Medical Journal of the
Islamic Republic of Iran

Volume 8
Number 4
Winter 1373
February 1995

Basic Science In Medicine

DEVELOPMENT OF A SIMPLE AND SENSITIVE ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR CLINICAL MEASUREMENT OF TESTOSTERONE USING PENICILLINASE AS LABEL

G.H.R. ASADY KARAM, M.Sc., AND M.T. RASAEE, Ph.D.

From the Department of Biochemistry, School of Medical Sciences, Tarbiat Modarres University, Tehran, Islamic Republic of Iran.

ABSTRACT

An enzyme-linked immunosorbent assay using a homologous combination of antiserum raised against testosterone-3-0-carboxymethyloxime-bovine serum albumin (T-3-0-CMO-BSA) and penicillinase-labelled T-3-0-CMO was developed. This assay was utilized to measure testosterone in serum samples of male and female subjects. The sensitivity of the assay is 50pg/well and the antibody developed cross-reacted in less than 20% with 5α-dehydrotestosterone (5α DHT) . Inter- and intraassay variations and all other validation factors such as recovery, test of parallelism, etc. were well in the acceptable ranges . Comparison of testosterone values of 32 plasma specimens obtained by solid phase ELISA method and radioimmunoassay (RIA) showed a good correlation (0. 96).
MJIRI, Vol. 8, No. 4, 243-248, 1995.

INTRODUCTION

The development of RIA has permitted the efficient determination of many hormones and other biologically-active substances, but from the view point of biohazard and problems of disposal resulting from the use of radionuclides, the development of enzyme immunoassay (EIA), is currently expected as an alternative to RIA. Using a solid support and performing ELISA for the detection of antigen and antibodies in body fluids has been an increasing practice during the last few years.[1-4] These have utilized either alkaline phosphatase,[5] glucoamylase,[6] or horseradish peroxidase[7] as the enzyme label. The use of penicillinase (E. C. 3.5.2.5.) as a marker enzyme in the development of EIA for some steroids and protein hormones[8,9] has certain advantages over other enzymes. These include its absence in biological fluids, greater stability (even at room temperature), higher sensitivity, higher conjugation ratio and a high turnover rate compared to other enzymes currently used in EIA. In this paper, we present our recent data on developing an ELISA for testosterone using penicillinase as a marker enzyme and 96 well microtitration plate solid support to separate the bound and free fraction of antigen.

MATERIALS AND METHODS

Testosterone-3-0-carboxymethyloxime (T-3-0-CMO), penicillinase (β-lactamase, E.C. 3.5.2.5), sodium dihydrogen phosphate, disodium hydrogen phosphate, soldium azide,

Table I. Cross-reactivities of anti-testosterone antisera with the structurally related C18, C19 and C21 steroids using T-3-0-CMO-enzyme by EIA and ELISA.

SN	Compound name	% Cross reaction byEIA	% Cross reaction by ELISA
1	4-Androstene,3,17 dione	0.50	0.25
2	4-Androstene,3,13 dione	<0.05	<0.05
3	Androsterone	<0.05	<0.05
4	Epiandrosterone	<0.05	<0.05
5	Progesterone	<0.05	<0.05
6	D-aldosterone	<0.05	<0.05
7	Corticosterone	<0.05	<0.1
8	Ethiocholane-3β, ol-17 one	<0.05	<0.05
9	Estriol	<0.05	<0.05
10	B-estradiol	<0.05	<0.05
11	17-ethenyl estradiol	<0.05	<0.05
12	Pregnanetriol	<0.05	<0.05
13	5β-pregnane, 3β,17α,diol, 20 one	<0.05	<0.05
14	Estrone	<0.05	<0.05
15	Cholesterin	0.30	<0.05
16	Hydrocortisone	<0.05	<0.05
17	Dehydroisoandrosterone	<0.05	<0.05
18	Danazol	<0.05	<0.05
19	Dihydrotestosterone	27.00	21.00
20	Testosterone	100.00	100.00

Table II. Recoveries of testosterone added to stripped plasma using anti-T-3-0-CMO-BSA and T-3-0-CMO-enzyme by ELISA.

Testosterone added pg/100µL*					
75 pg/100µL mean±SE	CV%	275 pg/100µL mean±SE	CV%	550 pg/100µL mean±SE	CV%
101.45±2.35	7.68	96.12±2.27	7.85	99.44±1.95	6.5

*Each concentration was assayed 11 times in 6 replicates.

polyethylene glycol (PEG), Freund's complete adjuvant, Freund's incomplete adjuvant, Sephadex G-25, dialysis tubing, DEAE Sephacel 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimid-HCL, N-hydroxysuccinimide and dimethylformamide were purchased from Sigma Chemical Company, St. Louis, Mo, USA. Testosterone-11β-carboxymethyl ether (T-11β-CME) was a gift from Prof. P.N. Rao, Chairman, Department of Organic Chemistry, Southwest Foundation of Biomedical Research, Texas, USA.[1,3,5,7] ^{3}H Testosterone (85.5 Ci/mmol), 2,5 diphenyl-oxazol (PPO), and 1, 4-bis 2-(4-methyl-1,5-phenoxyzolyl) benzene (POPOP) were supplied by New England Nuclear Corp, MA, USA. All other solvents and chemicals used were of analytical grade.

Preparation of testosterone-penicillinase conjugate

Testosterone-3-0-CMO was conjugated to penicillinase following an active ester procedure.[11] The conjugate was purified either on a Sephadex G-25 column or alternatively by dialysis and stored as described by Shrivastava, et al.[12]

Preparation and purification of antibodies

Production of antibody against T-3-0-CMO-BSA and its characterization by RIA is explained elsewhere.[10] Anti-rabbit gammaglobulin (ARGG) was prepared in goats and purified following the method of Levy and Sober,[13] serving as second antibody. The purification of anti-T-3-0-CMO-BSA using ion exchange chromatography was carried out

Table III. Inter- and intraassay variation of testosterone added to the charcoal stripped serum by ELISA.

Intraassay	n	Mean± SE Pool A† ng/mL	CV%	Mean±SE Pool B ng/mL	CV%	Mean±SE Pool C ng/mL	CV%
1	*6	73.5±5.1	13.9	270±11.7	8.6	500±33.6	13.46
2	6	69.8±3.1	10.9	266.6±10	9.6	585±8.06	3.30
3	6	84.3±5.0	14.7	265.6±10	10.0	556±31.3	13.80
4	6	73.3±7.6	10.4	270±15.2	9.7	623.3±11	3.30
5	6	71.1±4.4	6.24	231±6.4	17.3	573.3±15	6.40
6	6	72.1±8.0	7.30	273.3±80	7.2	508.3±20	9.90
7	6	72.5±4.6	16.5	200±17.7	21.8	531.6±25	11.90
8	6	83.3±9.5	3.24	271±12.4	11.2	521.6±16	7.90
9	6	79.5±2.8	8.80	275±10.2	9.1	541.6±15	6.80
10	6	83.1±3.3	9.95	281±12.7	11.9	538±13.7	6.26
11	6	78.8±2.2	7.00	268±13.0	11.7	538±13.7	6.26
12	6	81.1±1.1	2.91	278.7±30	2.4	534.8±40	1.68
Interassay	12	76.9±1.5	6.92	263.4±7	9.2	545.9±90	6.28

n= number of assays carried out
*Results of 6 replicates.
+Pool A= 75 pg testosterone added.
Pool B= 275 pg testosterone added.
Pool C= 550 pg testosterone added.

Table IV. Normal levels of testosterone in human plasma

Normal Plasma	RIA n†	RIA Mean±SE (ng/mL)	RIA Range±SE (ng/mL)	ELISA *n	ELISA Mean±SE (ng/mL)	ELISA Range±SE (ng/mL)
Female	20	0.5±0.10	0.2±1.00	7	0.56±0.10	0.2±1.00
Male	35	5.7±0.43	2.5±10.5	32	6.14±0.48	2.4±10.0

†n= Number of individuals from whom plasma was collected and assayed by RIA.
*n= Number of individuals from whom plasma was collected and assayed by ELISA

following the same procedure as previously described by us.[9]

Enzyme immunoassay of testosterone

Titer and dose-response assay was performed by EIA following our previously reported procedure.[14]

Enzyme-linked immunosorbent assay (ELISA)

ELISA was performed following our procedure reported elsewhere[9] with a minor modification. Briefly, different dilutions of purified gammaglobulin of anti-T-3-0-CMO-BSA were coated on the surface of the plate overnight, dissolving the antibody in a PBS buffer, pH 7.4 (except the first row which was coated with normal rabbit serum to serve as non-specific binding index (NSB). The plates were then washed with distilled water 4X, taped on a filter paper, coated with 3% gelatin buffer (PBS pH 7.7 containing 3% gelatin) for at least 30 minutes at room temperature and washed with D.D. water 4X (the plates coated in this way were stable for at least one week). For titer assay (checker board) 100µL of different dilutions of T-3-0-CMO-penicillinase conjugate (tracer) along with 100µL of buffer were added to each well and incubated at 37°C for 2 h, at the end of which the plates were dispensed and washed with distilled water 4X. To detect the enzyme activity, 200µL of substrate solution (penicillin V, 3.8 mg in 20 mL of 0.2 M PB, pH 7.2) were added to each well and incubated at 37°C for 1h. The content of each well was then transferred to a glass tube containing 1 mL of starch iodine solution (preparation of which is described elsewhere)[9] vortexed and incubated for 10 min. at 37°C. The enzymatic reaction was then stopped by adding 0.5 mL of a 5N solution of hydrochloric acid. The optical density was then measured at 620 nm on a Shimadzu UV-VIS 3100 spectrophotometer.

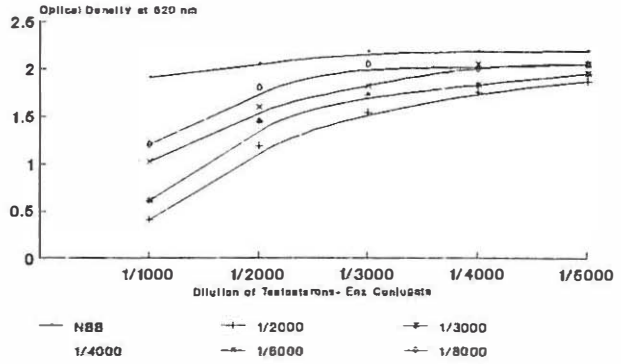

Fig. 1. AN (2), Optimum dilution of enzyme 1/1000 and antibody 1/4000.

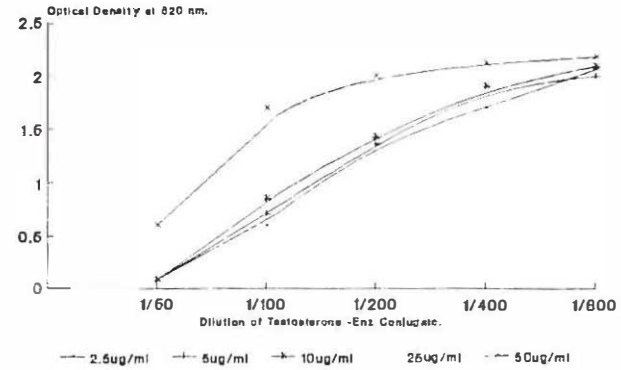

Fig. 2. AN (2), Optimum dilution of enzyme 1/100 and antibody 5μg/mL.

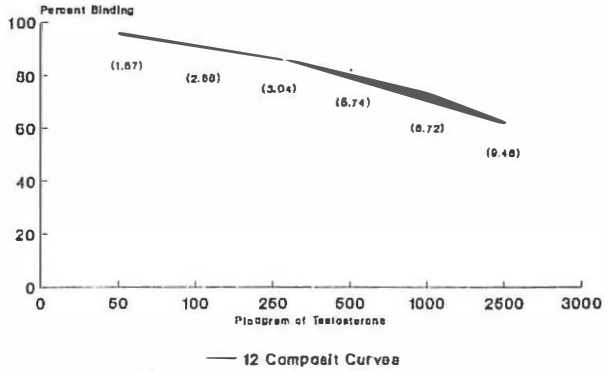

Fig. 3. AN (3), Bars = S
Values in parentheses= CV

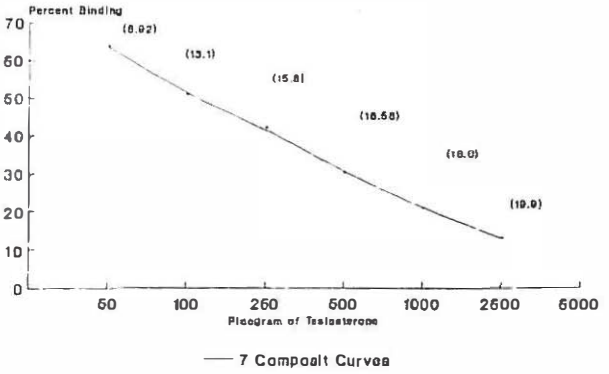

Fig. 4. AN (3), Bars= SD
Values in parentheses= CV

The sameprocedures were followed for standard and sample assay, where 100 μL of prepared standard or sample were added in place of buffer to each corresponding well and remaining steps followed similarly.

RESULTS

Figures 1 and 2 show a typical titer assay in our experiments following an EIA of double antibody procedure and ELISA respectively. In this way the optimum titer of antibody in the case of animal number one (AN 1), raised against testosterone -11β-CME-BSA was 1:2000 (i.e. initial dilution) and 1:1000 for tracer , while for AN 2 and 3 (antibody raised against T-3-0-CMO-BSA), the dilution of antibody was 1:4000 and that of tracer 1:1000 following an EIA procedure. In ELISA however, the optimum dilutions were found to be 10μg per well for the same antibody and 1:100 for enzyme conjugate. A typical dose response curve (result of seven experiments for EIA and ten for ELISA) are shown in Figs. 3 and 4. Here the CV values are all in the acceptable ranges and the assay sensitivity is from 25pg to 2500pg. The affinities for the three antibodies produced in this study were calculated using the Scatchard plot and the values obtained were 0.08×10^{-9} L/mol for anti-T-11β-CME by EIA, 0.094×10^{-9} L/mol for anti-T-3-0-CMO by EIA and 0.034×10^{9} L/mol for anti-T-3-0-CMO by ELISA (figures are not shown). The linear transformation of hyperbolic standard curve is shown in Figs. 5 and 6. The slope of standard curve is -1.36 and that of sample dilution, -1.46. Table I presents the data obtained on comparison of the cross-reactivities by EIA and ELISA. Here the cross-reactivity with 5α DHT slightly increases in EIA, although even this value is less than what was obtained in RIA (i.e. 32%). Table II shows the recovery data of a stripped plasmasample supplemented with a known amount of testosterone in three ranges (75, 175 and 550 pg/100 μl). The mean recovery ranged between 96 to 101 percent with a CV of not more than 7.5 percent. The result of inter- and intraassay variation of 36 assays performed simultaneously are discussed in Table III. In this case also all the statistical data are in full support of the assay's accuracy and reproducibility. Levels of testosterone in 20 female and 35 male serum samples were measured by ELISA and compared with the values obtained by standard RIA (Table IV). These are well in the range of reported testosterone values. Finally the correlation coefficient of the two procedures (ELISA and RIA) were calculated by SPSS program and the r value was found to be 0.96 which indicates a high correlation between RIA and ELISA (Fig. 6).

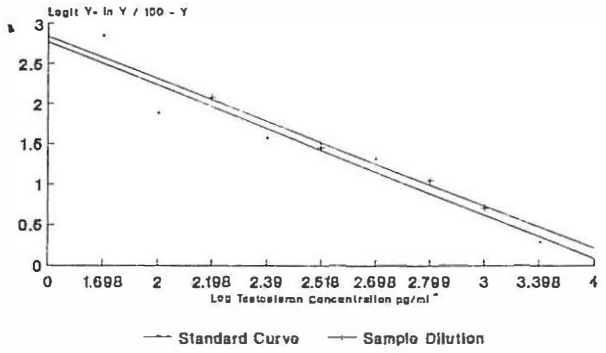

Fig. 5. AN(2), Slope of standard curve = -1.36, Slope of sample dilution= -1.46

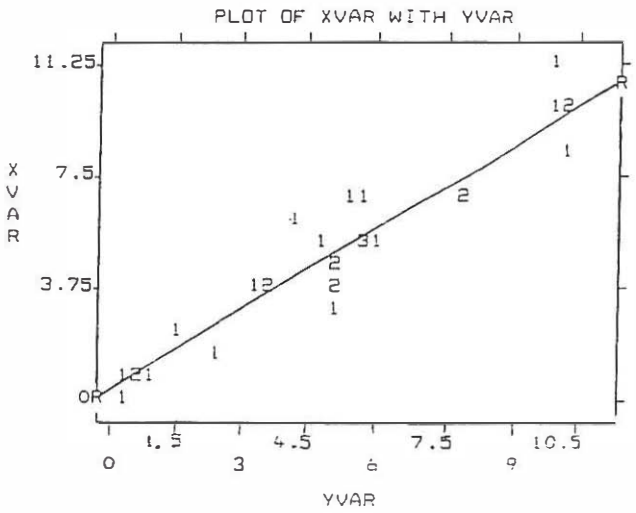

Fig. 6. Correlation coefficient of testosterone measurement following our procedure of RIA(X VAR) and ELISA developed in this study (Y VAR).
30 cases plotted. Regression statistics of XVAR on YVAR:
Correlation .96284 R Squared .92707 S.E. of Est .82948
Sig. -.0000
Intercept (S.E.) .06943 (.29443) Slope(S.E.) .94306 (.04999)

DISCUSSION

Testosterone is measured because of its importance in manifestations of various disorders. Radioimmunoassay is the method of choice in most laboratories of this country using a γ-emitting radioactive label because of the easy availability of kits imported from abroad and unwillingness of the private sector to alter the routine method. We have already reported a very low cost RIA for testosterone using home-made reagents with an excellent correlation with the imported ones. In that assay, ^{3}H is used instead of ^{125}I to decrease the risk ofhigh doses of radioactive contamination as well as to prolong the shelf-life of the labelled steroid. This strategy will lower the cost for one test by almost 100 times. However, to overcome some problems related to steroid immunoassays using a radioactive label (for example the cost of a counter) we planned to develop an ELISA in order to measure testosterone in serum samples using inexpensive equipment available in every laboratory. Recently we have also reported on the sensitivity and specificity of EIA using a heterologous combination of antibody and enzyme conjugate.[9] Here we used a homologous combination of the two and obtained acceptable sensitivities without compromising on other factors such as specificity. The antibody raised in this study cross-reacts with 5α DHT with a very low percentage. The end point detection is simple using an ordinary spectrophotometer and does not require an ELISA reader. All reagents were prepared locally and have been evaluated to meet the practical conditions of the laboratories in Iran. Considering the potential hazard involved in using the radioactive labels and very low cost of performing ELISA with an equal efficiency in comparison to RIA ,we therefore suggest using the present method in routine clinical work in Iran instead of RIA.

REFERENCES

1-Engvall E, Perlmann P: Enzyme-linked immunosorbent assay (ELISA) quantitative assay of immunoglobulin G.Immunochemistry 8:871-874,1981.

2- Engvall E, Perlmann P: ELISA III. Quantitation of specific antibodies by enzyme labelled anti-immunoglobulin in antigen-coated tubes. J Imnun 109:129-135,1972.

3- Van Weeman BK, Schuurs AHWW : Immunoassay using antigen-enzyme conjugates. FEBS Lett 15: 232-236,1971.

4- Maggio ET: Enzyme Immunoassay. Boca Raton, CRC Press, pp. 53-70,1980.

5- Ogihara T, Miyai F, Hishi K, Ishibashi K, Kumahara K:Enzyme labelled immunoassay for plasma cortisol. J Clin Endocrinol Metab 44:91-95,1977.

6- Tateshi K, Yamamoto H , Ogihara T, Hayashi C: Enzyme immunoassay of serum testosterone. Steroids 30:25-30,1977.

7- Rajkowski KM, Cittanova N, Desfosses B, Jayee MF: The conjugation of testosterone with horseradish peroxidase and a sensitive enzyme assay for the conjugate. Steroids 29:701-713, 1977.

8- Joshi UM, Shah HP, Sankolli GM : Penicillinase as a marker in enzyme-linked immunosorbent assay for steroid hormones. J Steroid Biochem 19:419-421,1983.

9- Rassaie MJ , Kumari GL, Panday PK, Gupta N, Kochopillai N, Grover PK: A highly specific heterologous enzyme-linked immunosorbent assay for measuring testosterone in plasma using antibody-coated immunoassay plates or polypropylene tubes. Steroids 57:288-294,1992.

10- Asady Karam GHR,Rasaee MJ, Aleaqua SA: Specific, sensitive and one-step extraction radioimmunoassay of testosterone for clinical diagnosis. *In press,* Med J Islam Repub Iran,

1993.

11- Hosoda H, Sakai Y, Yoshida H, Nambara T: Enzyme labelling of steroids by the activated ester method. Chem Pharm Bull (Tokyo) 27: 2147-2150, 1979.

12- Shrivastav TG, Kumari GL, Rao PN : Enzyme immunoassay of cortisol in human plasma using penicillinase as label. Clin Chem Acta 174:83-92, 1988.

13- Levy HB, Sober SA: A simple chromatographic method for preparation of gamma globulin. Proc Soc Exp Biol Med 103:250-252, 1960.

14- Rassaie MJ, Kumari GL, Rao PN, Shrivastav TG, Panday HP: Influence of different combinations of antibodies and penicillinase-labelled testosterone derivatives on sensitivity and specificity of immunoassay. Steroids 57: 112-118,1992.