Indian Journal of Clinical Biochemistry, (1999), 14 (1), 19-25

DEVELOPMENT OF A HIGHLY SENSITIVE DIRECT MICROTITER PLATE ELISA FOR HUMAN SERUM TESTOSTERONE

M. Banerjee, S. Bhattacharyya, and C. Das*

Department of Biochemistry*, All India Institute of Medical Sciences, New Delhi, 110 029 and Department of Biochemistry, Santosh World Medical Academy, Ghaziabad, U.P.

ABSTRACT

A simple, highly sensitive, direct, competitive ELISA for human serum testosterone has been indigenously developed. Specific antisera against testosterone were raised in rabbits using testosterone - 3carboxymethyl oxime (CMO) - bovine serum albumin (BSA) as the antigen. For the enzyme conjugate, testoterone - 3CMO was coupled with horse raddish peroxidase by the active ester method. The standard curve covered a wide range from 3.9 pg/ml to 500 pg/ml. The inter and intra-assay variation were found to be low and within the acceptable limits. Specificity and accuracy for the assay was established by having negligible crossreactivity with the related steroids and an excellent parallelism between the sample and standard dilution curve. Samples measured by RIA and ELISA showed very high degree of correlation (r = 0.991).

Key Words: Testosterone, Direct ELISA, Serum testosterone, Sensitive ELISA

INTRODUCTION

Testosterone, an androgen is elaborated essentially by the Leydig cells of the testis. Precise measurement of serum testosterone is of great value for the evaluation of testicular function in health and disease e.g. hypogonadism, Klinefelter's syndrome, testicular feminisation syndrome, hirsutism, Stein-Leventhal syndrome and testosterone producing tumors.

Radioimmunoassay (RIA) has remained a standard procedure for quantitation of serum testosterone for many years. However, it is associated with the disadvantages of radioactive hazards and their disposal. This has necessitated the development of comparable non-isotopic immunoassays in recent years. The first solid phase enzyme immunoassay (EIA) for testosterone was reported by Rajkowski et. al. (1). They used microcrystalline cellulose as the solid phase and horse raddish peroxidase (HRP) as the enzyme. The sensitivity of their assay was poor with respect to RIA and the procedure was tedious, time consuming and unsuitable for routine use.

In the past two decades, a number of enzyme linked immunosorbent assays (ELISAs) have been reported for the measurement of serum testosterone. Unfortunately, many of these

*Author for correspondence:
Dr. Chandana Das, Prof. & Head
at above address.

methods have a prerequisite of solvent extraction of the samples (2-5). Joshi et. al. (3) and Elder and Lewis (4) reported development of very sensitive ELISA for testosterone using the enzymes penicillinase and HRP respectively. The major flaw in these methods was in the design of the assay. In the first one, the final steps of the assay required transfer of aliquots from the ELISA plate to a seperate set of tubes and the latter method involved a large number of incubation and washing steps. These drawbacks render the assay less practical. Several other ELISAs developed for testosterone, use 2-2 azino- di (3 ethyl-benzthiazolin) sulphonic acid (ABTS) or O-phenylenediamine (OPD) as the substrate (5,6). Both are known mutagenic agents which may cause contact dermatitis, consequently increasing the health risk factor of the users.

The present study was thus undertaken to develop simpler, highly sensitive, direct ELISA for the measurement of serum testosterone using specific antibodies generated for the purpose.

MATERIALS AND METHODS

Materials

Testosterone -3 Carboxymethyl oxime, bovine serum albumin (BSA) RIA grade, Freund's complete adjuvant and the steroids were obtained from Sigma Chemical Co., USA. The source of N-hydroxysuccinamide, 1-ethyl-3 (3 dimethyl amino propyl) carbodimide and horse raddish peroxidase was Pierce Chemical Co., USA. TMB/ H_2O_2 was obtained from Genei, India. (1,26,7-^{3} H) Testosterone was procured from Amersham, UK. High binding microtiter ELISA strips were purchased from Costar, USA. All other chemicals were obtained from local sources and were of analytical grade.

Methods

Generation of testosterone Antiserum

Specific antisera against testosterone were generated by immunising New Zealand strain albino rabbits against testosterone - 3 carboxymethyloxime (CMO) - BSA. Primary immunization was done by injecting 500 μg of immunogen emulsified with complete Freund's adjuvant administered intradermally at multiple sites. Booster doses were given intramuscularly, with the same dose at monthly intervals. The antibody titer was checked after three booster injections. Out of ten rabbits immunized, only two produced antibodies which bound 30-50% of ^{3}H-testosterone at 10-100K dilution of the serum. These rabbits were bled retroribitally and the serum collected was stored at -20°C until further use. The serum of one of the rabbits having 35% binding of ^{3}H-testosterone at 40K dilution was subjected to cross reaction analysis with the related steroids e.g. 5 α - dihydroandrostenedione, 4 α - androstenedion, 5 α - androstanediol, 5 - α androstane 3b 17b diol.

Preparation of Testosterone - 3CMO-HRP conjugate

Testosterone - 3 CMO was coupled with horse radish peroxidase (HRP) by the active ester method of Hosoda et. al. (7) with some modifications. Briefly, 2-4 mg of testosterone - 3 CMO and 7-8 mg of N-hydroxysuccinamide were dissolved in 100 μl of distilled N, N dimethyl formamide (DMF). Two mg of 1 - ethyl - 3 (3 di- methyl amino propyl) carbodimide was

dissolved in 100 µl of distilled DMF and added dropwise to the first solution. To prepare the active ester the mixture was then incubated for one hour in ice bath followed by another hour at room temperature with constant stirring. The active ester was then added dropwise to the enzyme solution (8mg of HRP dissolved in 0.1 M PBS, pH - 7.4). The mixture was incubated for 2 hr at room temperature with constant stirring. The conjugate thus prepared, was purified on G-25 sephadex column (2 cm x 100 cm) pre-equilibrated with 0.1 M PBS. Fractions containing maximum protein activity were pooled, concentrated by Amicon filter (membrane with 10,000 MW cut out) and stored at 20°C in small aliquots with 1% BSA.

Checker Board Assay

The optimum dilution of the antibody and the conjugate to be used in the ELISA was determined by the checkerboard assay. High binding microtiter ELISA well strips were coated with testosterone antisera (200 µl) at dilutions of 100K, 150K, 200 K in 0.1 M PBS and incubated for 16 hr at room temperature (RT). The control wells were coated with normal rabbit serum (NRS) at equal dilutions. At the end of the incubation, the wells were washed thrice with 0.1 M PBS containing 0.5% Tween -20 (PBS-T). Then non-specific blocking was carried out by addition of 200 µl of blocking buffer (0.1 M PBS with 1% BSA) followed by incubation at 37°C for 30 mins. and three washings with PBS-T. Subsequently, 100 µl of three different dilutions of testosterone - 3CMO - HRP conjugate (5K, 10K, 20K) followed by 100 µl of 0.1 M PBS were added to different sets of wells. The wells were incubated at 37°C for 30 mins. and washed 3 times with PBS-T solution. Finally, 200 µl of the substrate (TMB/H_2O_2) was added to each well and the plate was kept in dark for 20 mins. The reaction was stopped by the addition 100 ul of 1N H_2SO_4 and the optical density (OD) taken at 450 nm in a ELISA reader.

Standard Curve

The wells of the microtiter plate were coated with testosterone antisera at a dilution of 100 K as stated above. Control/ NSB wells were coated with 100K dilution of NRS. Nonspecific blocking was carried out with PBS-BSA as discussed in the checker board assay. After washing the wells, 100 µl of doubling concentrations of testerone standard ranging from 3.9 pg/ml to 500 pg/ml were added to the respective wells. Subsequently 100 µl of conjugate (10K dilution) was added to all the wells and the plate was incubated at 37°C for 30 mins.. The wells were washed thrice with PBS-T to remove excess unbound conjugate. The enzyme activity was measured by addition of substrate (TMB/H_2O_2) as stated before. The reaction was stopped by addition of 100 µl of 1N H_2SO_4. The colour was read at 450 nm. The standard curve was drawn by plotting log of the standard concentrations in the x-axis and logit of percentage binding (B- NSB x 100/ Bo-NSB) in the Y- axis

Specificity of the antisera coated wells

Specificity of the antibody coated wells was determined by two ways, (i) performing the crossreaction studies with related steroids like 5α- dihydroandrostenedione 4- androstenedione, 5α- Androstanedoil 5α- androstane 3β, 17β diol and ii) by sample dilution curve, in which one

high value serum sample was sequentially double diluted and assayed for testosterone by ELISA.

Collection and Pretreatment of Human Samples

Peripheral venous blood sample between 10-11 A.M. from normal males of different age groups was collected and subjected to heat denaturation by placing the tubes in a waterbath at 60-62°C for 1 hour.

Quantitation of human samples by ELISA

The level of testosterone in the serum samples was quantitated by ELISA using the protocol as described earlier in the standard curve except that instead of standards 100 µl of heat denatured samples were added in the sample wells. The value of the samples were calculated from the standard curve conducted at the same time in the standard wells.

Radioimmunossay

The concentration of testosterone in the test sample was also quantitated by conventional radioimmunossay using the same standards and the antiserum as used in the ELISA.

Statistical Analysis

The intra-assay variation was calculated by assaying one sample each of low, medium and high testosterone values six times within an assay. The interassay variation was calculated by analysing eight different sera samples in six different ELISAs conducted on different days. Mean ± SD and the coefficient of variation for both intra and interassays were calculated. The correlation coefficient (r) of sample values obtained by ELISA or RIA was also calculated.

RESULTS

Checkerboard Assay

The specific optical density of each dilution of the antiserum was plotted against the log of conjugate dilution. Based on the optical density values an optimal antisera dilution of 100K and conjugate dilution of 10K was selected and used further for developing the ELISA.

Standard Curve of Testosterone ELISA

The composite standard curve of six different ELISAs conducted at different time periods show a sensitivity of 3.9 pg/ml of testosterone within the 95% confidence limit. The maximum range of the assay was 500 pg/ml. In comparison, the standard curve obtained by RIA was a little less sensitive with a sensitivity of 7.8 pg/ml.

Crossreactivity of the antisera by ELISA

Crossreaction studies revealed the ELISA to be very specific, having very little crossreactivity with the related steroids (Fig. 1). Only 5α-dihydrotestosterone, the tissue androgen crossreacted to an extent of 6.25%.

Correlation between ELISA and RIA

Testosterone levels in six sera samples quantitated by RIA and ELISA showed a very high degree of correlation with the coefficient (r) of 0.991 (Table 1).

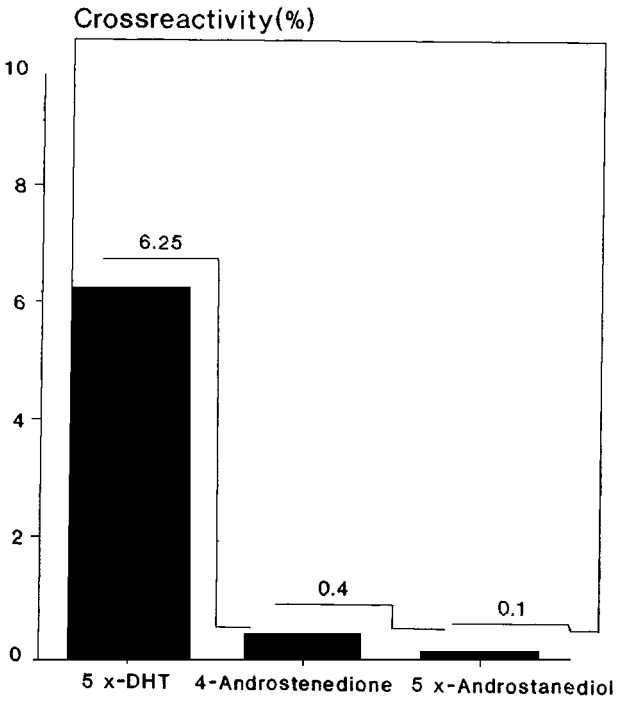

Fig. 1 Crossreactivity of testosterone antisera coated wells with related steroids.

Table 1. Correlation between the serum testosterone values determined by RIA/ELISA. Correlation coefficient (r) between RIA and ELISA is found to be 0.991.

	Sample Value in ELISA (ng/ml)		Sample Value in RIA (ng/ml)	
Sample	Mean	± SD	Mean	± SD
M1	8.17	0.65	8.80	0.56
M2	5.53	0.26	6.63	0.25
M3	7.18	0.60	7.57	0.27
M4	5.24	0.31	6.74	0.30
M5	9.63	0.70	10.1	0.17
M6	3.34	0.25	3.11	0.13
M8	1.86	0.23	2.34	0.01
M10	1.97	0.22	2.36	0.01

Intra and Interassay Coefficient of Variation

The intra and interassay coefficient of variation of the values quantitated by ELISA has been tabulated in Table 2. Low and medium value samples (M10 and M1) showed minimum variability (intra-assay : 0.05-5.96), interassay : 8.29) whereas high value sample (M1) showed little higher intra-assay (2.55 - 14.64%) and interassay (17.82%) variations.

Table 2. Intra and interassay variations of the sample values determined by ELISA.

Sample	Coefficient of variation (%)	
	Intra - Assay	Inter - Assay
Low Value (M10)	0.05 - 5.96	8.29
Medium Value (M2)	2.67 - 12.9	10.58
High Value (M1)	2.55 - 14.64	17.82

Samples dilution curve and parallelism with the standard curve

One of the serum samples was sequentially double diluted and the testosterone level in each dilution was determined by the developed ELISA. The values were plotted against the standard curve. Fig. 2 shows the parallelism between the standard curve and the sample dilution curve.

DISCUSSION

For the measurement of circulating hormones, recently efforts are being directed towards the use of simpler and cost-effective detection methods eliminating the hazards of

handling radioisotopes in the conventional RIA. We have demonstrated a sensitive, specific and a direct microtitre plate ELISA for testosterone using peroxidase as the enzyme marker.

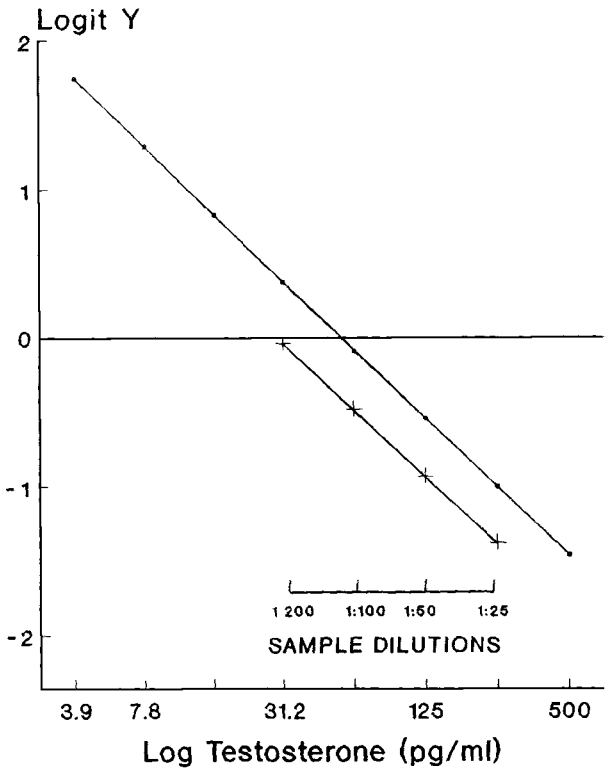

Fig. 2 Parallelism between the sample and standard dilution curve in ELISA.

Comparison of the standard curve obtained by the development ELISA with that of RIA revealed two important features. The sensitivity of the assay is twice higher in case of developed ELISA (3.9 pg/ml) as compared to that obtained by the RIA (7.8 pg/ml). Also the range of the assay is greater in the ELISA. Besides, the ELISA works at antisera dilution of 100K which is 2.5 times more than that required in RIA (40K). This would lead to less consumption of precious antiserum by the ELISA, indicating the cost effectiveness of the method.

The high degree of the specificity of the present assay is demonstrated by the fact that other steroids related to testosterone do not show any significant crossreaction except dihydrotestosterone (5-DHT) which cross reacts to about 6.25% (Fig. 1). This is much lower than most of the testosterone assays in use. Also, as 5a -DHT is primarily a tissue specific androgen, its circulating levels are very low (0.1 - 0.5ng/ml). Hence, cross-reaction of about 6% does not show any significantly increased testosterone values. Furthermore, the parallelism between the standard and sample dilution curve (Fig. 2) suggests the high specificity and accuracy of the developed ELISA.

The added attraction of the assay is that testosterone can be measured directly from the serum without prior solvent extraction. In contrast most of the RIAs for testosterone require prior solvent extraction, evaporation and solubilisation of the extracted residue in buffer. Moreover, TMB/H_2O_2 is used in this assay which is neither mutagenic nor induces contact dermatitis.

The precision of the present assay is quite satisfactory as evident by the consolidated displacement analysis of six different assays. The intra and interassay coefficient of variations being within the permissible limits (Table 2). The accuracy of the assay is further suggested by the reproducible values and acceptable standard deviations of the serum samples assayed on different days (Table 1). Finally, the evaluation of the human samples collected randomly from

healthy males by the ELISA exhibited an excellent correlation with the sample values obtained by RIA (r = 0.99) which indicates the validity of the present assay (Table 1).

Thus, a highly sensitive, specific and direct microtiter plate ELISA for testosterone is developed using the antiserum generated. This will be cost effective and better alternative to RIA for routine evaluation of clinical samples

Acknowledgement

This work was funded by a grant from Indian Council of Medical Research, Ansari Nagar, New Delhi - 110 029

REFERENCES

1. Rajkowski, K.M., Cittanova, N., Urios, P. and Jayle, M.F. (1977) An enzyme linked immunoassay of testosterone. Steroids 30 (1), 129-137.
2. Turkes, A., Turkes, A.O., Joyce, B.G., Read, G.F. and Riad-Fahmy, D. (1979) A sensitive solid-phase enzyme immunoassay for testosterone in plasma and saliva. Steroids 33, 347-359.
3. Joshi, U.M., Shah, H.P. and Sankolli, G.M. (1983) Pencillinase as a marker in enzyme-linked immunosorbent assays for steroid hormones. J. Steroid Biochem. 19 (1), 419-421.
4. Elder, P.A. and Lewis, J.G. (1985) An enzyme-linked immunosorbent assay (ELISA) for plasma testosterone. J. Steroid Biochem. 22 (5), 635-638.
5. Marcus, G.J. and Durnford, R. (1985) A simple enzyme-linked immunosorbent assay for testosterone. Steroids 46, 975-984.
6. Howard, K., Kane, M., Madden, A., Goling, J.P. and Fotrell, P.F. (1989) Direct solid-phase enzyme immunoassay of testosterone in saliva. Clin. Chem. 35 (10), 2044-2047.
7. Hosoda, H., Sakai, Y., Yoshida, H. and Nambara, T. (1979) Enzyme labelling of steroids by the activated ester method. Chem. Pharm. Bull. 27 (9), 2147-2150.