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A direct antigen heterologous enzyme immunoassay for measuring progesterone in serum without using displacer

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ABSTRACT

An antigen heterologous enzyme-linked immunosorbent assay (ELISA) for directly measuring progesterone in serum is described. Six combinations of antigens and enzyme conjugates were tested; the enzyme conjugate 17-αOH-progesterone-3-O-carboxymethyloxime-alkalinephosphatase (17-αOH-P-3-CMO-ALP) and the immunogen progesterone-3-carboxymethyloxime-bovine serum albumin (P-3-CMO-BSA) were found to be best. Fifty microliters of standard or serum sample and 100 μL of the 17-αOH-P-3-CMO-ALP enzyme conjugate were added to the antibody coated wells, and incubated for 1 h at 37 °C. Bound enzyme activity was measured by using *p*-nitrophenyl phosphate as substrate. The sensitivity of the assay was 0.11 ng/mL, and intra- and inter-assay CVs ranged from 5.1% to 9.6%. The analytical recoveries were 97–105%. The serum progesterone values obtained by this method correlated well with those obtained by radioimmunoassay; $r = 0.97$ ($n = 44$). Moreover, in this ELISA no displacing agent was used or special means was required to displace progesterone from corticosteroid binding globulin (CBG). Serum progesterone concentrations of subjects, with histories of recurrent spontaneous abortions were also measured, and correlated well with clinical history.

1. Introduction

Progesterone, a C_{21} steroid secreted by the corpus luteum, promotes the development of the endometrial lining. Serum levels of progesterone rise during the luteal phase of the menstrual cycle. If conception occurs, levels increase dramatically from the end of the first trimester to term during pregnancy. Because progesterone is required for the maintenance of pregnancy low levels are associated with luteal phase defect, ectopic gestation, and miscarriage. Many immunoassay techniques have been developed for measuring progesterone in serum. Most of these are radioimmunoassay (RIA), which involve the handling of radioactive materials. Given the inherent problems therein different non-radioactive methods have been developed for measuring progesterone, such as chemiluminescence immunoassays (CLIA) [1], time-resolved fluorescence immunoassays (TRFI) [2], fluorescence polarization immunoassays (FPI) [3], along with enzyme-linked immunosorbent assays (ELISA). Although CLIA and TRFIA compare favorably with RIA in terms of performance (sensitivity), whereas, instruments required for end product measurement are costlier. FPIs of progesterone, on the other hand, have not achieved desired levels of sensitivity [3,4]. Thus, the effectiveness of these techniques in the ordinary clinical setup is limited. Enzyme immunoassays (EIA) are still one of the most popular methods for measuring hormone concentrations. Homologous or site-heterologous EIAs using progesterone-11-hemisuccinate (P-11-HS) as the immunogen and P-11-HS

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or progesterone-3-O-carboxymethyloxime (P-3-O-CMO) as the enzyme conjugate have been developed [5–9], but failed to achieve proper sensitivity. Mitsuma et al. [10] used geometric isomers of P-3-O-CMO, for progesterone assay having sensitivity 0.16 pg/mL. Different homologous and heterologous EIAs for progesterone (bridge and site) were developed to achieve desired sensitivity and specificity [11]. The highest sensitivity achieved was 0.25 pg/mL with human serum using antibody generated against 7-α-carboxyethyl-thioether progesterone in combination with 6-β-progesterone-hemisuccinate-enzyme conjugate. Wu et al. [12] synthesized carboxylic acid derivatives of progesterone with different bridge lengths and conjugated them with ovalbumin, then tested them in an antigen immobilized progesterone EIA; the sensitivities ranged from 0.5 ng/mL to 50 ng/mL. In the above-mentioned assays, attempts were made to find combinations of immunogens and enzyme conjugates that led to progesterone EIAs effective in terms of both sensitivity and specificity. However, the performances of these EIAs were not up to mark in terms of sensitivity [11,12].

Generally, for the measurement of steroids directly from the serum, different reagents or combination of reagents were used as displacer in enzyme conjugate or sample dilution buffer to displace the steroid from the specific steroid binding protein. Danazol, dexamethasone, and cortisol were used as displacer in enzyme conjugate buffer for measuring progesterone directly from serum [13–15]. Trichloroacetic acid, dihydrotestosterone, saponin, and methyl isothiazolinone were used in sample dilution buffer for the same purpose [16,17]. In the present work, for the measurement of progesterone directly from the serum no steroid displacer either in enzyme conjugate buffer or sample dilution buffer was added. This is due to absence of specific binding globulin for progesterone, as 80% of progesterone is bound to albumin and 18% to transcortin and remaining 2% is free. The present study is to our knowledge, the first report of an antigen heterologous assay for the direct measurement of serum levels of progesterone by EIA.

2. Experimental

This study was conducted according to the Institutional Ethical Committee's norms.

2.1. Materials and methods

Progesterone, progesterone-3-O-carboxymethyloxime (P-3-O-CMO), progesterone-11-hemisuccinate (P-11-HS), 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide-HCL (EDAC-HCL), *N*-hydroxysuccinimide (NHS), bovine serum albumin (BSA), Freund's complete adjuvant (FCA), diethyl aminoethyl-sephadex (DEAE-sephadex), and sodium azide were all purchased from the Sigma Chemical Company, St. Louis, MO, USA. 17-αOH-progesterone-3-O-carboxymethyloxime (17-αOH-P-3-O-CMO) and other steroids were purchased from Steraloids Inc., UK. Alkaline phosphatase (Cat. No. EL-1L) was procured from Bangalore Genei, India. Breakable strip-based microwell plates were procured from Thermo Labsystems India Pvt Ltd. All other chemicals and buffer salts were purchased from the Sisco Research Laboratory (SRL), Bombay, India.

2.1.1. Preparation of immunogen and generation of progesterone antibody

Progesterone-3-O-carboxymethyloxime (P-3-O-CMO) and progesterone-11-hemisuccinate (P-11-HS) were conjugated to BSA according to the method of Basu et al. [18]. In brief, ten milligrams (10 mg) of P-3-O-CMO or P-11-HS were dissolved in 400 μL of dioxan and 400 μL of dimethyl formamide. In 200 μL of distilled water, 20 mg of *N*-hydroxysuccinimide (NHS) and 40 mg of 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride (EDAC-HCl) were dissolved, and the aqueous mixture was added to the steroid solution. The reaction mixture was vortex-mixed and incubated overnight at 4 °C to activate the –COOH group of the steroid. One hundred milligrams of bovine serum albumin (BSA) was then dissolved in 100 mL of distilled water. The activated steroid was added slowly to the aqueous solution of BSA. The reaction mixture was further vortex-mixed and incubated overnight at 4 °C. The steroid–BSA conjugate was then dialyzed against distilled water, and lyophilized. Polyclonal antibodies were generated in New Zealand white rabbits using P-3-O-CMO-BSA and P-11-HS-BSA immunogens as described elsewhere [18].

2.1.2. Preparation of alkaline phosphatase conjugate with progesterone derivatives

Alkaline phosphatase was conjugated to P-3-O-CMO, P-11-HS, and 17-αOH-progesterone-3-O-CMO (17-αOH-P-3-O-CMO) according to the method of Basu et al. [18], with modification. In brief, 4 mg of each progesterone derivative were dissolved in 50 μL of dimethyl formamide (DMF) and 50 μL dioxan. To each of the dissolved steroid derivatives, 100 μL of distilled water containing 5 mg of *N*-hydroxysuccinimide and 10 mg of 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride (EDAC-HCl) were added. The reaction mixture was vortex-mixed and incubated overnight at 4 °C. After overnight incubation, the reaction mixture was dried under a stream of N_2 gas. One hundred and twenty-five microliters of alkaline phosphatase (supplied in a solution of 50% glycerol, containing 5 mM Tris, 5 mM $MgCl_2$ and 0.1 mM $ZnCl_2$) and 875 μL of distilled water were then added. The reaction mixture was vortex-mixed and incubated overnight at 4 °C, then dialyzed against 5 mM Tris–aminomethane buffer (0.6 g of Tris–aminomethane, 1 g of $MgCl_2$, 20 g sucrose, and 1 g NaN_3 in 1 L of distilled water). The dialysate was centrifuged and the supernatant collected; 50% ethylene glycol (v/v), 1% BSA, 9% sucrose and 10% ammonium sulphate (w/v) were added to it and the mixture was stored at −30 °C. The dilutions of the ALP conjugate were prepared in 50 mM Tris buffer (pH 8.0) and working dilution was determined by checker board assay.

2.1.3. Immobilization of progesterone antibodies on polystyrene wells

Polystyrene wells of the micro-titer plate were coated either with P-3-O-CMO antibody or with P-11-HS antibody, according to the immuno-chemical techniques of Shrivastav et al. [19].