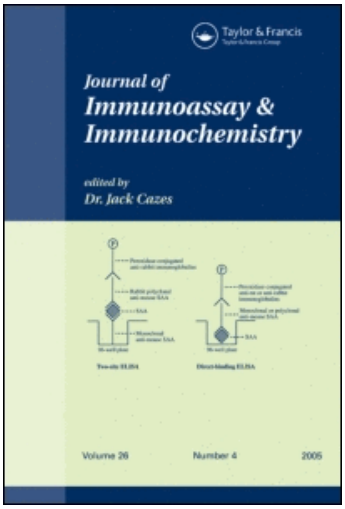

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One Step Enzyme Linked Immunosorbent Assay for Direct Estimation of Serum Testosterone

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ABSTRACT

One step competitive enzyme linked immunosorbent assay (ELISA) for direct estimation of testosterone in human serum is described. Testosterone-3-*O*-carboxymethyl-oxime-bovine serum albumin (testosterone-3-*O*-CMO-BSA), was used as immunogen and testosterone-3-*O*-carboxymethyl-oxime-adipic-acid dihydrazide-horseradish peroxidase (testosterone-3-*O*-CMO-ADH-HRP) was used as tracer. To the testosterone antibody coated micro-titer wells, standard or serum samples (100 μL), along with

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testosterone-3-*O*-CMO-ADH-HRP conjugate (100 μL) were incubated for 1 h at 37°C. Bound enzyme activity was measured by using tetra methyl benzidine/hydrogen peroxide (TMB/H_2O_2) as a substrate. In this new strategy, charcoal stripped pooled human serum spiked with non-cross reactive C_{18}, C_{19}, C_{21}, and C_{27} steroids, used for preparing the standards and blocking the sex hormone binding globulin (SHBG)/and other steroid binding globulins (SBG). The sensitivity of the assay was 0.015 ng/mL. The intra-assay and inter-assay coefficients of variation (CVs) were ranged from 7.8 to 11.8 and 4.8 to 10.4, respectively. The serum testosterone values, obtained by this method, were correlated well with those obtained by radioimmunoassay $r = .98$ ($n = 100$).

Key Words: Direct ELISA; Testosterone; One step assay; Bridge heterologous assay; Immunoassay.

INTRODUCTION

Testosterone is, quantitatively, a major androgen product of leydig cell of testis and thecal cells of graffian follicle of the ovary. Clinically, measurement of serum testosterone is useful in the investigation of infertility, impotence, and hypogonadism in the male and hirsuitism, virilism, and acne in the female. Several ELISAs have been reported for estimating the testosterone in saliva[1] and serum.[2–4] Most of the ELISAs for the estimation of testosterone in serum require extraction with organic solvent, which requires correction of recovery. Besides correction of recovery, the use of organic solvent increases the cost of ELISA by negatively affecting time, labour, and material. However, very few enzyme immunoassays have been reported requiring no extraction step for estimation of testosterones from serum samples.[6,7] These ELISAs are devoid of an extraction procedure, but require extra steps. These extra steps are addition of alkaline buffer for dilution of serum and heat denaturation of the serum sample.[6,7] In fact, these assays replace the extraction step by utilizing other steps. Thus, the simplicity in the procedure and direct use of sample in the assay system are still to be achieved.

We describe bridge heterologous ELISA for direct estimation of serum testosterone, based on the principle of competitive inhibition. In this new strategy, standards are prepared in pooled human serum from which the endogenous steroid has been stripped off by charcoal and, later on, spiked with non-cross reactive C_{18}, C_{19}, C_{21}, and C_{27} steroids for blocking the binding of testosterone to SHBG and other SBG.

Whereas, for releasing testosterone bound to SHBG in standard/unknown, 10 μg/mL of estradiol has been supplemented in conjugate buffer. We have also introduced labelling of steroid carboxylic derivative to a glycoenzyme, horseradish peroxidase, with spacer adipic acid dihydrazide using a periodate method. This ELISA method is not only simple and rapid, but is one-step and devoid of extra steps, for example, solvent extraction, heat denaturation, or addition of buffer. To the best of our knowledge, no similar method based on direct use of serum sample, has been reported for estimation of testosterone in serum.

EXPERIMENTAL

Materials

Testosterone, testosterone-3-*O*-carboxymethyl-oxime (testosterone-3-*O*-CMO), adipic acid dihydrazide (ADH), bovine serum albumin (BSA), 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide, HCl (EDAC), *N*-hydroxysuccinimide (NHS), gelatin, thimerosal, dextran T-70, freund's complete adjuvant (FCA), were all purchase from Sigma Chemical Company, St. Louis, MO, USA; Horseradish peroxidase and tetramethyl-benzidine/H_2O_2 solution were purchased from Pierce Chemical Company, USA, and Bangalore Genei, Bangalore, India, respectively. Microtiter plates were procured from Lab. System, India. All other chemicals and buffer salts were of analytical grade.

Buffers

1. The most frequently used buffer was 10 mM phosphate (10 Mm PB), pH 7.0, ($N_2PO_42H_2O$: 0.895 gm/L and $NaH_2PO_42H_2O$: 0.39 gm/L) containing 0.9% NaCl (10 m MPBS) 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 dextra T-70, 0.3%, BSA and 10 μg estradiol/mL.
3. Microtiter well blocking and stabilizing buffer was 10 mM PB containing 0.9% NaC1, 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.

Antibody Generation

The study was approved by the Institute's ethics review committee.

Primary Antibody

Testosterone-3-*O*-CMO was covalently linked to BSA by an activated ester method with modification.[8] The New Zealand white rabbits were immunized with this conjugate according to the procedure described elsewhere.[9]

Second Antibody

A group of three goats was immunized with an emulsion of 2.5 mL of FCA in 2.5 mL of saline containing 5 mg of normal rabbit-γ-globulin per goat following the method of Rao et al.[10] Blood was collected 12 days after the third booster injection and every 30 days thereafter and checked for titer.

Preparation and Dilution of Testosterone-3-*O*-CMO-ADH-HRP Conjugate

Horseradish peroxidase was activated and coupled to ADH by using the periodate method of Wilson and Nakane[11] with some modification. The testosterone-3-*O*-CMO was conjugated to ADH coupled HRP by an activated ester method with modification.[8] In brief, 10 μL of freshly prepared 0.1 M sodium meta periodate solution in water was added to 10 mg of horseradish peroxidase reconstituted in 1 mL of water. The reaction mixture was kept in the dark for 40 min. Activated HRP was passed through a G-25 column previously equilibrated with 10 mM ammonium carbonate, pH 9.3. The brown color activated HRP was directly collected in a vial containing 100 mg of ADH. The reaction mixture was kept at 4°C overnight. After overnight incubation, 10 μL of 5 M sodium cyanoborohydride in 1 M NaOH was added; the reaction mixture was kept at 4°C for 3 h. The above reaction mixture was passed through a G-25 column previously equilibrated with 10 mM PBS containing 0.1% thimerosal. The brown colored ADH-HRP conjugate was directly collected and kept at −30°C for future use.

Five milligrams of testosterone-3-*O*-CMO was dissolved in 400 μL of a 1:1 mixture of dimethyl formamide and dioxan. To the above 100 μL of

water was added 10 mg of NHS and 20 mg of EDAC; the reaction mixture was kept for activation at 4°C for 24 h. To the activated testosterone-3-*O*-CMO, 1 mL of ADH-HRP solution (approximately containing 1 mg of HRP) was added and the reaction mixture was further kept for 24 h at 4°C. After incubation, the reaction mixture was passed through a G-25 column, previously equilibrated with 10 mM PBS containing 0.1% thimerosal. The brown colored portion was pooled and, to it, a pinch of sucrose, ammonium sulfate, BSA, and an equal volume of ethylene glycol were added and the solution was kept at −30°C in aliquots for future use.

The optimal dilution of testosterone-3-*O*-CMO-ADH-HRP conjugate (1:4000) was found by checkerboard assay. The diluted conjugate was stored in the conjugate dilution buffer at 4°C for future use. This was stable for more than one year at 2–8°C.

Preparation of Testosterone Standard in Charcoal Stripped Serum

Deionized water was added to charcoal and stirred for 1 min and kept standing for 1 h. The water was decanted to remove fine charcoal. The sides of the beaker were wiped. The process was repeated until no more fine charcoal came to the surface of the water. We filtered the water by passing it through Whatman No.1 filter paper. The charcoal was heated overnight in an oven at 50°C and made into a powdery form by beating the clumps.

We added activated charcoal (50 mg/mL) to pooled serum and stirred for two hours at 45°C and centrifuged at $3000 \times g$ to remove the charcoal. Supernatant was collected and passed through a 0.45 μ membrane filter.

To the charcoal stripped filtrate, non-cross reactive C_{18}, C_{19}, C_{21}, and C_{27} steroids at their higher normal concentrations present in the subjects (male and female) were supplemented, and five, testosterone working standards, such as 0.2, 0.6, 2, 6, and 20 ng/mL, respectively, were prepared in it.

Coating of Microtiter Plate

Wells of a microtiter plate were coated with 225 μL of diluted testosterone-3-*O*-CMO antibody by an immunobridge technique described in a later article.[11] In brief, 250 μL of water-diluted normal rabbit serum (NRS) (1:1000) was dispensed into each well of the microtiter plate and

incubated overnight at 4°C. After incubation, contents of the plate were decanted and the plate was washed under running tap water. To the NRS coated wells of the microtiter plate, 250 μL of 1:4000 diluted second antibody in 10 mM PBS was added and incubated for 2 h at 37°C. After incubation, contents of the plate were decanted and plate was washed under running tap water. To the immunochemically immobilized second antibody through NRS coated wells of microtiter plate, 225 μL of testosterone antibody 1:4000 diluted in 10 mM PBS was added and incubated for 1 h at 37°C. After incubation, the contents of the plate were decanted and the plate was washed under running tap water. The unoccupied sites of the wells of the microtiter plate were blocked and the wells were stabilized by adding 250 μL of blocking and stabilizing buffer and incubating for 1 h at 37°C. The contents were decanted, and the plate was dried at room temperature and kept at 4°C for future use.

Preparation of Substrate Solution

Substrate solution was prepared from TMB/H_2O_2 solution (Bangalore Genei, India). According to the manufacturer's protocol, 100 μL of TMB/H_2O_2 solution was diluted to 2 mL (1:20 dilution) in water. This solution was freshly prepared just before its use.

One-Step ELISA Procedure

To the testosterone antibody coated wells, 100 μL of testosterone standards or serum sample were added in duplicate. The 100 μL of testosterone-3-*O*-CMO-ADH-HRP conjugate was added to all the wells and incubated for 1 h at 37°C. After incubation, the contents of the wells were decanted and washed under running tap water for five to six times by filling, decanting, and tapping. Finally, for measuring the bound enzyme activity, 100 μL of substrate solution was added to all the wells and incubated for 15 min at 37°C. The reaction was stopped by adding 100 μL of 0.5 M H_2SO_4 and the color intensity was measured at 450 nm in a Tecan-spectra ELISA plate reader.

Blocking of SHBG

Standards were prepared in pooled serum from which endogenous steroid has been stripped off by charcoal; non-cross reactive C_{18}, C_{19}, C_{21},

and C_{27} steroids were added at their higher normal concentration present in the subjects (male and female), for blocking the binding of testosterone to SHBG.

Release of Testosterone Bound to SHBG

The testosterone-3-*O*-CMO-ADH-HRP conjugate was diluted in HRP conjugate buffer which contains 10 μL estradiol/mL. The 100 μL of diluted conjugate was added to all the wells and incubated for 1 h at 37°C in the assay procedure. The estradiol present in the conjugate buffer binds to SHBG and releases the testosterone from it.

Radioimmunoassay (RIA) Procedure

The RIA of testosterone was performed following the method described by Abraham.[13]

Data Analysis

Quantification of testosterone in serum samples was performed by a computer program developed in our laboratory using a logit–log method. Regression analysis and the Mann–Whitney test were used to compare the values of samples obtained by this direct ELISA and the RIA.

RESULTS

The calibration curve in Fig. 1 represents the mean of ten standard curves obtained separately. Each standard concentration was used in duplicate in each assay. The coefficients of variation (CVs) of the standard(s) ranged from 3.2 to 5.6 per cent. On logit–log transformation of the curve, the equation was $Y = -1.14X + 0.71$.

Sensitivity

The lower detection limit of the assay, i.e., concentration equivalent to $B_0 - 2SD$, was 0.015 ng/mL of serum after forty-fold determination of B_0 binding.

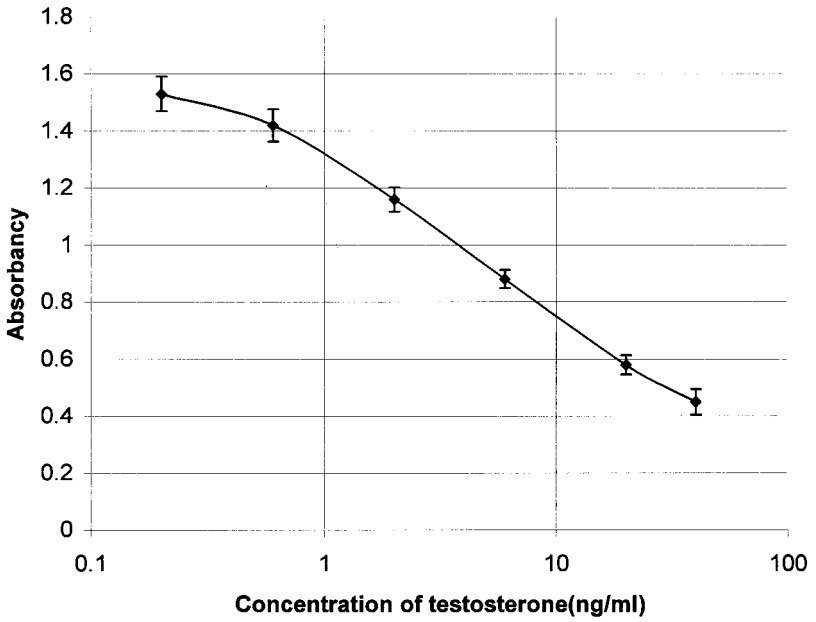

Figure 1. Composite standard displacement curve for 10 assays (in duplicate) of testosterone. Vertical bar at each point indicates the SD of each concentration.

Specificity of Antibody

Testosterone-3-*O*-CMO-BSA antibody had less than 0.1% cross-reaction with naturally occurring C_{18}, C_{19}, C_{21}, and C_{27} steroids, except 5α-dihydrotestosterone (10%).

Analytical Recovery

The ability of the assay to accurately quantify testosterone in serum samples was tested. Low, medium, and high concentration (2–15 ng/mL) of testosterone were added exogenously to three fractions of pooled female serum and two fractions of pooled male serum (low and medium). After addition, the concentration of testosterone was determined and recovery was calculated in each fraction of serum. The recoveries ranged between 97.2 and 102.7% (Table 1).

Table 1. Recoveries of testosterone from exogenously spiked pooled serum.

Type of pooled serum	Testosterone added (ng/mL)	Expected (ng/mL)	Obtained (ng/mL)	Recovery (%)
Female pool—Basal	0	—	1.4	—
Low	2	3.4	3.4	100
Medium	6	7.4	7.6	102.7
High	15	16.4	16.0	97.5
Male pool—Basal	0	—	8.6	—
Low	2	10.6	10.31	97.2
Medium	6	14.6	14.3	97.94

Intraassay and Interassay Variations

The analysis of 4 quality control serum for testosterone for intraassay ($n = 8$ replicate of each pool) gave CVs $\leq 11.8\%$ at all levels. The mean $\pm$ SD concentrations measured were as follows: serum A, 2.21 ± 0.91(ng/mL), serum B, 3.2 ± 0.25 (ng/mL) serum C, 7.1 ± 0.84 (ng/mL) and serum D,15.7 ± 1.62 (ng/mL). Interassay CVs for these 4 serums in 10 separate assays (8 replicate of each pool) were (10.4% at all levels. The mean $\pm$ SD values of these serums were 2.21 ± 0.23, 4.5 ± 0.34, 9.97 ± 0.48, and 17.7 ± 1.0 ng/mL, respectively.

Comparison of Direct ELISA with RIA for Testosterone Values

Testosterone values were measured in 100 serum samples by the new direct ELISA and by an in-house RIA method. Regression analysis of samples yielded the following equation:

$$Y(\text{ELISA}) = 1.01X(\text{RIA}) - 1.3 \qquad r = 0.98$$

The values obtained by ELISA and RIA were again tested by the Mann–Whitney U test procedure. These two methods were identical significantly ($Z = -1.1$, $p < 0.05$).

DISCUSSION

The developed ELISA for estimation of testosterone in human serum sample is direct, one-step, rapid, and simple. Only 100 μL of serum is

required and, within 1 h 20 min, the whole assay will be completed. The direct ELISA of serum testosterone has been reported,[5,6] which comprises two steps. In the first step, standard or serum samples are diluted two-fold with alkaline buffer and heated in water bath at 70–75°C for 30 min. In the next step, diluted and heated standard or serum samples were vortexed and used for assay.

One of the main problems associated with the direct assay is the matrix interference. Boots et al.[14] estimated that with total serum testosterone levels, using commercially available kits, a high degree of between-kit variability was observed which was attributed to level of SHBG.

In the present procedure, we introduced spiking of stripped pooled human serum (matrix) by non-cross-reactive C_{27}, C_{21}, C_{19}, and C_{18} steroids for preparing standards with improved accuracy. The serum matrix, stripped and spiked by steroids (non-cross reactive), can be employed for the preparation of standards for different steroid ELISAs, which may possibly eliminate the matrix effect.

In the present procedure, we have also introduced labelling of hapten (steroid) carboxylic group to HRP with spacer ADH using a periodate method. Adipic acid dihydrazide is a homobifunctional cross linking agent containing hydrazide group at both the ends. It provides a 10-atom bridge between cross linked molecules after conjugation. As the majority of amino groups in commercial horseradish peroxidase are blocked by allylisocyanate,[15] the enzyme is rather unreactive towards the carbodiimide activated hapten carboxylic derivative. This is because, during purification of HRP from the roots of horseradish plant, its component, sinigrin, is prone to releasing allylisothiocyanate,[16] and is likely to react with the enzyme's amine groups. Any resulting thioureas will be quite stable; so, these lysines are effectively lost as useful handles for derivatization. Perhaps, most importantly, changes in extraction conditions are likely to lead to batch-to-batch variation in the number of reactive amines that survive the process. Such differences in amine availability have been observed, which makes it difficult to establish reaction conditions which can be used for the one batch of HRP. The present procedure is the simplest method of attaching hapten to enzyme (HRP) with an increased bridge length, which is one of the desirable features of hapten immunoassay.[17] The enzymes being used in immunoassay are susceptible towards organic solvent, except HRP, which is robust towards organic solvent.[18–20] We noticed no change in enzyme activity when, for conjugating activated steroid, either enzyme added to activated steroid containing solvent or activated steroid to enzyme.

The analytical variables of the present ELISA, especially accuracy, which is regarded as a corner stone of the assay, are in agreement with the standardization of a method, which may be because of the use of steroid spiked stripped serum for blocking SHBG and for preparing standard in it, where the use of estradiol in conjugate buffer is to get release the testosterone from SHBG.

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