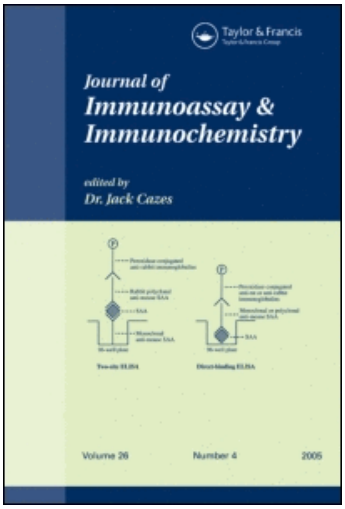

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Studies on the Influence of the Structural Modifications in the Tracer on the Immunoassay of Progesterone

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Abstract: The main objective of the present study was to examine the influence of different bridges in radioiodinated tracers on the assay performance of progesterone using antibodies. Three homologous and two heterologous immunoassay systems for the measurement of progesterone in human serum are described. Using an antiserum raised against progesterone-11α-hemisuccinate–bovine serum albumin (BSA), assays with homologous radioligands, namely progesterone-11α-hemisuccinate-^{125}I-tyrosine methyl ester (TME) and progesterone-11α-hemisuccinate-^{125}I-histamine, heterologous bridge radioligand, namely progesterone-11α-hemiphthalate-^{125}I-TME, and a heterologous site radioligand namely progesterone-3-(O-carboxymethyl) oxime (CMO)-^{125}I-histamine were optimized. A homologous assay system, using antiserum raised against progesterone-3-carboxymethyl oxime-BSA and progesterone-3-CMO-^{125}I-histamine as the radioligand was also optimized to develop a radioimmunoassay (RIA) for serum progesterone. Amongst the two homologous radioligands, viz., progesterone-11α-hemisuccinate-^{125}I-histamine and the corresponding TME conjugate tracer, the former yielded a standard curve with a higher slope (-0.6) as compared to the latter (-0.5). The heterologous bridge system with progesterone-11α-hemiphthalate-^{125}I-TME resulted in a more sensitive assay (slope of -0.8) than the homologous tracers, whilst the heterologous site radioligand, viz., progesterone-3-CMO-^{125}I-histamine gave the most sensitive assay (slope of -1.2). The homologous assay with antiserum against progesterone-3-CMO-BSA and progesterone-3-CMO-^{125}I-histamine tracer gave a standard curve having a slope of -0.97. The two antibodies developed against progesterone, viz., progesterone-11α-hemisuccinate-BSA and progesterone-3-CMO-BSA were characterized for their titre, sensitivity,

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and specificity. Considering the slope, sensitivity, cross-reactivity, and the quality of tracer, the assay system using antiserum against progesterone-11α-hemisuccinate-BSA and progesterone-3-CMO-^{125}I-histamine was found to be suitable for the development of RIA for serum progesterone. The bridges used in an immunogen for production of antibodies, as well as in the preparation of tracer, have a great influence on the assay characteristics.

Keywords: Heterologous tracer, Homologous tracers, ^{125}I-Labeled progesterone

INTRODUCTION

Progesterone (4-pregnen 3,20 dione) is a steroid hormone produced by the adrenal glands, gonads, corpus luteum, and placenta. As with other steroids, progesterone is also synthesized from cholesterol via a series of enzyme mediated biochemical reactions.[1] Specific sites of progesterone synthesis include adrenal cortex, corpus luteum, testes, and placenta. Of these, adrenal and testicular production is almost immediately shunted into other steroidogenic pathways and only a small amount is released into the blood. The main site of progesterone production is the corpus luteum in women during the luteal phase of the menstrual cycle and by the placenta during pregnancy. Thus, progesterone is an important steroid hormone and its estimation in serum is usually measured by radioimmunoassay for obtaining valuable diagnostic information.[2] Steroids, being haptens with no suitable groups for radioiodination, are modified, both to enable the preparation of immunogen as well as iodinated tracer. Although ^{3}H labeling would be the choice of radiolabel, the major advantages gained by replacing ^{3}H with ^{125}I as the emitting nuclide in the radioimmunoassay of small molecules are the higher specific activity, simplification of the assay procedures, and shorter counting times. Usually, the absence of convenient chemical groups for direct radioiodination of the molecule with ^{125}I or for the preparation of immunogen for the production of antisera and the presence of several structurally similar steroid hormones, including their metabolic products in the samples, presents radioimmunoassay for steroids as a challenge for analytical chemists to develop a sensitive and specific RIA.

The attachment of either a γ-emitting radioisotope or an enzyme to progesterone, elicitation of high quality antiserum and the development of assay systems for the estimation of progesterone in serum pose considerable technical problems. Labeling with ^{125}I can be carried out by direct iodination of the amino acid such as histamine or tyramine and then conjugating it to the steroid, or by coupling a reactive group such as tyrosine methyl ester (TME) and then radioiodinating the amino acid conjugate.[3] Antibodies are produced by the administration of steroid–protein conjugates. Attaching either a radioisotope or any non-isotopic label will require the preparation of steroid derivatives having carboxyl or amino groups for conjugation. Most

steroid RIA's involving iodinated steroids employ homologous systems where the same steroid derivative is used both for preparation of the immunogen for elicitation of antibodies, as well as for radioiodination. In such a system, some of the antibodies elicited by the immunogen recognize the bridge as well as the steroid, thus resulting in a radioligand having a higher affinity for the antibody than for the native steroid, resulting in an assay with a lower sensitivity.[4] Thus, the derivative used for the preparation of tracers and immunogen play an important role in influencing the sensitivity, which is reflected in the slope of the standard curve. Since many steroids have closely related structures, the site selected for conjugation of the protein in the preparation of the immunogen is important, as this will influence the specificity of the antiserum. Specificity is a primary requisite of an immunoassay, compared to the sensitivity and, therefore, the site selected for conjugation of the protein in preparation of the immunogen should be selected cautiously. When the same derivative is used for the preparation of tracer, more to avoid the synthesis of another derivative, the sensitivity will be poorer due to the increased affinity of the antiserum for the tracer, as the assay system would demand a higher amount of the analyte to inhibit the binding due to the common bridge binding. To circumvent this problem, either a different site on the steroid could be selected or a different bridge could be derivatized at the same site. The studies carried out by Corrie et al.[5] show that a homologous system could give a sensitive standard curve, provided one uses a poorly antigenic bridge such as a glucoronide linkage. In a heterologous bridge assay system, the immunogen and the derivative used for the preparation of tracer differ in the nature of the bridging groups, whereas, in a heterologous site assay system, the carrier protein in immunogen and the iodinating species are attached at two different sites on the steroid. However, because of the commercial importance, details of such assay systems for progesterone are not found in literature.

This paper describes the preparation, purification, and characterization of four different tracers, viz., progesterone-11α-hemisuccinate-^{125}I-TME, progesterone-11α- hemisuccinate-^{125}I-histamine, progesterone-11α-hemiphathalate-^{125}I-TME, and progesterone-3-(O-carboxymethyl) oxime (CMO) -^{125}I-histamine tracer. Evaluation of these tracers in the assay systems employing antiserum against progesterone-11α-hemisuccinate-BSA and progesterone-3-CMO-BSA, which were prepared and characterized in-house, are also described.

EXPERIMENTAL

Progesterone-11α-hemisuccinate, tyrosine methyl ester hydrochloride, histamine, progesterone reference standard, 11α-hydroxy progesterone, phthalic anhydride, progesterone-3-CMO, progesterone-3-CMO-BSA conjugate, progesterone-11α- hemisuccinate–BSA conjugate, chloramine-T,

isobutyl chloroformate, bovine serum albumin, bovine γ-globulin, and sodium metabisulphite were obtained from Sigma Chemicals, USA. Carrier free ^{125}I as sodium iodide, specific activity 15–17mCi/μg (550–625 MBq/μg), 100 mCi/mL (3700 MBq/mL) was obtained from Izotop, Hungary. Whatman 3 mm 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 GF 254 from Acme Chemicals, Mumbai. 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 used were of AR grade.

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.

Preparation of Radiolabeled Progesterone Derivatives

Progesterone-11α-hemisuccinate–TME Conjugate

Progesterone-11α-hemisuccinate–TME conjugate was prepared according to a method reported by Kothari et al.[6] A solution of progesterone-11α-hemisuccinate (130 mg) in 5 mL of dry dioxane and tri-butyl amine (75 μL) was cooled to 8–10°C. Isobutyl chloroformate (40 μL) was added and the solution was incubated further for 20 minutes at 8°C. TME (69 mg) in 20 mL dioxane: water (1 : 1), was added to the activated progesterone hemisuccinate and the reaction mixture was stirred continuously at 4°C for 4 hours. During this reaction, the pH was maintained at ~8 by the addition of 1 M 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 150 mL of ice-cooled distilled water and the progesterone-11α-hemisuccinate–TME conjugate was precipitated. The precipitate was spun down at 6,000 rpm in the centrifuge and washed sequentially with 30 mL distilled water, 30 mL of 1 M HCl, 40 mL of 1 M $NaHCO_3$ and, finally, with 40 mL of distilled water. The precipitate was dried in a vacuum dessicator. The purity of this compound was checked by TLC using the solvent system benzene : acetone : methanol (5 : 5 : 2). The yield of the conjugate was determined and stored at −20°C.

Progesterone-11α-hemiphthalate-TME Conjugate

Progesterone-11α-hemiphthalate-TME conjugate was synthesized according to the procedure of Allen et al.[7] 11α-Hydroxy progesterone (0.5 g) was refluxed along with phthalic anhydride (1 g) in 10 mL of dry pyridine for

7–8 hours. The reaction mixture was cooled, acidified with 2 N HCl, and extracted in chloroform. The chloroform was removed using a rotary evaporator. TLC in the solvent system, chloroform : methanol : water (90 : 10 : 1) was performed to identify the presence of any of the reactants.

The procedure for the preparation of the TME conjugate of this derivative was the same as mentioned for the TME conjugate of progesterone-11α-hemisuccinate (11α-HS) derivative. TLC of the reaction mixture was performed using chloroform : methanol (80 : 20) as a solvent system to identify the formation of the conjugate. The purity of progesterone-11α-hemiphthalate-TME (11α-hphth–TME) conjugate was checked by TLC using benzene : acetone : methanol (5 : 5 : 2), as well as chloroform : methanol (80 : 20) as solvent systems. The TME conjugates of the progesterone derivatives were characterized by HPLC.

Progesterone-11α-HS-^{125}I-TME and Progesterone-11α-hemiphthalate-^{125}I-TME

Ten μL (2.5 μg) of ethanol solution of respective TME conjugates of progesterone, along with 40 μL of 0.5 M phosphate buffer, pH-7.4 and 10–20 μL (1.5 mCi) (50 MBq) of $Na^{125}I$ was taken in a culture tube. 5 μL (25 μg) chloramine-T, dissolved in 0.05 M-phosphate buffer, pH-7.4, was added and the reaction was stopped after 2–3 minutes by the addition of 5 μL (150 μg) sodium metabisulphite in 0.05M-phosphate buffer, pH-7.4. The reaction mixture was diluted with 0.3 mL of 0.05 M-phosphate buffer, pH-7.4. The reaction mixture was spotted onto paper electrophoresis in 0.025 M phosphate buffer, pH-7.4, to estimate the radioiodination yield and the specific activity. Specific activities of the tracers were also calculated by using a RIA displacement method[9,10] for assuring the quality of the tracers to be used for optimizing the assay system.

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 : distilled water (90 : 10 : 1). The radioactivity in every 1 cm zone of the preparative plate was measured using a NaI(Tl) scintillation counter. Silica gel from the fractions showing high counts was extracted with 2 × 2 mL of ethanol in glass test tubes. The ethanol layer was separated by centrifugation and stored at −20°C. The tracers were evaluated for specific activity, radiochemical purity, immunoreactivity, and stability when stored at 4°C. Immunoreactivity of the tracers was evaluated by reaction with excess of specific antibody.

Progesterone-11α-HS-^{125}I-histamine and Progesterone-3-CMO-^{125}I-histamine

Progesterone-11α-HS-^{125}I-histamine and progesterone-3-CMO-^{125}I-histamine were prepared with slight modification of a reported procedure by Grace et al.[8] wherein the previously radioiodinated histamine is conjugated to

activated progesterone-11α-hemisuccinate and progesterone-3-CMO, respectively. One mg of the acid derivative, dissolved in 50 μL of dry dioxane and 10 μL of 20% tributylamine, was reacted with 10 μL of 10% isobutyl chloroformate for 30 minutes at 4–8°C with constant stirring. The mixed anhydride formed was diluted to 1.4 mL with dioxane.

Radioiodination of histamine was carried out simultaneously. To 10 μL (2.2 μg) of histamine in 50 μL of 0.5 M phosphate buffer, pH 8, and 20 μL (2 mCi) (74 MBq) of ^{125}I, 10 μL (50 μg) of chloramine-T was added. The contents were kept stirring gently for about 2–3 minutes. The reaction was quenched by the addition of 10 μL (300 μg) of sodium metabisulphite. Radioiodination yield of histamine was estimated by paper electrophoresis of the reaction mixture.

Coupling of ^{125}I-histamine with the acid derivatives was carried out by the addition of 50 μL (35.7 μg) of progesterone mixed anhydride of the respective derivatives to the radioiodinated histamine. The pH of the reaction mixture was maintained at 8.0. The reaction was carried out for 2 h at 4°C with constant stirring.

The reaction mixture was acidified with 0.9 mL of 0.1 M HCl and extracted with 1 mL of toluene. The organic phase was discarded and, to the aqueous phase, was added 0.9 mL of 0.1 M sodium hydroxide solution along with 1 mL of sodium metabisulphite (1 mg/mL) in 0.5M-phosphate buffer, pH 7. The iodinated compound was extracted from the aqueous phase with 0.5 mL 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-11α-hemisuccinate-^{125}I-histamine tracer or progesterone-3-CMO-^{125}I-histamine tracer. 200 μL of the toluene extract was spotted at 4.0 cm from the lower end of a TLC preparative plate. The plates were developed in benzene : ethanol : acetic acid (75 : 24 : 1), dried, and 1 cm zones were measured for radioactivity in a NaI(Tl) scintillation counter. Silica gel from the identified radiotracer zones were extracted with 2 × 2 mL portions of ethanol. The iodinated product was diluted to about 185 KBq/mL in 0.05 M phosphate buffer containing 0.1%BSA. These tracers were evaluated for radiochemical purity, immunoreactivity, and stability when stored at 4°C. All the four radiotracers were also characterised by HPLC.

Preparation of In-House Antibodies Against Progesterone

All the animal experiments were carried out in strict compliance with the relevant national laws relating to the conduct of animal experimentation.

Antiserum Against Progesterone-11-α-hemisuccinate-BSA Conjugate

Progesterone-11α-hemisuccinate-BSA conjugate was dissolved in normal saline and was emulsified with Freund's complete adjuvant, in the ratio 1 : 2.5

to give a concentration of 1 mg of conjugate per 0.5 mL. Four New Zealand white rabbits (~1 kg) were injected intradermally with 0.5 mL of emulsion per rabbit. Five booster injections of emulsions, prepared at a concentration of 250 μg per 0.5 mL 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 selected antiserum was used for the development of the liquid phase progesterone assays for the estimation of progesterone in human serum. This antiserum was dispensed into 1 mL aliquots, freeze-dried, and stored at 4°C until further use.

Antiserum Against Progesterone-3-carboxymethyloxime-BSA Conjugate

Two young rabbits (~1 kg) were immunized with progesterone-3-CMO-BSA conjugate in normal saline and emulsified with Freund's complete adjuvant in the ratio of 1 : 2.5. 0.5 mL of the emulsion containing ~800 μg of the progesterone-3-CMO–BSA conjugate was injected intradermally at multiple sites on the shaved back of the two rabbits. Booster injections were given intramuscularly with one-third of the original amount at monthly intervals, until a suitable antiserum was obtained. The rabbits were bled ~10–20 days after every booster and the antiserum was evaluated for titre, specificity, and sensitivity. The % crossreactivity was calculated as the ratio of progesterone concentration to the crossreactant, which gives 50% inhibition in the maximum binding.

Progesterone Standards

The standards were prepared in methanol and calibrated spectrophotometrically. Further dilutions were made in 0.05 M phosphate buffer, pH 7.5, and working standards were prepared in human serum, free of progesterone, which was prepared by treating serum with activated charcoal.

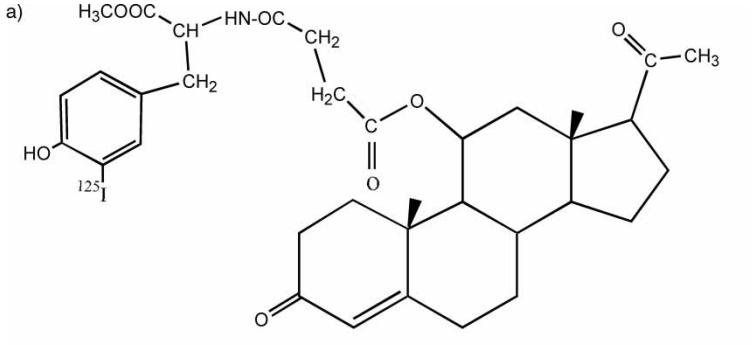
Assay Procedure

In a polystyrene tube, (50 μL) progesterone standard or sample, (100 μL) progesterone antibody, (300 μL) progesterone tracer (185 KBq) were added and incubated for 2 h at room temperature. The separation system used in the present study was goat anti-rabbit IgG aided polyethylene glycol (PEG). Second antibody (100 μL) and (1 mL) 10% PEG solution were added and incubated further for 15 minutes at room temperature and centrifuged at 2,000 × g for 20 minutes. The precipitate was measured for radioactivity in a NaI(Tl) scintillation counter. Five homologous and heterologous standard curves were constructed with the different tracers and two antibodies.

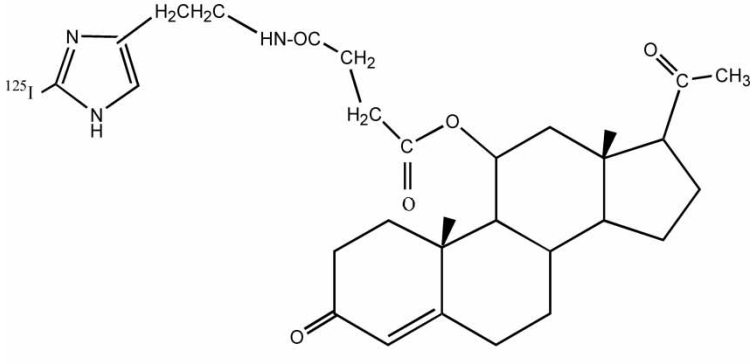
RESULTS AND DISCUSSION

Preparation of Radiolabeled Progesterone Derivatives

The structures of four tracers are shown in Figures 1a and 1b. The progress of the formation of the TME conjugates of the corresponding progesterone derivatives were monitored by TLC of the reaction mixture. The conjugates were obtained by precipitation in aqueous medium, followed by centrifugation at 6,000 rpm. A single component in the TLC of the purified product ascertained the purity. In the solvent system benzene : acetone : methanol (5 : 5 : 2), the R_f of progesterone-11α-hemisuccinate was 0.73 and that of TME was 0.52. The third spot from the reaction mixture, at R_f 0.84, was confirmed later to be progesterone-11α-hemisuccinate–TME conjugate.

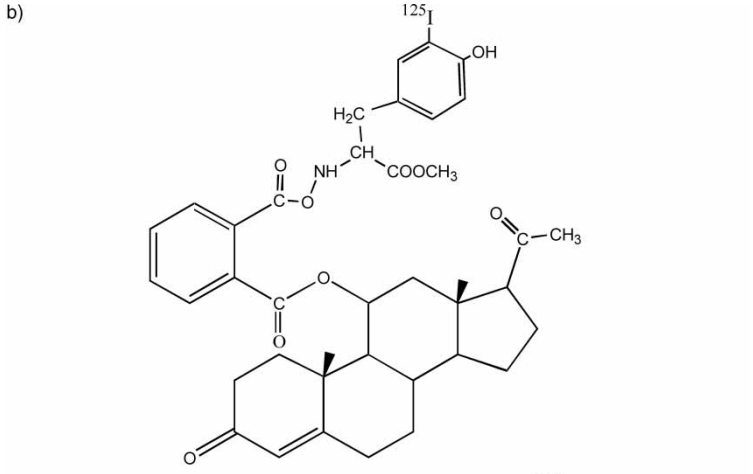


Progesterone-11α-hemisuccinate-^{125}I-TME



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

Figure 1. a) Progesterone-11α-HS-^{125}I-TME and progesterone-11 α-HS-^{125}I-histamine. b) Progesterone-11α-hphth-^{125}I-TME and progesterone-3-CMO-^{125}I-histamine. (*continued*)



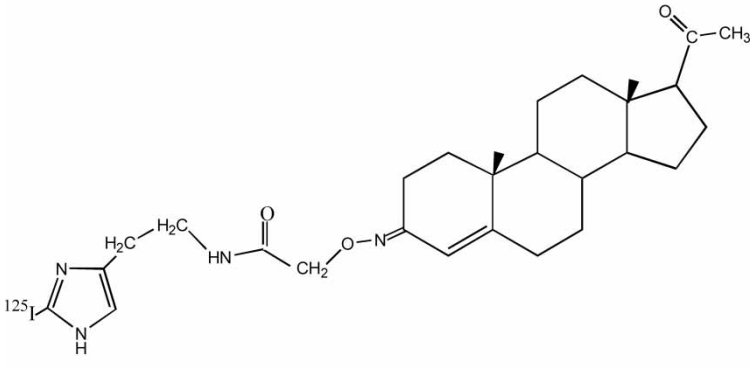


Figure 1. Continued.

14 mg of the product was obtained, corresponding to a 7% yield and was stored at −20°C. The lower yield was probably due to the difficulty in separating the precipitate, which could be improved with a refrigerated centrifuge at a speed of 10,000 rpm.

TLC of the reaction mixture of progesterone-11α-hemiphthalate synthesis in the solvent system chloroform : methanol : water (90 : 10 : 1) 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 which was for progesterone-11α-hemiphthalate derivative. The reaction mixture was extracted with chloroform and then the chloroform was removed *in vacuo* to yield 640 mg of the product, corresponding to a 43% yield.

TLC of the reaction mixture of TME conjugation of the hemiphthalate derivative in the solvent system chloroform : methanol (80 : 20) showed three spots, one at R_f of 0.58 which was due to unreacted progesterone-11α-hemiphthalate, a second at R_f of 0.3 due to unreacted TME, and the third spot at 0.7, which was due to the progesterone-11α-hemiphthalate-TME conjugate. TLC of the purified phthalate-TME conjugate using solvent system benzene : acetone : methanol (5 : 5 : 2) showed the R_f of progesterone-11α-hemiphthalate-TME conjugate as 0.84, whereas the R_f of progesterone-11α-hemiphthalate was 0.77 and that of TME is 0.5. TLC of the purified conjugate after precipitation from water, using the solvent system chloroform : methanol (80 : 20) showed a single spot at R_f 0.72. Both the solvent systems could be used for characterization of the conjugate. The yield of the conjugate obtained was 5% ($\sim$10 mg). The change in functional group, viz., hemiphthalate or hemisuccinate did not have any marked difference in Rf value in TLC when solvent system benzene : acetone : methanol (5 : 5 : 2) was used. The labeling studies with histamine generally resulted in overall low yields ($\sim$25%) with respect to the iodine activity used. The conjugate was stored at -20°C until further use, for radioiodination. The HPLC pattern for purified progesterone-11α-hemisuccinate–TME conjugate and progesterone-11α-hemiphthalate-TME conjugate, along with TME, is shown in Figure 2. The single peak in HPLC pattern further confirmed the purity of the TME conjugates.

Paper electrophoresis of the reaction mixture of radioiodinated progesterone-11α-hemisuccinate-TME showed a radiolabeling yield of 90% with the remaining activity as free iodide (Figure 3a). Specific activity of the tracer, when calculated from the iodination yield, was found to be $\sim$285 μCi/μg

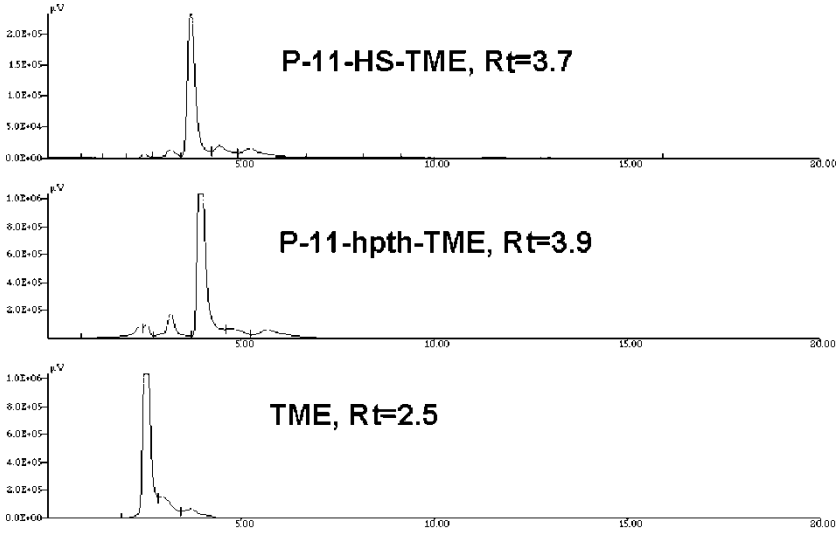


Figure 2. HPLC of progesterone-TME conjugates.

(10 MBq/μg). The radioiodinated progesterone tracer was extracted into chloroform. The chloroform layer was subjected to further purification on a 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

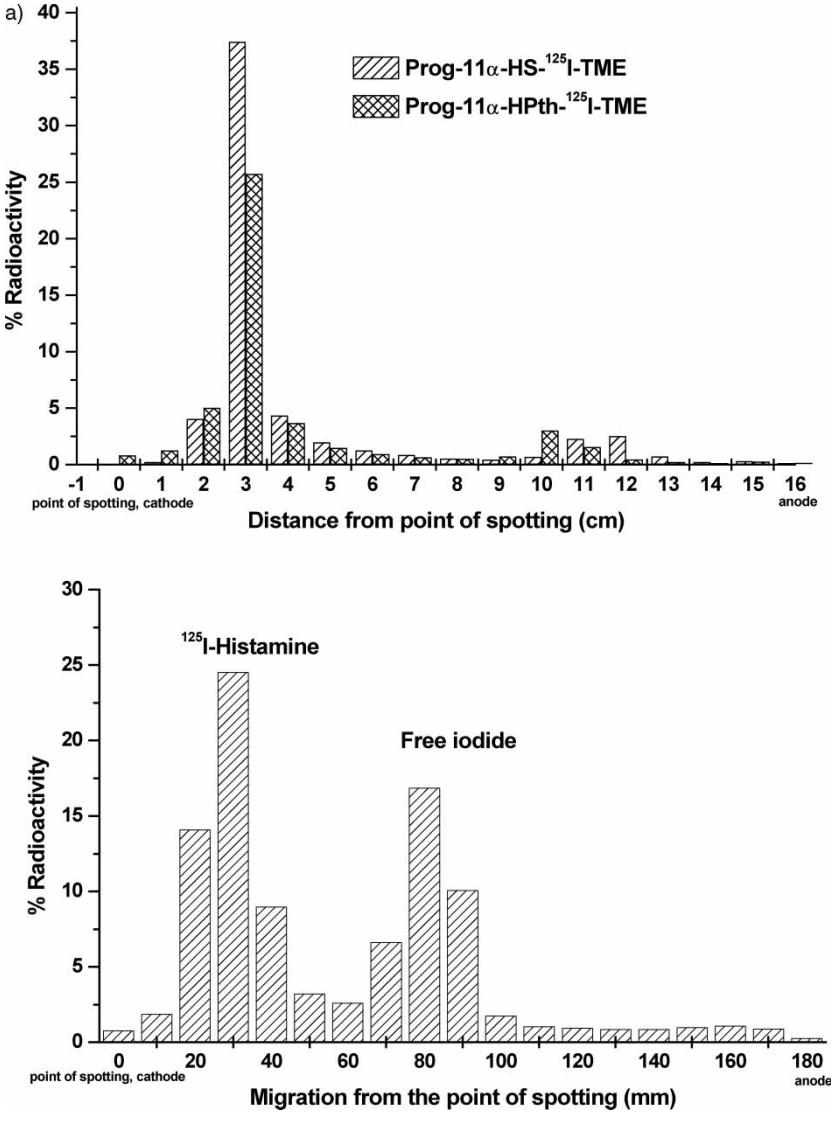


Figure 3. a) Paper electrophoresis of radioiodinated progesterone–TME conjugates and histamine. b) HPLC of radioiodinated progesterone tracers.

(*continued*)

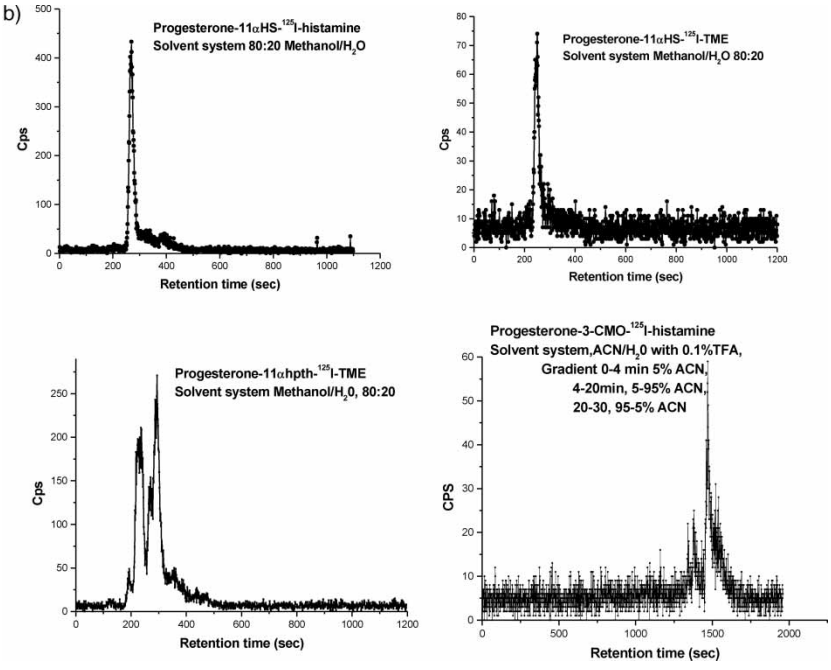


Figure 3. Continued.

0.7 showed an immunoreactivity of ∼75–80% with excess antibody and a non-specific binding of ∼6–7%. The R_f of cold progesterone-11α- hemisuccinate-TME conjugate was 0.53 in the same solvent, thus separating the cold and the radioactive conjugate efficiently. Specific activity of the tracer calculated by the displacement analysis method was found to be ∼741 μCi/μg (30 MBq/μg). The binding remained constant over a period of two months with no significant increase in the non-specific binding, indicating that this tracer was stable for at least two months.

The radiolabeling yield of progesterone-11α-hemiphthalate-^{125}I-TME was 75–78%, as estimated from paper electrophoresis (Figure 3a). Preparative TLC of the purified radiotracer using the solvent system 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 (17 MBq/μg) whereas, when calculated by the RIA displacement method, it was found to be ∼300 μCi/μg (11 MBq/μg). The difference in the specific activity may be due to the dissimilarity in the immunoreactivity of the iodinated conjugate and cold progesterone. Immunoreactivity of the extracted fraction with an excess of progesterone antibody showed a specific binding of ∼65–70% and a non-specific binding of ∼8–9%. The tracer was found to be stable for only six weeks when stored at 4°C.

Radioiodination yield of histamine, as estimated by paper electrophoresis, was about 45–65% (Figure 3a). The iodinated histamine was coupled to the preactivated progesterone derivative. The extraction of the reaction mixture in toluene, at an acidic pH, removes unconjugated progesterone derivative into the organic phase, leaving the free iodide, the radioiodinated histamine not coupled to progesterone derivative, and the radioiodinated progesterone conjugate in the aqueous phase. The extraction at neutral pH extracts, predominantly, the iodinated progesterone-histamine conjugate. The yield of the labeled compound extracted in toluene was 15–25%. TLC of the toluene extract further purifies the iodinated conjugate from the traces of impurities. In TLC, using a solvent system such as benzene : ethanol : acetic acid (75 : 24 : 1), the radioiodinated progesterone-11α-hemisuccinate-^{125}I-histamine and progesterone-11α-hemisuccinate gave R_f values of 0.56 and 0.4, respectively. 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 the purified radiotracer was 98%. The specific activity of the tracer by displacement analysis was found to be only $\sim$460 μCi/μg (17 MBq/μg), probably due to the close R_f values of the progesterone-hemisuccinate and the radiolabeled compound. However, the immunoreactivity results show a binding of $\sim$85% with excess of progesterone antibody where the non-specific binding was 6–7%. The labeling studies with histamine generally 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 for the assay.

In TLC, using a solvent system such as benzene : ethanol : acetic acid (75 : 24 : 1), the radioiodinated progesterone-3-CMO-^{125}I-histamine and progesterone-3-CMO showed R_f of 0.54 and 0.33, respectively. This separation is 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 the RIA displacement method was found to be 1,000 μCi/μg (37 MBq/μg). The radiochemical purity of the purified radiotracer was 98%. The immunoreactivity results show a binding of $>$80% with excess of progesterone antibody, whereas, the non-specific binding was $<$10%. The HPLC pattern of the four progesterone iodinated tracers are given in Figure 3b. Among the four radiotracers, HPLC of progesterone-11α-hemiphthalate-^{125}I-TME tracer showed two peaks, which rendered this tracer unacceptable for further use.

Preparation of Progesterone In-House Antibodies

The titre curves obtained from three rabbits (R4, R8, and R10) immunized with progesterone-11α-HS-BSA after the fourth booster are shown in Figure 4. It is seen that the titre curves of R4 and R8 exhibited a steep fall, indicating the possibility of developing sensitive assays. The antiserum

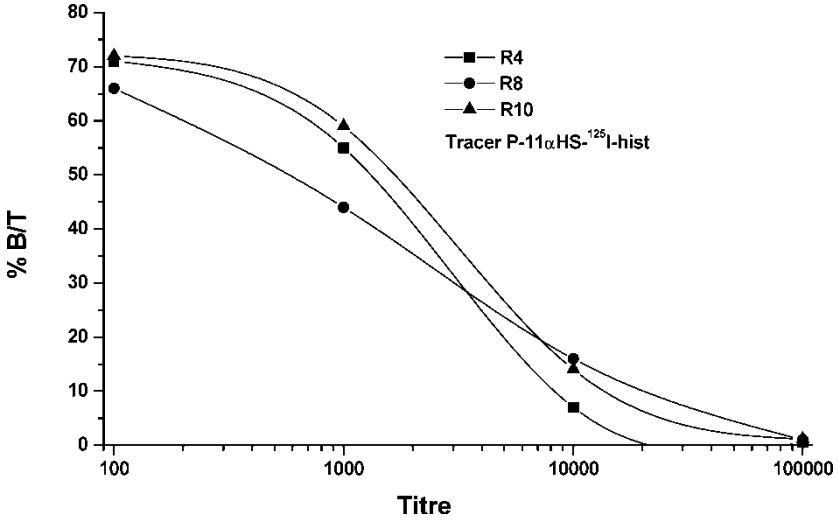


Figure 4. Titre curve for progesterone-11αHS–BSA antiserum from three rabbits.

from the rabbit R4 was used in further experiments for assay development, as these resulted in better dose-response curves compared to R8. The titres after five boosters from the R4 rabbit are shown in Figure 5. It can be seen that the first three booster crops have low titres, while the fourth and fifth booster crops have high titres. However, between the fourth and fifth booster crops, the

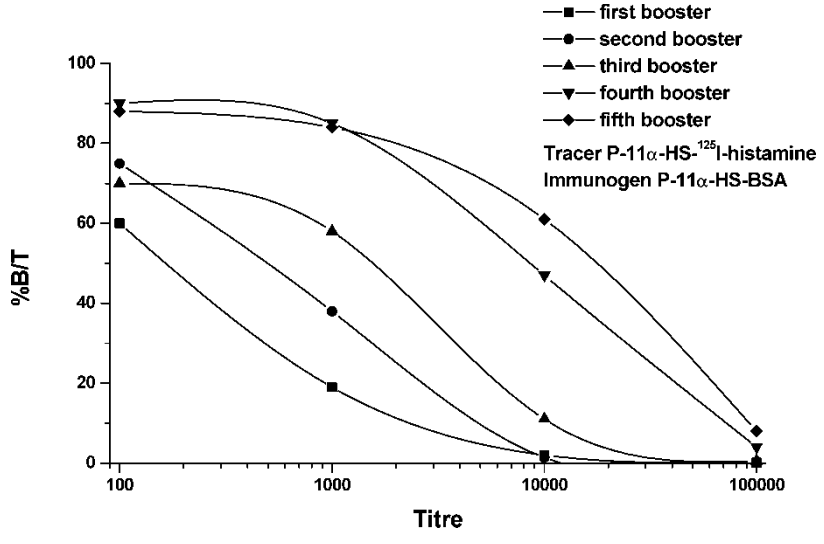


Figure 5. Titre curve for R4 antiserum with progesterone-11αHS-^{125}I-Histamine.

former has shown a sharper fall, indicating a marginally more sensitive response. Hence, the fourth booster crop was further used for development of the assay. The titre obtained with the four tracers with antiserum from R4, fourth booster, is shown in Figure 6. The optimum dilution of the antibody obtained is 1 : 12,000 with progesterone-11α-hemisuccinate-^{125}I-histamine tracer, 1 : 7,000 with progesterone-11α-hemisuccinate-^{125}I-TME tracer, 1 : 1,000 with progesterone-11α-hemiphthalate-^{125}I-TME tracer and 1 : 800 with progesterone-3-CMO-^{125}I-histamine. The higher titre in the case of the homologous tracers was due to the antibodies produced against the common bridge at position C-11, whereas, the titres were much lower with the other two tracers. Rabbits (R14 and R18) immunized with progesterone-3-CMO-BSA gave a titre of 1 : 200 with progesterone-3-CMO-^{125}I-histamine after the third booster (Figure 7). No binding was observed with the tracers, progesterone-11α-hemisuccinate-^{125}I-histamine and progesterone-11α-hemiphthalate-^{125}I-TME. This could be due to the different sites of conjugation in the tracer (11-position) and the immunogen (3-position).

The affinity constant, 'K', which was calculated by plotting B/F vs. total bound fraction on a Scatchard plot for progesterone-11α-HS–BSA antibody for different assay systems is given in Table 1. These, along with other assay parameters, namely, dose at 90%B/B_0 and slope of the standard curves calculated from the logit-log graph for different assay systems, are also given in Table 1. It is observed that both of the tracers, P-HS-^{125}I-histamine and P-HS-^{125}I-TME, which are homologous with respect to both site and bridge, have shown poor slope of the standard curves, thus indicating

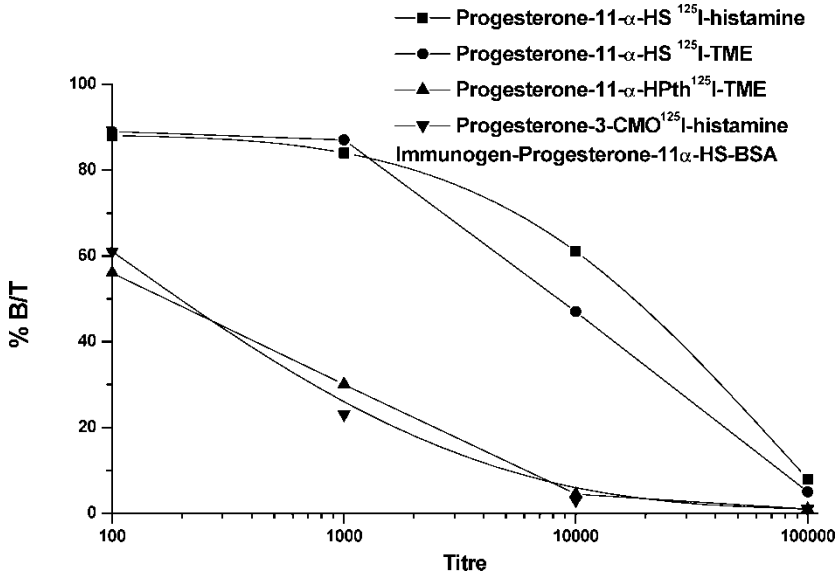


Figure 6. Titre curves for R4 antiserum with four progesterone tracers.

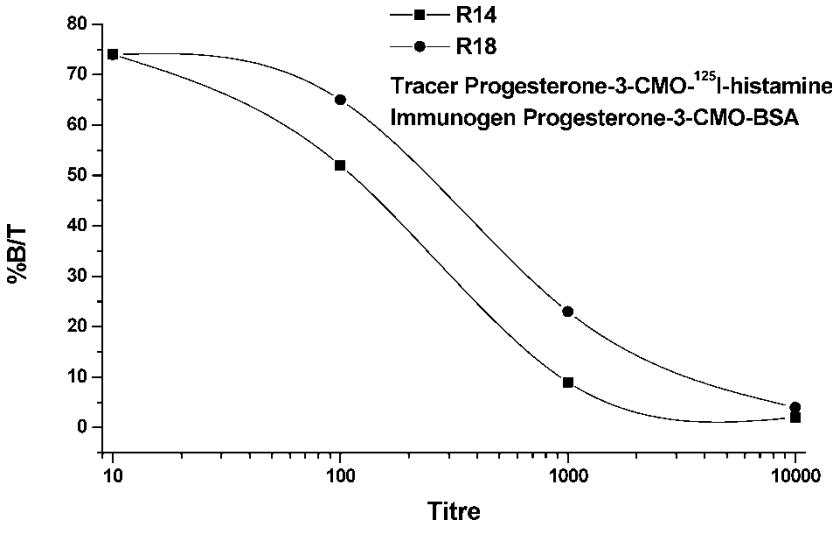


Figure 7. Titre curve for progesterone-3-CMO– BSA antiserum.

poor sensitivity. But the tracer with heterologous bridge (hphth-^{125}I-TME) and heterologous site (3-CMO-^{125}I-histamine) has shown a marked increase in the slopes of the standard curves and, hence, an improved sensitivity. Sensitivity generally depends upon the affinity of the antibody in an antigen-antibody reaction. However, the marked difference in sensitivity between heterologous versus homologous is governed by the ability to replace the tracer from binding to the antibody by cold progesterone, which is far easier in the heterologous system. The affinity constant, along with other parameters given above for progesterone-3-CMO-BSA antibody and progesterone-3-CMO-^{125}I-histamine radiotracer, is also given in Table 1.

Cross reactivity of progesterone analogues when an assay system using progesterone-11α-hemisuccinate-BSA antibody and the four radiotracers (both, homologous and heterologous assay systems) and using progesterone-3-CMO-BSA antibody and progesterone-3-CMO-^{125}I-histamine tracer (homologous assay) are tabulated in Table 2a and 2b. No significant crossreactivity was observed with antiserum against progesterone-11α-hemisuccinate-BSA. Antiserum raised against progesterone-3-CMO-BSA showed a high crossreactivity of 150% with pregnane-5β, 3,20 dione, rendering the antiserum unsuitable for the assay, although the standard curve showed a slope of -0.97.

The titre of the second antibody, i.e., goat ant rabbit antibody used, was higher with a higher titre of primary antibody and lower with a lower titre of primary antibody. Danazol was included in the assay to avoid the binding of progesterone to binding proteins and sex hormone binding globulin.[11]

Table 1. Characteristics of optimum progesterone assays

Progesterone tracers	Immunogen	System	Affinity constant (K) L/M	Slopes from logit-log plot
Progesterone 3-CMO-^{125}I-Histamine	Progesterone-11α-hemisuccinate-BSA	Heterologous site and heterologous bridge	2.3×10^9	-1.2
Progesterone-11α-hemiphthalate^{125}I-TME	Progesterone-11α-hemisuccinate-BSA	Heterologous bridge and homologous site	1.38×10^9	-0.8
Progesterone-11 α-hemisuccinate- ^{25}I-Hist	Progesterone-11α-hemisuccinate-BSA	Homologous bridge and homologous site	0.8×10^9	-0.6
Progesterone -11 α- hemisuccinate^{125}I-TME	Progesterone-11α-hemisuccinate-BSA	Homologous bridge and homologous site	0.63×10^9	-0.5
Progesterone 3-CMO-^{125}I-Histamine	Progesterone-3-CMO-BSA	Homologous bridge and homologous site	1.55×10^9	-0.97

Table 2a. Cross reactivity of progesterone11α hemisuccinate–BSA antiserum

	Progesterone 11α hemisuccinate–BSA antibody	
Cross reactants	Progesterone 11α hemisuccinate-^{125}I -Histamine	Progesterone 3 CMO-^{125}I-histamine
Progesterone	100%	100%
17α-hydroxy progesterone	2%	1.2%
Cortisol	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%

The standard curves for different homologous and heterologous assays are shown in Figure 8, and Figure 9 shows another homologous assay for progesterone. It was observed that the slopes of the standard curves in the case of the heterologous system was higher than the homologous system. However, the assay range was higher for the homologous system. The assay system with antiserum against progesterone-11α-HS-BSA and a progesterone-3-CMO-^{125}I-histamine (heterologous site and bridge) as tracer, was found to be the most suitable assay system, considering the slope, sensitivity, crossreactivity, and the assay range.

CONCLUSION

The main objective was to study the influence of the bridge as well as the site at which the hapten is modified for preparation of immunogen or a tracer on

Table 2b. Cross reactivity of progesterone 3 CMO–BSA antiserum

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

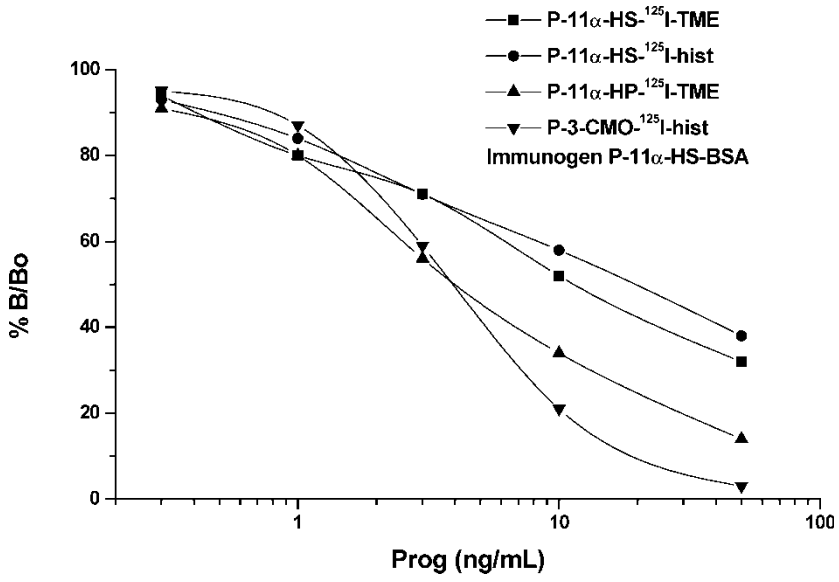


Figure 8. Standard curves with homologous (C-11 position) and heterologous tracers.

the assay performance. In this study, radioimmunoassays for progesterone in human serum have been developed, employing four different combinations of the vital reagents, namely the tracer and the antiserum, and their influence on assay parameters such as sensitivity and slope are evaluated. As expected, the

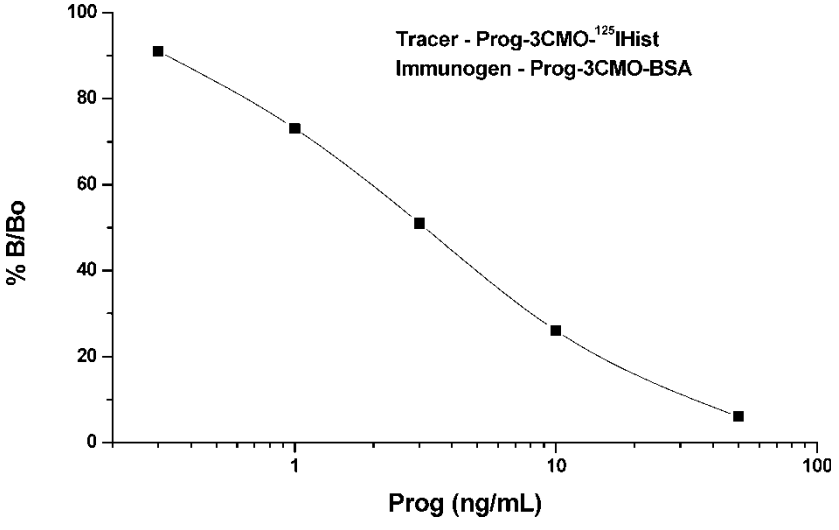


Figure 9. Standard curve for homologous (C-3 position) system.

homologous assay system using a common hemisuccinate bridge for both tracer (progesterone-11α-HS-^{125}I-histamine and progesterone-11α-HS-^{125}I-TME) and immunogen (progesterone-11α-HS-BSA) resulted in standard curves with poorer slopes (-0.5, -0.6) than with a heterologous bridge assay system with the hemisuccinate linkage in immunogen (progesterone-11α-HS-BSA) and the phthalate linkage in the tracer (progesterone-11α-hphth-^{125}I-TME, slope -0.8). Although a good slope of -0.8, the tracer with phthalate linkage could not be taken as the tracer of choice, as the HPLC pattern showed contamination in the quality of the tracer. It was also observed that a heterologous site system with a hemisuccinate linkage in the immunogen (progesterone-11α-HS-BSA) and a carboxymethyl oxime linkage in the tracer (progesterone-3-CMO-^{125}I-histamine) resulted in standard curves with better slopes (-1.2); this combination was selected for the optimization of the assay system. On the other hand, the homologous assay system using the 3-CMO linkages in both, immunogen as well as the tracer, resulted in a standard curve with a comparatively higher slope (-0.97). However, the antiserum showed a high crossreactivity with pregnane-5β- 3,20-dione and, hence, this homologous assay could not be used for estimation. These studies indicate that the selection of a conjugation site and a bridge for introduction of either a radioisotope or a non-isotopic label such as enzyme, as well as for preparation of immunogen, is crucial for the optimization of the assay system for progesterone. Moreover, the actual performance of the assay cannot be predicted and will have to be ascertained experimentally.

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