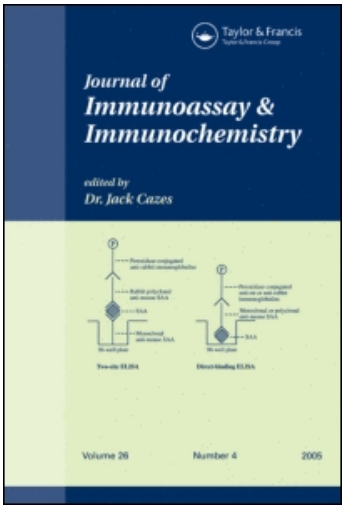

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Development of Rapid and Sensitive One-Step Direct Enzyme Linked Immunosorbent Assay for 17-α-OH-Progesterone in Serum

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Abstract: Using a homologous combination of immunogen and enzyme conjugate, a highly specific and sensitive Enzyme Linked Immunosorbent Assay (ELISA) was developed to measure 17-α-hydroxy-progesterone (17-α-OH-P) in human serum. The antiserum was raised against 17-α-hydroxy-progesterone-3-O-carboxy-methyloxime bovine serum albumin (17-α-OH-P-3-O-CMO-BSA) in New Zealand white rabbits. The enzyme conjugate was prepared by labeling 17-α-hydroxy-progesterone-3-O-carboxymethyloxime with horseradish peroxidase (HRP). Checkerboard assay was performed to determine the working dilutions of antiserum and enzyme conjugate. Dose-response studies were carried out by incubating 25 μL enzyme conjugate along with 50 μL of standards on the primary antibody coated wells for 1 hour. 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 detection limit of the assay was 180 pg/mL. The assay was more

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specific as compared to most other reported immunoassays for 17-α-OH-P. Cross reaction with analogous C_{18}, C_{19}, and C_{21} steroids was less than 0.1% except for progesterone which showed 2.1% cross reaction. The intra- and inter-assay coefficients of variation ranges from 3.7–7.5% and 6.9–11.7%, respectively. The developed ELISA correlated well with established RIA, with a correlation coefficient of 0.9 ($n = 30$).

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

INTRODUCTION

Congenital Adrenal Hyperplasia (CAH) is an autosomal recessive disorder of the steroid biosynthetic pathway observed due to the malfunctioning of enzymes involved in the conversion of cholesterol to cortisol. The classical form of CAH is characterized by severe salt wasting and presence of phenotypically altered genitalia by birth, whereas non-classical CAH manifests postnatally and mainly presents symptoms of hirsuitism and amenorrhoea.[1] This wide variation in clinical symptoms of the disease is attributed to different mutations in the corresponding CYP218 gene.

A mutation in cyt P450 component of enzyme 21-hydroxylase is the most prevalent cause of CAH. This obstructs the conversion of 17-α-OH-P to 11-desoxycortisol, thereby raising 17-α-OH-progesterone levels in (plasma/serum) too many folds as against the normal value of ≤ 20 nM/L.[2] A moderate increase in 17-α-OH-P is also observed with defective 11-β hydroxylase deficiency. This makes 17-α-OH-P an effective indicator for monitoring 21-hydoxylase functioning. The disorder can be confirmed biochemically by assaying the serum/plasma levels of 17-α-OH-P in all age groups, including infants, in addition to the obvious clinical symptoms of the disease.

Traditionally, radioimmunoassay (RIA) has been employed to quantify serum levels 17-α-OH-P by using more specific antibodies that 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 and cumbersome sample preparation steps, made them less popular a choice for routine analysis.[3–5] During the last two decades, immunoassays employing various non-isotopic labels, depending upon their end point measurement, have been developed as an alternative to RIA. The analysis of 17-α-OH-P in dried blood-spotted filter disc has been done by time resolved fluorescence and chemiluminescence enzyme immunoassays, but the requirement for a closed system and costly equipment has limited their use to some laboratories.[6,7] The use of physical techniques, e.g., liquid chromatography, has been described to achieve a very good sensitivity of 2.5 μg/L, but is again subject to interference from CBG and has low through-put as well.[8] Further, the retention time changes with the concentration of solvent in the mobile phase and may present biased results. More recently, the introduction of liquid chromatography-mass-spectrometry (LC-MS) and

tandem mass spectrometry in clinical steroid analysis has allowed a spectrum of steroid hormones to be measured simultaneously.[9–11] Most of the published methods based on gas-chromatography (GC-MS)[12] and some of the published methods based liquid chromatography-tandem mass spectrometry (LC-MS/MS)[13] require prior derivatization of the samples and are aimed at screening blood spots or serum matrix that precludes measurements within the normal ranges. Also, the technique requires a time consuming setup, expertise in handling, and large volume of samples (1–2 mL) for extraction, as compared to enzyme immunoassays. Thus, these more sophisticated techniques could replace the colorimetric enzyme immunoassays (EIA) only in some specialized and well equipped laboratories making later a popular choice even in today's scenario.

Not many EIAs have been reported for 17-α-OH-P measurement in serum. The EIA developed by Huble et al. was not sensitive enough to assay 17-α-OH-P in dried filter discs.[14] Also, a commercially available EIA kit for 17-α-OH-P determination was found to be inadequate for the purpose because of its high cross-reaction with 17-α-OH-5-pregnenolone sulfate.[15] The significant variation in the results obtained with the commercial kits further strengthens the need for a definitive technique in this respect.

The lack of any easy, reliable, and specific EIA system for 17-α-OH-P analysis has prompted us to carry out the present work. This paper describes development of a rapid and very sensitive homologous ELISA for 17-α-OH-P 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 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 tetramethylbenzidine/H_2O_2 solution were purchased from Bangalore Genei, Bangalore, India, and Arista Biochemical, USA, respectively. Microtitre plates were procured from Greiner, Germany.

Buffers

1. 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.

2. 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.

3. 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.

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

17-α-OH-Progesterone was coupled to BSA by an active ester method with some modification.[16] To 5 mg of 17-α-OH-progesterone, 200 μL each of 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 hours at 4°C. Activated 17-α-OH-progesterone solution was added to the aqueous solution of BSA (1 mg/0.3 mL), vortex mixed, and kept for 24 hour incubation at 4°C. The 17-α-OH-progesterone-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.

Immunization

17-α-OH-progesterone-BSA (1 mg) was dissolved in saline (0.5 mL) and emulsified with Freund's complete adjuvant (0.5 mL). The subcutaneous injections were given to New Zealand white rabbits at multiple sites, initially, as 5 weekly injections followed by the monthly booster injection. The rabbits were bled 10 days after the booster injection. Antiserum was collected after centrifugation at 2,500 rpm for 10 minutes and stored at −30°C.

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.[17] 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 hours 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, the 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 onto Microtitre Plates

The 96-well microtitre plate was coated using the immunobridge technique for primary antibody immobilization, as described elsewhere.[17] 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 (1:32,000) 17-α-OH-P antiserum in buffer "1" was dispensed. The plate was kept at 37°C for 2 hours. Unbound antibody was then washed off and 250 μL of buffer "3" was then added to block the unoccupied sites of the plate. The plate was kept at 37°C for 1 hour. Thereafter, the plate was decanted, dried at room temperature (RT), and kept at 4°C for future use.

Assay Procedure

To the 17-α-OH-P-BSA antiserum (1:32,000) coated microtitre wells, 50 μL of standards (0–50 ng/mL) were added in duplicate, followed by the addition of 25 μL of 17-α-OH-P-HRP enzyme conjugate (1:5,000). 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.

RESULTS

Sensitivity

The lower detection limit of the assay, i.e., concentration equivalent to A_0-2SD, was 180 pg/mL, after 20-fold determination of A_0 binding. A graphical representation of a calibration curve for 17-α-OH-P is illustrated in Fig. 1.

Specificity

The specificity of the 17-α-OH-P-BSA antibody was estimated as the percentage of cross-reaction with commercially available steroids with analogous structures. 17-α-OH-progesterone-BSA antibody showed 2.1%

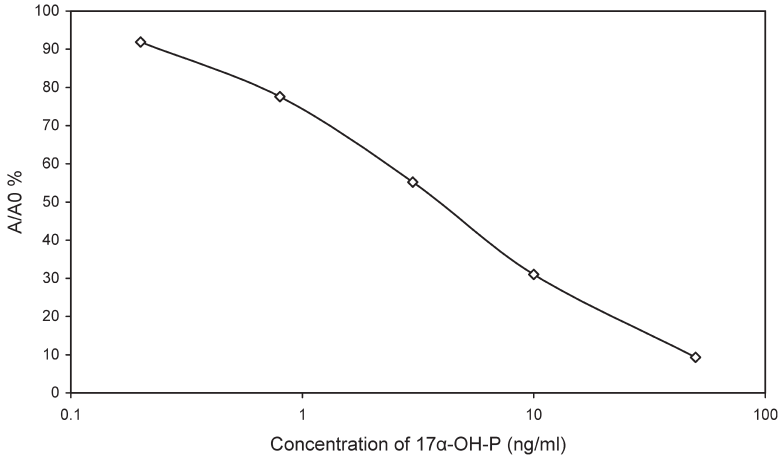

Figure 1. Calibration curve for 17-α-OH-progesterone.

cross-reaction with progesterone and less than 0.1% cross-reaction with other structurally related C_{18}, C_{19}, and C_{21} steroids.

Analytical Recovery

The ability of an assay to accurately quantify 17-α-OH-progesterone in serum was tested. The pooled serum specimens were fortified with known amounts of low, medium, and high concentrations (2.5–15 ng/mL) of 17-α-OH-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 variation

Table 1. Recovery of 17-α-OH-progesterone from exogenously spiked pooled serum

Sample	Observed value	Expected value	Recovery (%)
Pool A (Basal)	4.56	—	—
Pool B (2.5 ng/ml)	6.61	7.06	93.6
Pool C (5.0 ng/ml)	10.24	9.56	107.1
Pool D (15.0 ng/ml)	21.22	19.56	108.4

range from 3.7–7.5% and 6.9–11.7%, respectively. Table 2 shows the precision profile for 17-α-OH-progesterone assay.

Correlation Coefficient

Thirty human serum samples were analyzed for 17-α-OH-P, both by the in-house ELISA and a well-established RIA kit. The correlation coefficient was 0.9 ($r = 0.9$, $n = 30$). 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.

DISCUSSION

We have described a rapid, simple, and cost effective direct ELISA for the quantification of serum 17-α-OH-P levels. This assay is based on a homologous combination of immunogen and enzyme conjugate. Homologous assays have been proven to be relatively less sensitive because of the similar linkage between hapten-label and hapten-immunogen conjugates that is strongly recognized by antibodies.[18] However, we have achieved an appreciable detection limit of 180 pg/mL, which is much superior to the other reported enzyme and even some fluorescence immunoassays. The enzyme immunoassay reported by Maeda et al. has a lower detection limit of 1 ng/mL, in spite of utilizing a heterologous combination of immunogen and enzyme conjugate that is believed to improve the assay sensitivity.[19] The sensitivity of the automated solid phase ELISA developed by Elder et al. was 0.3 ng/mL, which is, again, significantly lower, as compared to the sensitivity reported in the present study.[20] The lower detection limit of

Table 2. Inter and intra assay coefficient of variation for the measurement of 17-α-OH-progesterone

Variation	Sample value	Coefficient of variation (%)
Intra-assay N = 8	2.4 ± 0.34	14.1
	4.95 ± 0.52	10.2
	15.58 ± 1.17	7.5
Inter-assay 5[a]	2.57 ± 0.19	7.4
	5.53 ± 0.49	8.8
	15.77 ± 0.44	2.7

N = Number of times same sample analyzed for intra-assay variation.

[a]=Number of times assays carried out for inter-assay variation.

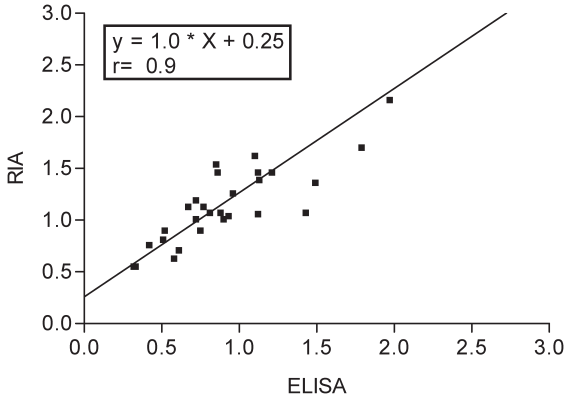

Figure 2. Linear regression graph of correlation between ELISA and RIA.

the present assay is even better than the radioimmunoassay developed by Dyas et al., which achieved a sensitivity of 248 pg/mL by employing magnetisable solid phase antiserum.[21] The assay is even more sensitive compared to the time resolved fluroimmunoassay developed for 17-α-OH-P in serum by El-Gamal et al. that showed a sensitivity of 0.16 ng/mL.[22] and strictly comparable with the one developed by Gonzalez et al. with a lower detection limit of 33 pg/mL.[7]

The raising of highly specific antiserum has always been a central point while developing an immunoassay. Various studies have been carried out by employing the C_3, C_6, and C_7, positions of 17-α-OH-P to covalently link it to the carrier protein, but many of them failed to achieve the desired level of specificity.[23,24] El-Gamal et al. have developed a specific antiserum in sheep against 17-α-OH-P, utilizing its C_3 position.[25] We have also used the same site of the steroid to couple it with BSA for raising the antiserum in New Zealand white rabbits. The specificity of the antiserum thus produced is better than that reported by El-Gamal, which showed 5.6% cross-reaction with 17-α-OH-pregnenolone.[25] An automated solid phase ELISA for 17-α-OH-P by Elder et al. has been reported to give a high cross-reaction with 17α-hydroxy pregnenolone, progesterone, and 11-deoxy-cortisol.[19] Similarly, the radioimmunoassay developed by Barron et al. is reported to give very high cross-reaction with progesterone and 11-deoxycortisol.[3] In the present study, the % cross-reaction of 17-α-OH-P-3-CMO-BSA antiserum with other analogous C_{18}, C_{19}, and C_{21} steroids was less than 0.1% except progesterone, which showed 2.1% cross reaction.

The performance of the current ELISA is even remarkable in terms of the assay duration. The enzyme immunoassay reported by Maeda et al.[19] needed overnight incubation, followed by a 2 hour enzyme-substrate reaction; similarly, the ELISA developed by Elder et al.[20] required 5 hours for its completion. This time limit is reduced to only one hour in our assay system, with

an additional twenty minutes required for substrate incubation. The rate of antigen-antibody interaction and establishment of equilibrium between the free and bound Ag-Ab complex dictates the optimum time required for an immunoassay. The high level of immunoreactivity of the antibodies helps in achieving a quick equilibrium. The use of Keyhole limpet haemocyanin as a carrier protein has been found to produce very high titres of antiserum as compared to BSA, which was explained to be due to the high steroid:protein ratio.[25] On the contrary, we have used BSA as a carrier protein in the present study and realized high titres of antiserum and used a working dilution of 1:32,000. This could be due to the variation in the degree of the immune response produced using BSA in different animal models, producing high titres in rabbits as compared to sheep. The volume of reagents used by us in our assay system is also less, i.e., 100 μL of antiserum and 25 μL enzyme conjugate, which further stresses the high immunoreactivity of the antibodies in the present study.

Finally, the assay also fulfills other criteria of validation that include precision and recovery. The intra- and inter-assay precision lie in the range of 3.7–7.5% and 6.9–11.7%, respectively. The exogenously spiked serum samples gave 93.60–108.4% recovery. The precision and recovery determined in some published reports were at high 17-α-OH-P concentrations (starting with lower spiking dose 8–9 ng/mL), indicating that these assays may not be suitable in lower concentration ranges.[18,24] We have, in the present study, obtained very good recovery for a concentration as low as 2 ng/mL. A good agreement is observed in 17-OH-P values with a well-established RIA kit for 30 serum samples.

In summary, we report a fast and reliable homologous ELISA suitable for measuring 17-α-OH-P in serum which can be readily used for mass screening of CAH. We believe that this assay will overcome the problems of low specificity, as revealed by the literature and obviates the need for lengthy sample preparation steps.

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