a 146 amino acid (16 kDa) protein [1]. Crystal structure and NMR studies have characterized leptin as a four-helix bundle cytokine containing one disulfide bond (Cys_{96}-Cys_{146}) that is essential, both to its structure and to its function. There is no known close homology between leptin and other proteins but it is structurally similar to the long chain helical cytokine family [2,3].

Leptin is bound to receptors, mainly in hypothalamus. Receptors, similar to those of the class I cytokine receptor super family, have been identified in the central nervous system as well as in peripheral tissue locations [4]. In the hypothalamus, leptin has been reported to suppress neuronal levels of neuropeptide Y (NPY) and agouti-related peptide (AGRP), while stimulating the production of proopiomelanocortin (POMC) [5]. Together, these actions and other less well characterized neuroendocrine signalling pathways bring about a suppression of feeding behaviour as well as changes in energy expenditure and body weight. Although it has been shown that plasma leptin levels correlates well with body fat in humans and rodents [6,7], the mechanisms that modulate leptin levels remain unclear. There are correlations between leptin concentrations and insulin concentrations [8,9]. In humans, glucose does not appear to modulate leptin levels [10]. The majority of the current information on leptin, however, is restricted to work on rodents and studies of human obesity [1-13].

We report the development and validation of a radioimmunoassay for the quantitative measurement of human leptin in serum.

2. MATERIALS

RhLeptin, BSA and chloramineT were obtained from Sigma-Aldrich, USA. $Na^{125}I$ was from Institute of Isotopes, Budapest. Iodogen was procured from Pierce, USA. TLC plates and Congo Red were from Merck and Triton X-100, NaN_3, polyethylene glycol, phosphate salts and acetone from REANAL, Hungary, Sephadex G-50 from Pharmacia, Sweden and Freund's adjuvant from DIFCO Laboratories. Horse serum and anti-rabbit IgG raised in sheep, were obtained from University of Veterinary Medicine, Kaposvár, Hungary.

3. METHODS

3.1. Radioiodination of recombinant human leptin antigen with ^{125}I

10 μg recombinant human leptin (rhLeptin) was labeled with 37–56 MBq ^{125}I (by two methods, using chloramineT or iodogen as oxidising agents. 10 μg rh-leptin and 37–56 MBq ^{125}I are taken in a test tube to which 10 μL chloramineT (1 mg/mL) is added and vortexed for 15 sec. The reaction is stopped by the addition of 20 μL sodium metabisulfite (1mg/mL). 50 μL KI (10 mg/mL) and 400 μL phosphate buffer 0.05M, pH: 7.4 are also added. The efficiency of labeling was determined from thin layer (TLC) chromatography developed in acetone.

The purification of tracer was performed by gel filtration on a Sephadex G-50 column, where the eluant was 0.05 M phosphate buffer, pH 7.4 containing 0.1% BSA and 0.1% sodium azide. Fractions of 0.5 mL were collected. The purified tracer was diluted to a concentration of 25000 cpm/100μL and stored at 4°C. The dilution buffer was 0.05 M phosphate buffer pH 7.4 containing 1% BSA, 0.05% Triton X-100, 0.1% NaN_3 and 0.01 mg/ml Congo-Red colouring agent.

3.2. Antiserum production

Two New Zealand white rabbits were immunized intradermally on multiple sites, each with 200 μg rhLeptin mixed with complete Freund's adjuvant. The injections were repeated three times at three weeks intervals with 100 μg, and the rabbits were bled after the boosters. The specificity of antiserum was examined with ten antigens.

3.3. Standard solutions

Standard solutions were prepared with rhLeptin diluted in sterile horse serum, aliquots of 0.5 ml were lyophilized and stored at 4°C. For the assay, quantities of 100 μL of standards, unknown samples, antiserum and tracer were used.

Development of the radioimmunoassay procedure

Kinetic study
The antigen–antibody reaction kinetics was analysed between 3 and 48 h at two temperatures viz. 4°C and room temperature. Effects of shaking and pre-incubation with the antibody were also tested.

Separation
The separation of free and bound fractions was performed using a 1 mL mixture of anti-rabbit IgG and 8% PEG After incubation of 20 minutes at room temperature, the mixture was centrifuged at 2000 g for 20 minutes and the supernatant decanted. The radioactivity in the pellets was counted to determine bound radioactivity.

Sample implementation

39 serum samples of different concentrations were measured with the developed method as well as commercially available kit from Linco (USA). Leptin concentrations in serum samples of 35 lean (BMI 17–26 kg/m^2), 45 overweight (BMI 27–35 kg/m^2) and 44 obese (BMI 36–68 kg/m^2) fasting adult patients (50 men and 74 women, Szent Imre Hospital, Budapest) were measured with our assay. The results were compared by means of unpaired, two-tailed t-tests and the correlation between leptin concentration and BMI for men and women separately was also established. To study the relation between serum leptin concentration and body fat percentage, leptin concentrations of 90 patients were measured.

4. RESULTS AND DISCUSSION

Labeling efficiency and immunological stability were the main criteria for selecting the best labeling method. Both the labeling methods proved to be satisfactory with respect to labeling efficiency, which was 75–90%, as calculated from TLC strips (Figure 1).

Leptin labeled by the iodogen method gave a low maximum binding of only 30–35% in contrast to leptin labeled by the chloramineT method, where the binding was 45–50%. For this reason, we chose chloramineT labeling method for preparing our tracer. Fractions of high radiochemical purity (> 98%) as estimated by TLC were obtained after purification by gel filtration (Figure 2). The specific activity of the tracer was between 2.8 and 3.3 MBq/μg (mean 3 MBq/μg, n = 10). The tracer was stable for at least 5 weeks.

Two polyclonal antibodies were obtained from the immunized rabbits, with titers of 1:6000 and 1:5000, respectively. The first one was selected and its specificity analysed with 10 different antigens, dd not show any significant cross-reaction (Table I)

It was observed that to reach the kinetic equilibrium 18–24 h of incubation at 4°C were required. Shaking the mixture did reduce the reaction time. The sensitivity of the assay can be increased by pre-incubating overnight with the antiserum and incubating with tracer at room temperature.

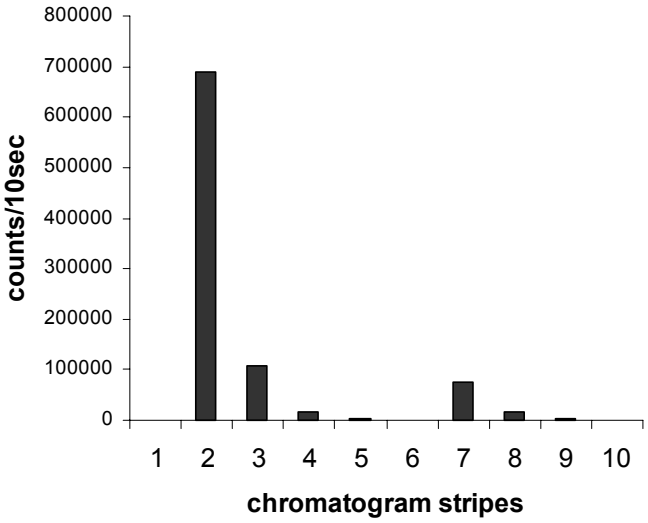

FIG. 1. Thin layer chromatogram of the labeling mixture, determination of labeling efficiency.

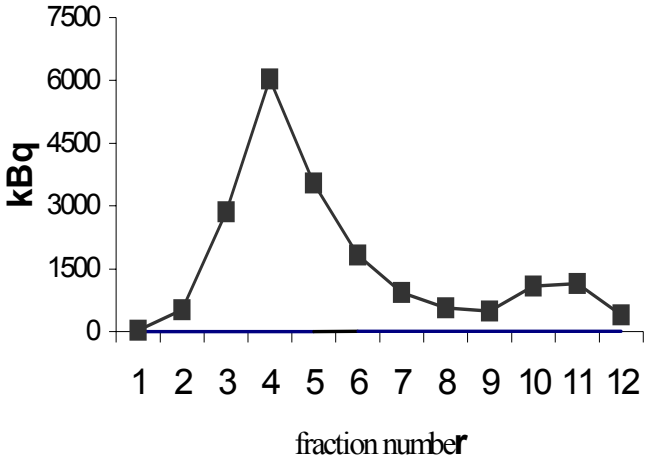

FIG. 2. Purification of ^{125}I-leptin, Elution curve on Sephadex G-50.

TABLE I. SPECIFICITY OF ANTI-LEPTIN POLYCLONAL ANTIBODY

Antigen	Concentration	Cross-reaction
Glucagon (Serono)	800 pg/mL	nd
IGF-1 (Schering-CIS)	1050 ng/mL	nd
Insulin (Institute of Isotopes)	400 mUI/L	0.03%
C-Peptide (Institute of Isotopes)	30 ng/mL	nd
TNF-α (Calbiochem)	200 ng/mL	nd
IL-1β (Calbiochem)	40 µg/mL	nd
HPL (Aalto Bio Reagents)	20 µg/mL	nd
FSH (Pharmacia)	150 mUI/mL	nd
PRL (Calbiochem)	400 ng/mL	nd
HCG (in-house)	**100 µg/mL**	**nd**

nd=not detectable

However, this "improvement" would bring a displacement of the measuring range towards the lower concentrations, due to which samples with a leptin concentration between 50 and 100 ng/mL (as in the case of most overweight and obese patients) cannot not be accurately measured. For this reason, we chose a one-step assay with an overnight incubation at 4°C. Under these conditions, a wide measuring range (0.5–100 ng/mL) makes it possible to determine leptin concentrations in both lean subjects and obese patients with good sensitivity (0.5 ng/mL).

For assessing the imprecision of our assay, we determined the intra-assay and inter-assay coefficient of variation from 10 replicates of three and four serum samples respectively. A dilution test was performed using four serum samples and the percent recovery was calculated. The calculated intra-assay and inter-assay coefficient of variations were <6% and 8%, respectively (Table II). Recoveries obtained from the dilution test were between 88% and 106% (Table III).

Comparison of the concentrations of serum samples of different concentrations with our assay and with Linco RIA showed a good correlation (Figure 3). The linear correlation coefficient between our

in-house developed leptin RIA and LINCO Leptin-RIA was 0.87. Mean serum leptin concentrations of lean overweight and obese patients are shown in Table IV. Serum leptin concentrations were significantly higher in lean women than in lean men ($p < 0.000001$) and in overweight women than in overweight men ($p < 0.0001$) but this difference was not statistically significant in the case of obese men and women ($p = 0.09$).

The linear correlation coefficient between BMI and leptin concentration was 0.71 for men (Figure 4) and 0.52 for women (Figure 5).

TABLE II. INTRA-ASSAY AND INTER-ASSAY IMPRECISION

INTRA-ASSAY				INTER-ASSAY				
Sample:	A	B	C	Sample:	D	E	F	G
	4.30	18.52	45.97		2.50	4.79	10.01	51.43
	4.10	17.95	46.16		2.70	5.14	10.81	45.78
	4.30	18.28	46.98		2.28	4.41	10.01	49.67
	4.16	15.75	39.60		2.53	5.62	10.18	50.30
	4.37	18.70	43.85		2.36	5.1	10.53	47.38
	4.54	16.76	45.37		2.66	4.98	9.36	47.83
	4.12	18.21	47.30		2.59	5.31	10.73	46.36
	4.50	17.39	45.31		2.44	5.05	9.62	52.02
	4.10	17.06	42.01		2.8	4.84	9.36	44.97
	4.37	18.24	44.28		2.9	5.52	10.64	50.04
Mean	4.29	17.69	44.68	Mean	2.58	5.08	10.13	48.58
SD	0.16	0.93	2.37	SD	0.19	0.36	0.55	2.45
CV%	3.79	5.27	5.31	CV%	7.53	7.02	5.43	5.04

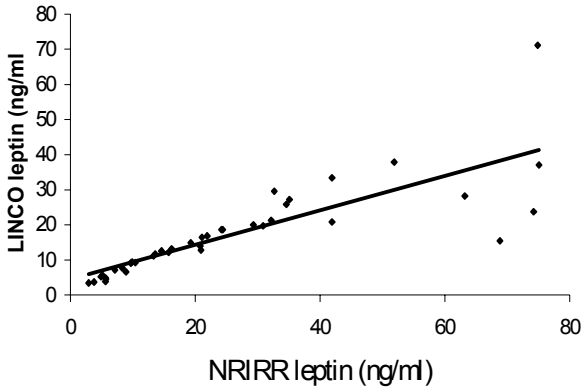

FIG. 3. LINCO Leptin-RIA and NRIRR Leptin-RIA comparison.

TABLE III. DILUTION TEST

Dilution	Measured (ng/mL)	Expected (ng/mL)	Recovery	Measured (ng/mL)	Expected (ng/mL)	Recovery
Sample			H			I
1x	49.23			34.27		
2x	21.9	24.62	89%	15.1	17.14	88%
5x	9.05	9.85	92%	6.27	6.85	91%
10x	5.22	4.92	106%	3.04	3.43	89%
Dilution	Measured (ng/mL)	Expected (ng/mL)	Recovery	Measured (ng/mL)	Expected (ng/mL)	Recovery
Sample			J			K
1x	87.4			76.27		
2x	42.08	43.70	96%	33.88	38.14	89%
5x	16.63	17.48	95%	14.57	15.25	96%
10x	9.12	8.74	104%	8.03	7.63	105%

TABLE IV. LEPTIN CONCENTRATIONS MEASURED IN SERUM SAMPLES OF LEAN (BMI 17-26). OVERWEIGHT (BMI 27-35) AND OBESE (BMI 36-68) PATIENTS

BMI(kg/m^2)	Men(n)	Leptin (ng/mL)Mean ± SD	**Women (n)**	Leptin (ng/mL) Mean ± SD
17 – 26	14	6.63 ± 3.37	21	17.65 ± 6.16
27 – 35	18	20.42 ± 17.46	27	51.39 ± 24.94
36 – 68	18	53.46 ± 39.23	26	80.36 ± 62.73

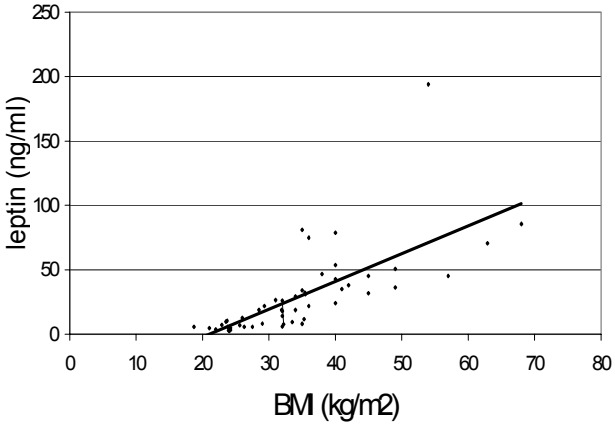

FIG. 4. Correlation between BMI and serum leptin concentrations in men.

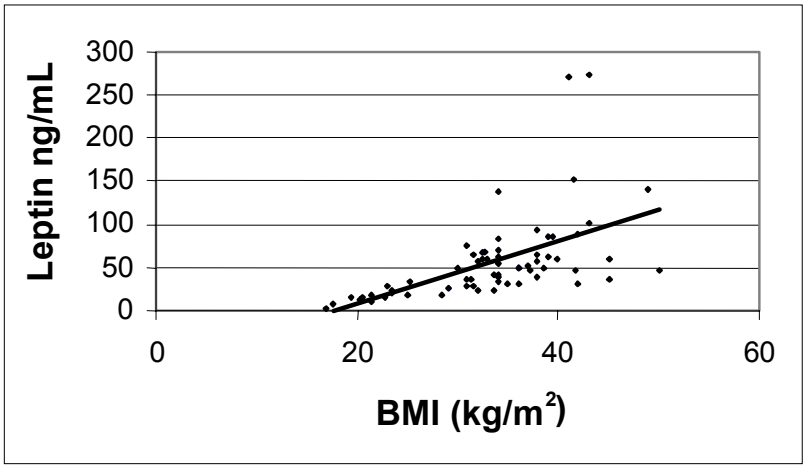

FIG. 5. Correlation between BMI and serum leptin concentrations in women.

Measuring the leptin concentrations of 90 obese subjects with known body fat percentage we found that the quadratic model provided the best fit (Figure 6). These results are the same as described by Considine [11].

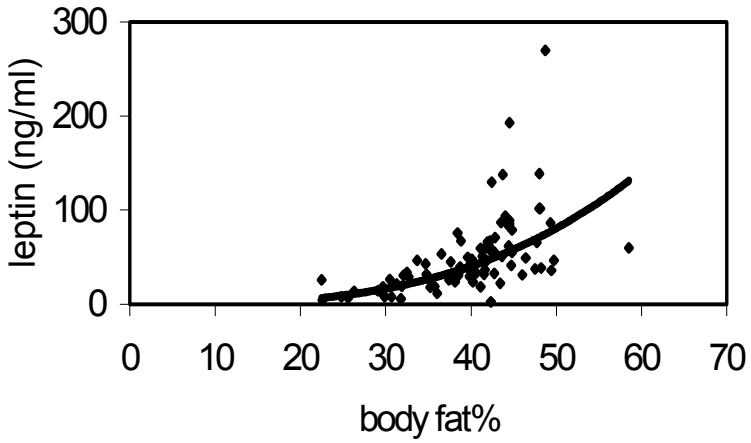

FIG. 6. The relation between the % body fat and the serum leptin level in 90 obese subjects.

5. CONCLUSION

A specific, sensitive and accurate method for the quantitative determination of the human leptin in serum was developed [12]. Preliminary data on lean, overweight and obese human patients shows that the leptin radioimmunoassay could be a useful tool in clinician's hands to monitor the efficacy of treatment of obese patients.

A significant interest is being shown in leptin hormone measurements in serum samples of different mammals by basic researchers, veterinary and medical doctors and there is a core sequence comprised of eight amino acids (GLDFIPGL) that is totally conserved in leptin molecules from all the evaluated species [12]. Thus, future developments are planned to investigate a multi-species radioimmunoassay which is available for leptin hormone measurements in laboratory animals, companion (pet) and livestock animals as well as in humans.

REFERENCES

[1] ZHANG, Y., PROENCA, R., MAFFEI, M., BARONE, M., LEOPOLD, L., FRIEDMAN, J.M., Positional cloning of the mouse obese gene and its human homologue. Nature **372 (**1994) 425-432.

[2] ZHANG, Y., BASINSKI, M.B., BEALS, J.M. et al. Crystal structure of the obese protein leptin E-100. Nature **387 (**1997) 206-209.

[3] KLINE, A.D., BECKER, G.W., CHURGAY, L.M. et al., Leptin is a four-helix bundle: secondary structure by NMR. FEBS Lett. **407 (**1997) 239-242.

[4] TARTAGLIA, L.A., DEMBSKY, M., WENG, X, et al., Identification and expression cloning of a leptin receptor. Cell **83 (**1995) 1263-1271.

[5] FLIER, J.S., MARATOS-FLIER, E., Obesity and the hypothalamus: novel peptides for new pathways. Cell **92 (**1998) 437-440.

[6] WING, R.R., SINHA, M.K., CONSIDINE, R.V., LANG, W., CARO, F., Relationship between weight loss maintenance and changes in serum leptin levels. Horm Metab Res **28** (1996) 698-703.

[7] FRIED, S.K., RICCI, M.R. AND RUSSELL, C.D., Laferrere B. Regulation of leptin production in humans. J Nutr. **130** (2000) 3127S-3131S.

[8] CAPRIO, M., FABBRINI, E., ISIDORI, A.M., AVERSA, A. AND FABBRI, A., Leptin in reproduction. TRENDS Endocrinol Metab. **12** (2001) 65-72.

[9] MATSUMURA, K., TSUCHIHASHI, T., FUJII K. AND ILIDA, M., Neural regulation of blood pressure by leptin and the related peptides. Regul Pept. **114**. (2003) 79-86.

[10] HOSODA, K., MASUZAKI, H., OGAWA, Y., MIYAWAKI, T. et al., Development of Radioimmunoassay for human leptin. Biochem Biophys Res Commun. **221** (1996) 234-239.

[11] CONSIDINE, R.V., MADHAR, K., SINHA, M.K., HEIMAN, M.L. et al., Serum immunoreactive-leptin concentrations in normal-weight and obese humans. New Engl J Med **334** (1996) 292-295.

[12] RICHARDS, M.P., CAPERNA, T.J., ELSASSER, T.H., ASHWELL, C.M., MCMURTRY, J.P:, Design and application of a polyclonal peptide antiserum for the universal detection of leptin protein. J Biochem Biophys Meth **45** (2000) 147-56.

[13] LAGARDE, A.R., NAGY, K., FORGACH, T., JANOKI, G.Y.A., Development of a radioimmunoassay for the measurement of human leptin in serum. Nucl Med Rev Cent East Eur. **6** (2003) 105-109.

PUBLICATIONS ARISING OUT OF THE CRP

NIKOKIRI-KOUVALAKI, H., PETROU, P., KAKABAKOS, S., SIAFAKA, A., AND CHRISTOFIDIS, I., Development of an enzyme immunoassay for the determination of progesterone in serum and cow milk Book of Abstracts, 11th Pan-Hellenic Pharmaceutical Congress, **29** 31/3/2003, Athens, Greece p 140.

ANA R. LAGARDE, KATALIN NAGY, TIBOR FORGÁCH AND GYŐZŐ A. JÁNOKI, Development of a radioimmunoassay for the measurement of human leptin in serum. Nucl. Med Rev. Cent. East Eur., **6** (2003) 105-109.

TARVEEN KARIR, GRACE SAMUEL, KANCHAN KOTHARI, N. SIVAPRASAD AND MEERA VENKATESH, Studies on the influence of the structural modifications in the tracer on the immunoassay system of progesterone. J. Immunoassay (in press).

KRISHNA MOHAN, ARUNA KORDE, GRACE SAMUEL, SACHIN HAZARE AND MEERA VENKATESH, Analysis of food samples for Aflatoxin B1 Contamination using Radioimmunoassay,. Proceedings of the DAE-BRNS Symposium on Nuclear and Radiochemistry NUCAR 2005, 619-620.

BYSZEWSKA-SZPOCINSKA, E., AND MARKIEWICZ, A., The new RIA kit for the determination of progesterone in cow`s milk. International Conference: Isotopic and Nuclear Analytical Techniques for Health and Environment. Vienna, Austria 10-13 June 2003. Abstract IAEA- CN-103/050.98.

ABBREVIATIONS

Ab2	Second antibody
ABTS	2,2’ azino-biz (3ethylbenzylthiazoline-6-sulfonic acid
BγG	Bovine gamma globulin
BSA	Bovine serum albumin
CMO	Carboxymethyl oxime
CPM	Counts per minute
HAC-NaAC	Acetic acid-sodium acetate
Hpth	Hemiphthalate
HRP	Horseradish peroxidase
HS	Hemisuccinate
Mab	Monoclonal antibody
MES	2-(N-morpholino)ethanosulfonic acid
NSB	Non-specific binding
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
RCP	Radiochemical purity
TMB	Tetramethyl benzidine
TME	Tyrosine methyl ester

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