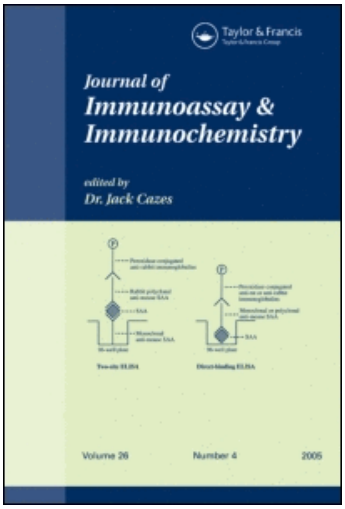

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Development of ELISA for Measurement of Progesterone Employing 17-α-OH-P-HRP as Enzyme Label

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Abstract: The present study was aimed to develop a highly specific and sensitive Enzyme Linked Immunosorbent Assay (ELISA) to measure progesterone in human serum using a heterologous combination of immunogen and enzyme conjugate. The antiserum was raised against Progesterone-3-O-carboxymethyl-oxime bovine serum albumin (P-3-O-CMO-BSA) in New Zealand white rabbits. The enzyme conjugate was prepared by labeling 17-α-hydroxy-progesterone-3-O-carboxymethyloxime (17-α-OH-P-3-O-CMO) with Horseradish Peroxidase (HRP) to form 17-α-OH-P-3-CMO-HRP. A Checkerboard assay was performed to determine the working dilutions of antiserum and enzyme conjugate. Dose-response studies were carried out by incubating 100 μL enzyme conjugate along with 50 μL of standards in the primary antibody coated wells for 1 hour.

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The bound enzyme activity was measured colorimetrically using tetramethyl benzidine/hydrogen peroxide (TMB/H_2O_2) as substrate. The enzyme substrate reaction was terminated with 100 μL of 0.5 M H_2SO_4 after 20 min and the intensity of the color was measured using Tecan ELISA reader at 450 nm. The assay was validated in terms of sensitivity, specificity, precision and recovery. The lowest detection limit of the assay was 0.2 ng/mL. Cross-reaction with analogous steroids pregnenolone and 17-α-OH-P were found to be 6.8 and 6.1%, respectively. For other analogous steroids, it was less than 0.1%. The intra- and inter-assay coefficient of variation ranges from 4.52–7.39% and 4.65–9.55%, respectively. The developed ELISA correlated well with established RIA, with a correlation coefficient of 0.91 (n = 40).

Keywords: 17-α-OH-Progesterone, ELISA, Heterologous system

INTRODUCTION

Progesterone is the principal secretory product of the corpus luteum and is responsible for progestational effects, i.e., cell differentiation and induction of secretory activity in the endometrium in the estrogen-primed uterus. It is also required for implantation of the fertilized ovum and maintenance of pregnancy, as blastosist is dependent on progesterone produced by corpus luteum.[1] Insufficient levels of progesterone, or if it is produced for too little time, does not allow the egg to survive inside the hostile uterine lining. Thus, it is needed to facilitate implantation and prevent the rejection of developing embryo. In case of pregnancy, 6–8 weeks after implantation, the placenta becomes the major source of progesterone, and helps to protect the fetus from rejection by the maternal immune system.[2–5] Therefore, estimating the plasma or serum progesterone concentration is one of the most useful parameter to assess the ovulatory and corpus luteum function.

Many immunoassay techniques had been developed for determination of progesterone in serum. A competitive protein-binding assay for the estimation of progesterone in plasma, using corticosteroid binding globulin (CBG) as the binding reagent and ^{125}I labeled progesterone as tracer, was first developed by Murphy.[6] The first RIA of progesterone was reported by Abraham et al.,[7] which comprised [1,2-^3H]-17-hydroxy-progesterone as tracer and the antiserum was raised against 11-deoxy-cortisol-21-hemisuccinate. The use of more specific antiserum obviated the problem of non-specificity arising from corticosteroid binding globulin (CBG), to some extent. However, the problems associated with RIAs, such as their inherent radiation hazards, disposal problem, need for licensing, and cumbersome sample extraction steps, made them less popular a choice for routine analysis.

Dray et al.[8] first developed the non-radioactive immunoassay of progesterone using β-galactosidase as enzyme label. Since then, a number of non-radioactive methods like chemiluminescence immunoassay,[9] time-resolved fluorescence immunoassay,[10] fluorescence polarization immunoassay,[11] along with enzyme linked immunosorbent assay, have been developed in parallel with radioimmunoassay for measuring progesterone in serum to improve the assay performance.

Although chemiluminescence and time-resolved fluorescence immunoassay are comparable with radioimmunoassay in terms of assay performance, they need specialized instrumentation to get the final result, thus limiting the use of these techniques in ordinary routine clinical setups. On the contrary fluorescence polarization immunoassays of progesterone are not good enough to get sensitive assays.[11,12] Enzyme immunoassays (EIA) are still among the popular methods for the quantitative estimation of hormones. In the above-mentioned assays, attempts have been made to find better combinations of immunogen and enzyme conjugate to develop a progesterone EIA with good assay performance in terms of sensitivity and specificity, simultaneously, which is still lacking.

In the present study, an antigen heterologous ELISA has been developed for the measurement of progesterone in serum by using different antigens for immunogen and label preparation. The lack of any easy, reliable, cost effective, and specific EIA system for progesterone analysis has prompted us to carry out this work. This paper describes development of a rapid and sensitive heterologous ELISA for progesterone, which is highly specific and cost-effective too.

EXPERIMENTAL

Chemicals and Reagents

All solvents, chemicals, and salts used in the present study were of analytical grade and were used without prior purification. All steroids used for the synthesis and cross-reactivity were obtained from Sterloids, Inc., Newport, USA. Bovine serum albumin, N-hydroxy succinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC), complete Freund's adjuvant, gelatin, and thimerosal were purchased from Sigma Chemical Company, St. Louis, MO, USA. Horseradish peroxidase and tetramethyl- benzidine/H_2O_2 solution were purchased from Bangalore Genei, Bangalore, India, and Arista Biochemical, USA, respectively. Microtitre plates were procured from Greiner, Germany.

Buffers

A. The most frequently used buffer was 10 mM phosphate (10 mM PB) pH 7.0, ($Na_2HPO_42H_2O$: 0.895 gm/L and $NaH_2PO_42H_2O$: 0.39 gm/L) containing 0.9 % NaCl (10 mM PBS) and 0.1% NaN_3.
B. HRP conjugate dilution buffer was 10 mM acetate buffer (10 mM AB) pH 5.6, (CH_3COONa: 0.84 gm/L and 1 N CH_3COOH 1.5 mL/L), containing 0.1% thimerosal and dextran T-70, 0.3% BSA.
C. Microtitre well blocking and stabilizing buffer was 10 mM PB containing 0.9 % NaCl, 0.2% BSA, 0.1% gelatin, thimerosal, dextran T-70, ethylene diamine tetra acetic acid: di-potassium salt (EDTA: K salt), and 0.01% gentamicin sulfate.

Methodology

Preparation of Progesterone-3-O-CMO-BSA

P-3-CMO was coupled to BSA by an active ester method with some modification.[13] To 5 mg of P-3-CMO, 200 μL of each, dioxan and dimethyl formamide was added. To this solution 100 μL of water containing 10 mg NHS and 20 mg EDAC was added. The reaction mixture was activated for 24 hour at 4°C. Activated P-3-CMO solution was added to the aqueous solution of BSA (1 mg/0.3 mL), vortex mixed and kept for 24 hour at 4°C. The P-3-O-CMO-BSA conjugate was dialyzed against 3–4 changes of water. The dialysate was frozen, lyophilized and kept at 4°C in aliquots of (1 mg) for immunization.

Preparation of 17-α-OH-Progesterone-HRP Conjugate

17-α-OH-Progesterone was directly conjugated to HRP by an activated ester method with some modification.[14] In brief, 5 mg of 17-α-OH-P was dissolved in 200 μL of dimethyl formamide and 200 μL of dioxan. To this solution 100 μL of water containing 10 mg NHS and 20 mg EDAC were added; the reaction mixture was activated for 24 hour at 4°C. Activated 17-α-OH-P solution was added to the aqueous solution of HRP (1 mg/mL) and kept for 24 hour incubation at 4°C. Thereafter, reaction mixture was passed through a G-25 column, previously equilibrated with 10 mM PBS containing 0.1% thimerosal. The brown colored fractions containing enzyme activity were pooled and, to it, 1% of sucrose, ammonium sulfate, BSA and an equal volume of ethylene glycol were added. The solution was kept at −30°C in aliquots for future use.

Coating of Antibody to Microtitre Plates

The 96-well microtitre plate was coated using the immunobridge technique for primary antibody immobilization according to immunobridge techniques of Shrivastav et al.[14] In brief, 250 μL of normal rabbit serum (NRS) diluted (1:250) in water was dispensed into each well and incubated at 37°C overnight. Following incubation, the plate was washed under running tap water 15-20 times. To the NRS coated wells, 250 μL of 1:1,000 diluted goat anti rabbit gamma globulin (ARGG) was added and incubated for 2 hours at 37°C. Thereafter, the plate was decanted and washed under running tap water. To the ARGG coated microtitre plates, 100 μL of appropriately diluted (1:30,000) P-3-CMO -BSA antiserum in buffer "A" was dispensed. The plate was kept at 37°C for 2 hours. Unbound antibody was then washed off and 250 μL of buffer "C" was then added to block the unoccupied sites of the plate.

Immunization

Intramuscular injections were given to New Zealand white rabbits according to the Shrivastav et al. method.[15] In brief, P-3CMO-BSA (1 mg) was dissolved in saline (0.5 mL) and emulsified with Freund's complete adjuvant (0.5 mL). The emulsion (250 μL) was injected intramuscularly in the limbs of rabbits. The 5 weekly primary injections were followed by the monthly booster doses. The booster doses were given in Freund's incomplete adjuvant and the rabbits were bled 10 days after the booster injection of each month. Antiserum was collected after centrifugation at 2,500 rpm for 10 min and stored at −30°C.

Preparation of Progesterone Standard

Six progesterone-working standards (0 ng/mL, 2.5 ng/mL, 5 ng/mL, 10 ng/mL, 20 ng/mL, and 50 ng/mL) were prepared in Buffer "B".

Assay Procedure

To the P-3-O-CMO-BSA antiserum (1:30,000) coated microtitre wells, 50 μL of standards (0-50 ng/mL) were added in duplicate followed by the addition of 100 μL of 17-α-OH-P-HRP enzyme conjugate (1:4,000). The wells were then incubated for 1 hour at RT. The bound and unbound enzyme conjugate was separated by washing the wells 15–20 times under running tap water by filling, decanting, and flicking. 100 μL of TMB/H_2O_2 substrate was added to the wells and incubated for 20 min at RT. The reaction was terminated with 100 μL of 0.5 M H_2SO_4. The

yellow color developed was measured by a Tecan Spectra automatic micro-well reader at 450 nm wavelength.

Radio Immunoassay (RIA) of the Samples

RIA of the samples was performed by the Immunotech RIA kit (Imunotech, France) utilizing progesterone antibody coated tube and I^{125} labeled progesterone, according to the manufacturer's guideline.

RESULTS

Sensitivity

The lower detection limit of the assay, i.e., the concentration equivalent to A_0-2SD, was 0.2 ng/mL, after 20-fold determination of A_0 binding. A graphical representation of a calibration curve for progesterone is illustrated in Fig. 1.

Specificity

The specificity of the P-3-CMO-BSA antibody was estimated as the percentage of cross-reaction with commercially available steroids with

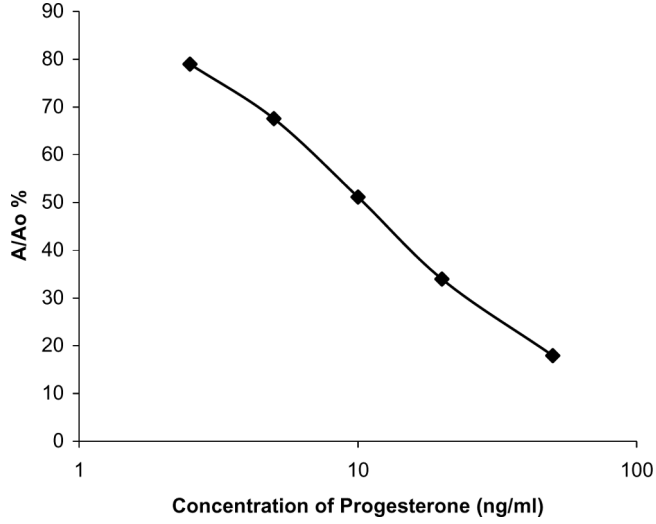

Figure 1. Calibration curve for Progesterone using P-3-CMO-BSA antibody and 17α-OH-P-3-CMO-HRP enzyme conjugate.

analogous structure. P-3-CMO-BSA antibody showed 6.8% cross-reaction with pregnenolone and 6.1% with 17-α-OH-P and less than 0.1% cross- reaction with other structurally related steroids.

Analytical Recovery

The ability of an assay to accurately quantify progesterone in serum was tested. The pooled serum specimens were spiked with known amounts of low, medium, and high concentrations (5–30 ng/mL) of progesterone. The % recovery of spiked pools was determined; results are summarized in Table 1.

Precision

The level of imprecision is estimated by performing multiple analyses on pooled serum samples. The intra- and inter-assay coefficients of variations ranged from 4.52–7.39% and 4.65–9.55%, respectively. Table 2 shows the precision profile for progesterone assay.

Correlation Coefficient

Forty human serum samples were analyzed for progesterone, both by the in-house ELISA and by a well-established RIA kit. The correlation coefficient was 0.91 ($r = 0.91$, $n = 40$). The graph for linear regression of ELISA with RIA, in Fig. 2, was plotted with Graph Pad Prism version 3.00 for Windows, Graph Pad Software, San Diego, California USA. The correlation coefficient was 0.91. Regression analysis of the samples yielded following equation, $(\text{ELISA}) = 0.50 \times (\text{RIA}) + 0.94$ ($r = 0.91$, $P < 0.0001$).

Table 1. Recovery of Progesterone from exogenously spiked pooled serum

Serum pool no.	Progesterone added (ng/mL)	Progesterone expected (ng/mL)	Progesterone observed (ng/mL)	Recovery (%)
Pool A	–	–	10.64	–
Pool B	5.0	15.64	14.42	95.2
Pool C	10.0	20.64	19.13	92.6
Pool D	15.0	25.64	25.11	97.9
Pool E	30.0	40.64	31.98	78.6

Table 2. Inter and intra assay coefficient of variation for the measurement of progesterone

Variation	Sample value	Coefficient of variation (%)
Intra-assay n = 8	10.80 ± 0.68	6.36
	11.87 ± 0.70	5.95
	13.23 ± 0.97	7.39
	15.25 ± 0.68	4.52
Inter-assay N = 5	10.44 ± 0.48	4.65
	11.83 ± 0.74	6.31
	12.43 ± 0.70	5.70
	14.74 ± 1.40	9.55

n = Number of times same sample analyzed for intra-assay variation.
N = Number of times assays carried out for inter-assay variation.

DISCUSSION

In the present study, a direct, rapid, and a simple ELISA has been reported. This developed assay permits the direct addition of the serum sample into the assay, which requires only one hour and fifteen minutes for completion.

Achieving appreciable sensitivity and specificity, simultaneously, is a problem in enzyme immunoassay, specifically in the case of progesterone.[16] Different homologous and heterologous assays have been

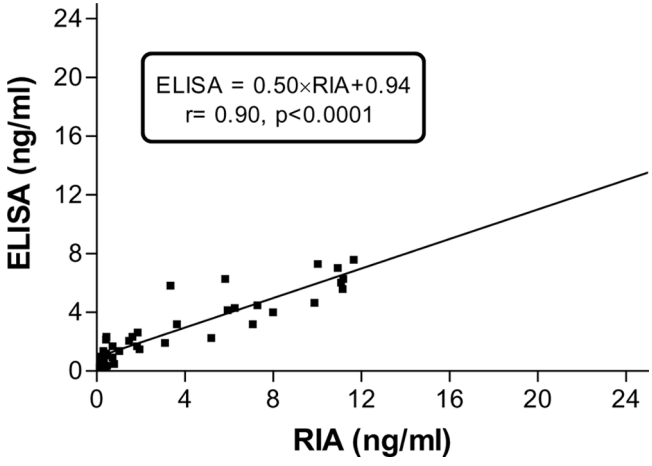

Figure 2. Linear regression graph of correlation between ELISA and RIA.

designed in the past for progesterone. But, most of them do not offer good sensitivity and specificity simultaneously.[17] Most of the assays reported so far have utilized different kinds of heterologies, e.g., bridge, site and antigen for developing the sensitive assay, but they have compromised on the specificity of the assay.

Wang et al. described a homologous assay using a P-11-HS bridge, having a sensitivity of 0.5 pg/well and a 32% cross-reaction with 11-α-OH P and 62.5% cross reaction with 5 α- pregnane-3, 20-dione.[18] Sauer et al. described a bridge heterologous assay with P-11-HS antibody and P-11-glucuronide enzyme conjugate with a sensitivity of 25 pg/well, and 8% cross-reaction with 5-β-Pregnane-3 -20 dione.[19] A site and bridge heterologous progesterone assay developed by Saha and Das[20] using the P-11-HS antibody with the P-3-O-CMO enzyme conjugate, had a sensitivity of 3.8 pg/well, whereas Munro and Stabenfeldt[21] developed an assay earlier using the same combination with a sensitivity of 0.25 pg/well and cross reaction with 11-α-OH P (21.4%) and 5-α-Pregnane-3-20 dione (62.5%).

Different homologous assays (P-3-O-CMO, P-11-HS, P-6-β-HS) and heterologous assays (P-3-O-CMO-BSA with P-6-β-HS-HRP, P-11-HS–BSA with P-3-O-CMO-HRP, P-6-β-HS-BSA with P-3-O-CMO-HRP and P-7-α-carboxethyl-thio ether-BSA with P-6-β-HS-HRP) were described by Hatzidakis et al.[22] and only P-7-α-carboxethyl-thio ether-BSA with P-6-β-HS-HRP was selected among them with a sensitivity of 0.25 ng/well and a 10% cross reaction with 5-β-pregnane-3-20-dione.

Introduction of antigen heterology is supposed to improve the sensitivity to a greater extent, compared to bridge and site heterology, but at the expense of specificity, as reported in the literature.[23] In our laboratory, we have already published the antigen heterologous assay of progesterone using alkaline phosphatase (ALP) as a label.[24] With the use of HRP a label, cross-reaction with pregnenolone was reduced to almost half (6.8%) compared to what it was with ALP (11%). Moreover, the cross-reaction with 17-α-OH-P was also marginally improved, keeping the sensitivity comparable (0.2 ng/mL) with ALP assay. In the present study, we have shown that selection of the enzyme at the time of label preparation may be crucial to the specificity of the assay, as is evident from the better specificity with HRP compared to ALP.

The idea of using HRP instead of ALP for label preparation was to reduce the overall assay time and make it more cost effective, since the ALP reaction kinetics is considerably very slow and it is more expensive than HRP. Moreover, the color pattern/gradient of HRP is sharper than ALP, which can be distinguished even with naked eye. ALP may give false positive results while working with serum, while HRP, because of its plant origin, is more popular to test samples from human or animal origin.[25]

In short, we report a fast and reliable antigen heterologous ELISA suitable for measuring progesterone in serum, which has several

advantages. The present assay achieves an appreciable sensitivity and simultaneously specificity, which has been a problem for progesterone assays in the past.

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