Medical Journal of the
Islamic Republic of Iran

Volume 7
Number 2
Summer 1372
August 1993

A SPECIFIC, SENSITIVE, AND ONE-STEP EXTRACTION RADIOIMMUNOASSAY OF TESTOSTERONE FOR CLINICAL DIAGNOSIS

G.H.R ASADIKARAM,* M.J. RASAEE,* S. ALE AGHA,**
G.L. KUMARI,# AND P.N. RAO##

*From the *Department of Biochemistry, School of Medical Sciences, Tarbiat Modarres University, Tehran, I.R. Iran; the **Quality Control Department, Razi Research Institute, Karaj, I.R. Iran; the #Department of Reproductive Biomedicine, National Institute of Health and Family Welfare, New Delhi, India; and the ##Department of Organic Chemistry, South West Foundation of Biomedical Research, San Antonio, Texas, U.S.A.*

ABSTRACT

Testosterone was measured using antibodies raised against testosterone 11B-carboxymethyl ether bovine serum albmin (T-11B-CME-BSA) and testosterone 3-O-carboxymethyl oxime-BSA as immunogen. The antibody produced in this study exhibits minimal cross reactivity with the structurally related steroids specially 5 dihydrotestosterone (5 DHT). This allows to ommit the clean up step and measure testosterone in female serum samples accurately with a high sensitivity, precision, and specificity. The coefficent of variation (CV), standard deviation (SD) and standard error of mean (SE) were all in acceptable ranges. Antibody-bound and free steroids were separated by addition of dextran coated charcoal. The method was applied to a set of clinical samples, the results of which are discussed in this communication. The assay was compared with the available imported kits using 125 I as tracer. The correlation coefficient obtained is calcualted to be r= 0.96, showing that the results obtained by these two methods are fully comparable and the assay may be replaced with the similar preparations imported from abroad.

MJIRI, Vol. 7, No.2, 101-108, 1993.

Keywords: Radioimmunoassay, Testosterone, Steroid assay, Hormones.

INTRODUCTION

Radioimmunoassay (RIA) has been the predominant analytical technique used in endocrinology during the past 20 years. Since the introduction of the first RIA by Yallow and Berson[1] many investigators have been involved in developing a simpler assay system. The measurement of testosterone in serum is necessary for the diagnosis and management of a number of disorders, including hypogonadism, Klinefelter's syndrome and testicular feminization in man, hirsutism, Stein-Leventhal syndrome, and testosterone producing tumors, both of adrenal and ovarian origin, in women.

Over the last two decades procedures for all steroid assays including testosterone have been investigated with different techniques such as gas liquid chromatography[2,3] and high performance liquid chromatography.[4,5] However, use of high affinity antibodies has eliminated most of the tedious purification, derivatization and column chromatographic steps leading to rapid, efficient and

Reprint requests should be addressed to: MJ Rasaee, Department of Biochemistry, School of Medical Sciences, Tarbiat Modarres University, Tehran Iran.

This work has been supported by Tarbiat Modarres University Project Department.

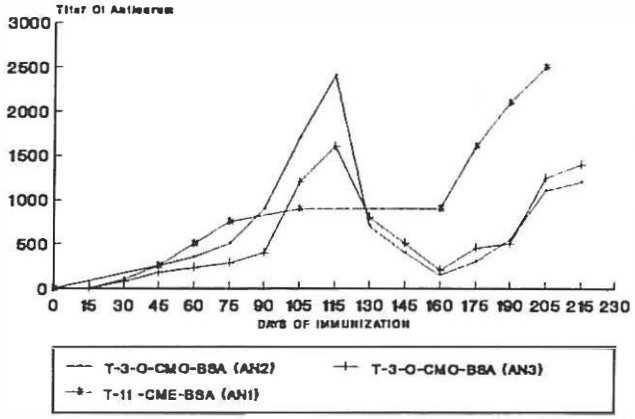

Fig. 1. Effect of immunization on antibody development.

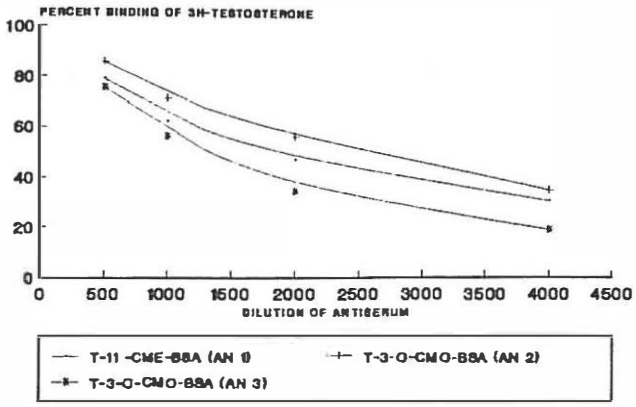

Fig. 2. Titration curves of antibody raised against testosterone.

sensitive assay with large through-put of samples. The most important component of an immunoassay is the availability of a high affinity and well characterized antibody. Since the first report of Nugent and coworkers,[6] a number of attempts have been made on the preparation of specific testosterone antisera for use in RIA. The specificity of such antibodies is influenced by the position of the testosterone molecule used for conjugation to the carrier. It has been suggested that the stereochemistry of the steroid molecule is one of the important determining factors to obtain a specific antibody.[7,8] Hence, many positions on steroid molecule have been tried to fulfill the above criteria.[9,10] In this paper, we report a very specific antibody and a simple one-step extraction procedure for clinical assessment of testosterone by RIA using two antibodies for different sexes.

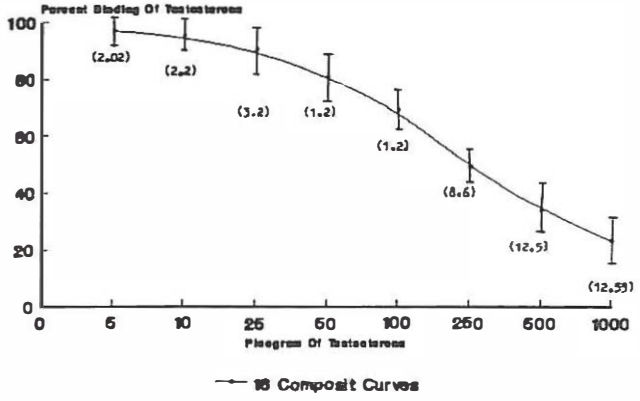

Fig. 3. Standard dose response curve using anti-testosterone-11B-CME-BSA*.

MATERIAL AND METHODS

N-Hydroxy succiniimide ester, dioxan, bovine serum albumin, 1-ethyl-3 (3-dimethylamino propyl) carbodiimide-HCl charcoal, dextran-T70, sodium dihydrogen phosphate, disodium hydrogen phosphate, testosterone, dihydrotestosterone, 2,5 diphenyl-oxazole (PPO), 1/4, bis-2-(4-methyl-1-5-phenoxyzolyl) benzene (POPOP), complete Fround's adjuvant (CFA), incomplete Fround's adjuvant, testosterone-3-O-CMO-BSA (T-3O-CMO), and all structurally related steroids used in this study were obtined from Sigma Chemical Company, St. Louis, USA. (1.2.6.7-3H testosterone) (3HT) was supplied by Amersham International PLC, Bucks, UK. All other reagents were of analytical grade and were purchased from E. Merk Chmical Co, Germany.

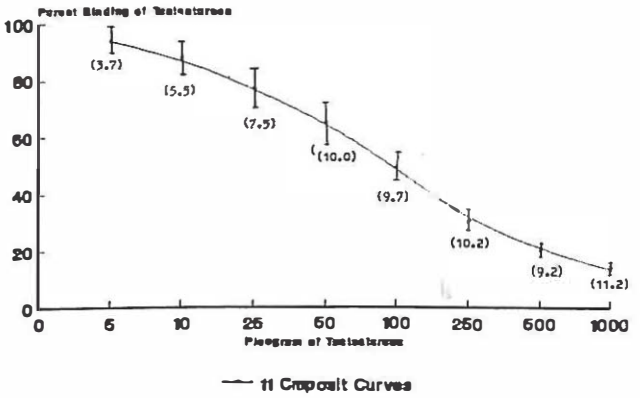

Fig. 4. Standard dose response curve of anti-testosterone-3-O-CMO-BSA*.

Prepration of Immunogen

Testosterone 11B-carboxymethyl ether (T-11B-CME)

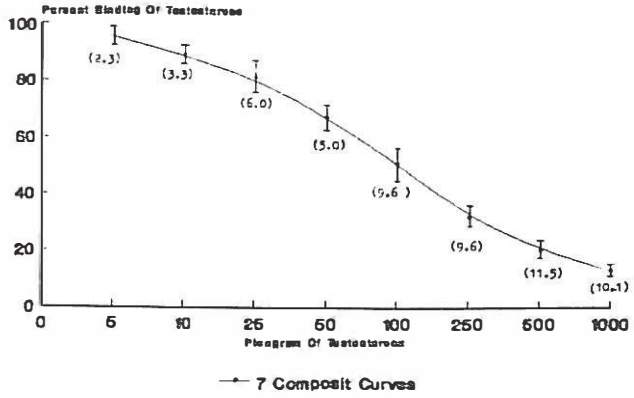

Fig. 5. Standard dose response curve of anti-testosterone-3-O-CMO-BSA*.

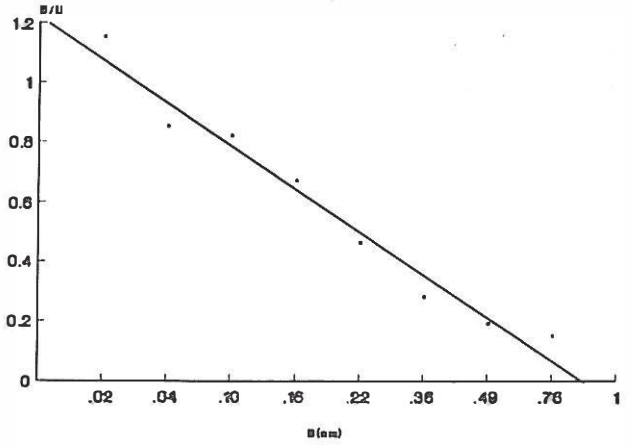

Fig. 6. Scatchard plot of anti-testosterone- 11B-CME-BSA*.

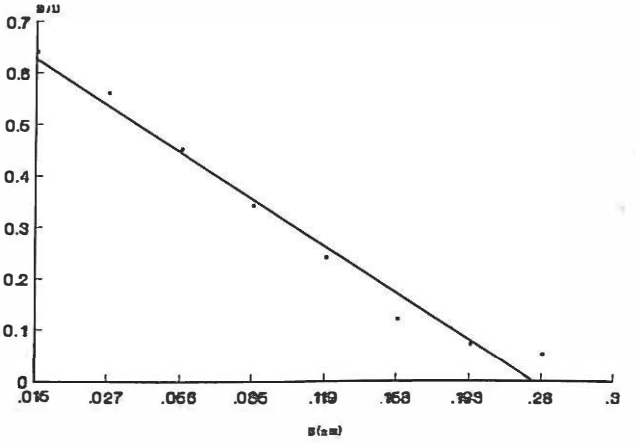

Fig. 7. Scatchard plot of anti-testosterone- 3-O-CMO-BSA*.

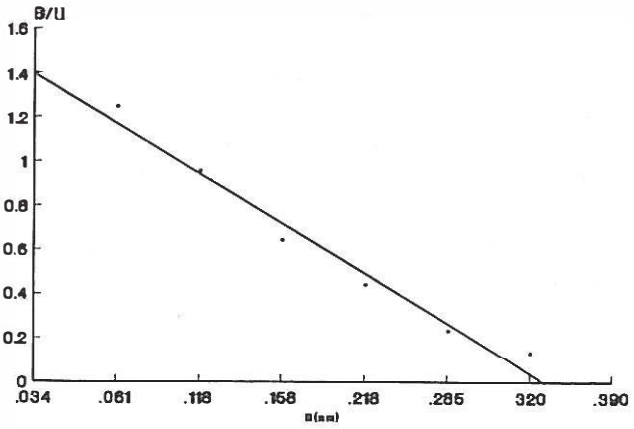

Fig. 8. Scatchard plot of anti-testosterone- 30-CMO-BSA*.

and T-3O-CMO were coupled to BSA following the procedure of Hosoda et al.[11] with a minor modification. Briefly, 18 mg of the steroid was solubilized in minimum amount of dioxan, 10 mg of N-hydroxy succiniimide ester and 13 mg of carbodiimide-HCl were added, the reaction mixture was left to stirslowly at room temperature for 2 hr; the activated ester was then added to a BSA Solution (63 mg/ml) prepared in phosphate buffer saline PBS (10 mmol, pH 7.0) containing 0.1% sodium azide. The reaction was left to stir over night at 4°C and dialyzed against one liter of PBS (10 mmol pH 7.2) containing 0.1 % sodium azide. The dialysate was freez-dried and the number of testosterone molecules conjugated to each protein molecule was determined photometrically at 250 nm.[12]

Antiserum

Three female New Zeland white rabbits of four months age were immunized following the procedure of Vitukoitis et al.[13] Briefly, 500 µg of canjugate was dissolved in 1 ml of saline and emulsified with 1 ml of (CFA). The resulting emulsion was injected intradermally on the back of the rabbits over40 places. This was followed by an intramuscular booster injection 30 days later (250 µg of immunogen emulsified in 1 ml saline and IFA v/v). This was repeated at 15 days intervals and the animal was bled from marginal ear vein seven days after each booster injection, and all bleedings were checked for titer, specificity, and sensitivity. Testosterone-3-O-CMO-BSA was injected following a similar procedure.

RIA Procedure

Titer: Radioimmunoassay was performed following the procedure explained by Abraham et al.[14] Briefly, serum separated from each bleeding was diluted in PBS (10 mmol/l, pH 7.2), containing 0.1% gelatin and 0.1% sodium azid (called RIA buffer hereafter) to obtain a 1/500 up to 1/16000 dilution of antibody. Tritiated testosterone

Table I. Cross reactivities of anti-testosterone antisera with the structurally related C18, C19, and C21 steroids

S.N.	Compound name	%cross reaction anti-T- 11B-CME	%cross reaction anti-T- 3-O-CMO	
		AN 1	AN2	An3
1	4-ANDROSTENE 3,17 DIONE	2.5	0.50	<0.05
2	4-ANDROSTENE 3,13 DIONE	1	<0.05	<0.05
3	ANDROSTERONE	<0.05	<0.05	<0.05
4	EPIANDROSTERONE	<0.05	<0.05	<0.05
5	PROGESTERONE	0.50	<0.05	<0.05
6	D-ALDOSTERONE	<0.05	<0.05	<0.05
7	CORTICOSTERONE	0.62	0.08	<0.1
8	ETHIOCHOLANE-3B OL-17 ONE	<0.05	<0.05	<0.05
9	ESTRIOL	<0.05	<0.05	<0.05
10	B-ESTRADIOL	<0.05	<0.05	<0.05
11	17-ETHENYL ESTRADIOL	<0.05	<0.05	<0.05
12	PREGNANTRIOL	<0.05	<0.05	<0.05
13	5B-PREGNAN 3,17 DIOL, 20 ONE	<0.05	<0.05	<0.05
14	ESTERONE	<0.05	<0.05	<0.05
15	CHOLESTERIN	<0.05	<0.05	<0.05
16	HYDROCORTISONE	0.30	<0.05	<0.05
17	DEHYDROISOANDROSTERONE	<0.05	<0.05	<0.05
18	DANAZOL	<0.05	<0.05	<0.05
19	DIHYDROTESTOSTERONE	2.30	32.00	36.00
20	TESTOSTERONE	100.00	100.00	100.00

Table II. Analytical recoveries of testosterone from charcoal treated plasma using anti-T-11B-CME-BSA and anti-3-0-CMO-BSA (AN 2)

concentration	testosterone added ng/100 µl	%recoveries* mean ± SE	CV%	%recoveries** mean ± SE	CV%
Ether blank	0	No Inter	------	---------	------
Serum blank	0	ND	------	---------	-------
Very low	0.2	102.7 ± 6.6	1.97	100.1 ± 0.71	2.02
Low	0.5	101.2 ± 1.0	3.32	97.67 ± 1.35	1.35
Medium	0.75	105.4 ± 1.4	5.28	97.23 ± 1.16	3.38
High	1.5	105.4 ± 1.2	3.34	97.14 ± 1.20	3.50

No Inter= No interference by ether used for extraction
ND= No detectable testosterone from serum stripped off endogenous steroids
*= Results of 8 experiments all in 6 replicates for anti-11B-CME-BSA
**= Results of 8 experiments all in 6 replicates for anti-3-O-CMO-BSA

was diluted in the same system in order to obtain 10,000 CPM/100 µl (called tracer solution hereafter). The tubes were set such that the first two tubes recieved 800 µl of RIA buffer and 100 µl of tracer (designated as total counts). The next two tubes recieved 600 µl of buffer and 1000 µl of tracer (named as non-specific binding, NSB). Other tubes recived 500 µl of RIA buffer, 100 µl of tracer, and 100 µl of antibody of different dilutions, in duplicates. The reaction mixture was vortexed and incubated at 4°C for 16 h, at the end of which 200 µl of dextran coated charcoal (prepared by adding 0.65 g Norit A and 0.0625 g dextran T-70 in 100 ml of RIA buffer) was added to every tube except tube no 1 and 2, and incubated for 10 min at 4°C. The tubes were then centrifuged at 3000 rpm for 10 min at 4°C and the supernatant was transferred immediately to a scintillation vial containing 5 ml of popop scintillation coctail (prepared by adding 7.5 g ppo and 0.25 g of dissolved in 2.5 liter of toluene): These were then counted inan LKB model 1410 liquidscintillation spectrometer.

Table III. Precision profiles of testosterone using anti-T-11B-CME-BSA

EXP NO	N	POOL A** MEAN ± SE	CV%	POOL B** MEAN ± SE	CV%	POOL C** MEAN ± SE	CV%	POOL D** MEAN ± SE	CV%
intraassay									
1	6	22.83 + 0.54	5.78	47.00 ± 0.96	5.02	78.50 ± 1.63	5.07	147.1 ± 2.64	1.67
2	6	23.16 ± 0.53	5.69	49.50 ± 0.22	1.09	75.83 ± 2.42	7.79	149.5 ± 2.64	4.50
3	6	21.20 ± 0.88	10.1	52.66 ± 0.92	4.27	78.30 ± 2.48	3.16	149.6 ± 2.38	3.80
4	6	20.83 ± 0.94	11.0	53.16 ± 1.74	8.01	72.00 ± 1.37	4.64	151.5 ± 1.50	2.71
5	6	20.16 ± 0.16	1.98	47.60 ± 1.77	9.09	73.00 ± 0.87	2.92	147.8 ± 1.64	2.71
6	6	19.66 ± 0.61	7.62	49.16 ± 3.00	15.50	75.00 ± 0.51	1.68	155.8 ± 1.45	2.27
interassay									
	6*	21.30 ± 0.57	6.60	49.84 ± 1.04	5.11	75.93 ± 1.87	2.79	150.8 ± 1.45	2.08

N= number of times same sample analyzed for intraassay variation
*= number of times same sample analyzed for interassay variation
CV= coefficient of variation
**= pg/100 µl of extract

Table IV. Precision profiles of testosterone using anti-T-3-O-CMO-BSA

EXP NO	N	POOL A** MEAN ± SE	CV%	POOL B** MEAN ± SE	CV%	POOL C** MEAN ± SE	CV%	POOL D** MEAN ± SE	CV%
intraassay									
1	6	16.4 ± 0.50	7.9	55.16 ± 1.0	4.6	79.60 ± 2.80	8.6	160.0 ± 3.9	6.06
2	6	21.0 ± 0.92	10.8	45.40 ± 2.0	0.11	71.20 ± 0.58	1.99	144.4 ± 2.10	4.10
3	6	21.0 ± 0.63	7.30	50.9 ± 0.96	4.64	70.90 ± 3.00	10.5	143.3 ± 2.90	4.48
4	6	20.0 ± 0.31	3.38	47.1 ± 1.49	7.77	67.50 ± 2.90	10.6	151.5 ± 1.5	2.71
5	6	19.7 ± 0.63	7.86	48.8 ± 1.21	6.12	73.00 ± 0.87	2.92	147.2 ± 4.80	8.49
6	6	20.0 ± 1.13	13.6	49.6 ± 1.20	5.90	64.20 ± 1.80	7.05	140.2 ± 4.80	8.49
interassay									
	6*	19.6 ± 0.69	8.60	49.50 ± 1.37	6.81	71.03 ± 2.15	7.40	147.9 ± 4.86	2.94

N= number of times same sample analyzed for intraassay variation
*= number of times same sample analyzed for interassay variation
CV= coefficient of variation
**= pg/100 µl of extract

Standard Curve

After selecting the optimum titer, the antibody dilution was prepared as before and a serially diluted standard testosterone (5 pg-1 ng, prepared from a stock ethanolic solution of testosterone in RIA buffer) was added to each of the duplicate tubes. The assay was terminated as explained before and the standard curve was constructed.

Cross Reaction

Number of structurally related steroids (C19, C21 and C18) were checked from a range of 25 pg to 100 ng following a similar procedure as explained before for testosterone measurement.

Assay Validity

Analytical recoveries: Normal human male and female serum were stripped off steroid content by charcoal treatment. A known amount of testosterone was then added in four ranges (very low, low, medium, and high) and recovered by a one-step ether extraction. Briefly, 100 µl or more of serum was diluted to 1 ml, with distilled water and extracted with 3 ml ether, 2 times. The ether layer was dried and reconstituted with 1 ml of RIA buffer and the assay performed as explained before using 100 µl of the reconstituted dry residue.

Inter and Intra-Assay Validation

Pool extracts were prepared by mixing the extracts of recovery step making four different concentrations (very

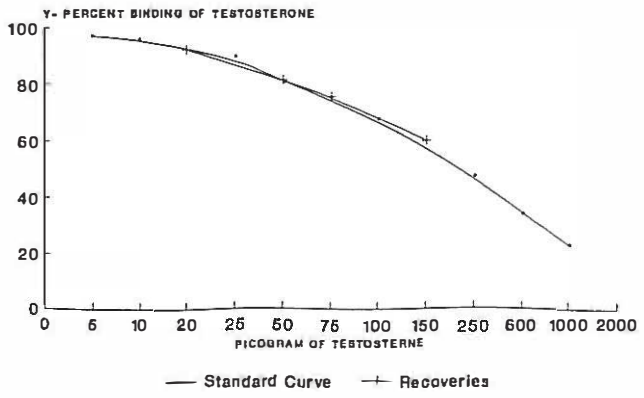

Fig. 9. Recoveries of testosterone using anti-T-11B-CME-BSA.

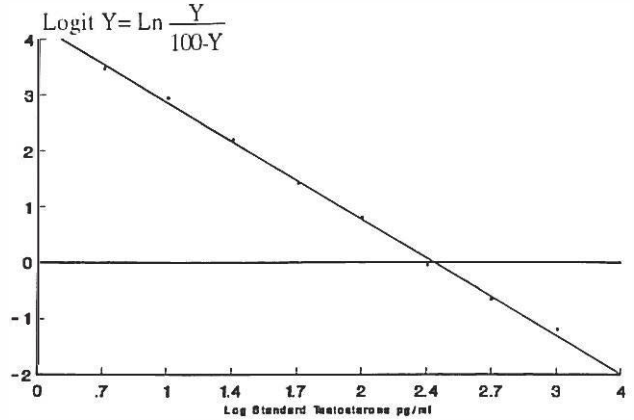

Fig. 10. Logit-log transformation of the dose response curve for anti-T-11B-CME-BSA*.

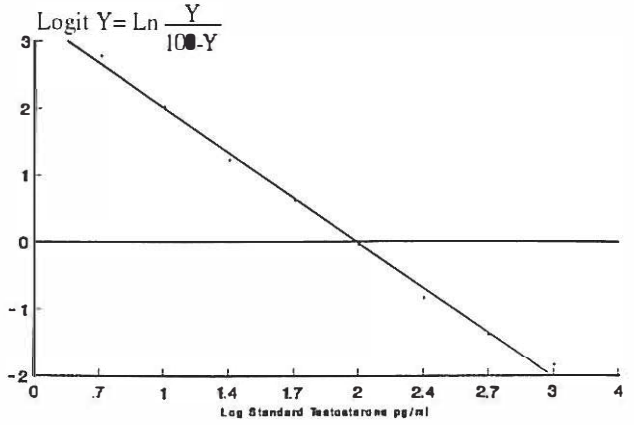

Fig. 11. Logit-log transformation of the dose response curve for anti-T-O-CMO-BSA*.

low, low, medium, and high) and six successive assays of each pool in six replicates were performed.

Test of Parallelism

A normal serum sample was extracted as explained before and diluted serially so that six different dilutions were obtained. These were then checked with a standard curve using both antibodies.

Normal and Pathological Sample

Testosterone content of 55 normal male and female samples were measured following this procedure using anti T-11-CME-BSA for male samples and anti-T-3-O-CMO-BSA for female samples. A total of 50 pathological samples were also measured for testosterone content following similar strategy.

RESULTS

The number of steroid molecules conjugated to each BSA malecule was calculated to be 14.5 for T-11B-CME and 20 for T-3O-CMO. Figure 1 shows the immunization schedule of the two immunogen used to raise antibody. Normally the optimum titer was obtained after 4 to 6 months of immunization. Figure 2 denotes the optimum titer of antibodies prepared in this study. This was selected as 50% binding of tracer to antibody and calculated to be 1/2200 for animal numberone (AN1), 1/3200 for AN2 and 1/1400 for AN3. Results of composite curve for 18, 11, and 7 successive RIAs to construct the respective standard curves for anti-T-11B-CME (AN1), anti-T-3O-CMO (AN2) and anti-T-3O-CMO (AN3) are shown in Figures 3, 4 and 5. The values in parentheses indicate the respective percent coefficient of variation (CV) while bars drown show the standard deviation (SD). Figures 6,7 and 8 show the affinity (equilibrium constant, Ka) of the anti-T-11B-CME-BSA and anti-T-3O-CMO-BSA (AN 2 and 3). The Ka values obtained for the three antibodies are 1.13×10 l/mol, 2.3×10 l/mol, and 4.24×10 l/mol, respectively. These values are well in the range of high affinity antibodies. Percent cross reaction of antibodies with the related steroids are shown in Table I. These results indicate that the antibody raised against T-11B-CME is highly specific, while antibody raised against T-3-O-CMO had 30-32% cross reaction with 5 DHT. However, the percent cross reactivity of androstenedione increased in the case of anti-T-11B-CME. Table II represents the results obtained on recovering known amount of testosterone added to stripped serum. Percent recoveries are between 97-105% using both antibodies and the CVs calculated were less then 5%. Precision profiles of RIA measurement are shown in Tables III and IV. Results of 6 experiments in which each pool was measured in 6 replicates are shown here. Within and between assay CVs are calculated to be in the acceptable ranges. Logit log transformation of the standard curve (linearization of the hyperbolic curve) is shown in Figures 10,11 and 12. These results show the slope of the standard curve to be -1.46, -2.4, and -1.41 for antibody raised against T-11B-CME (AN1)

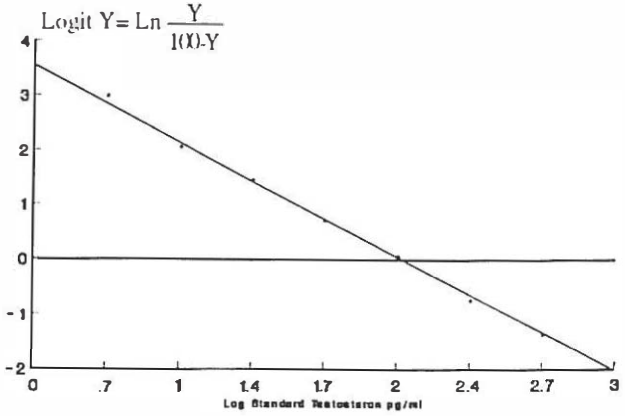

Fig. 12. Logit-log transformation of the dose response curve for anti-T-3-O-CMO-BSA*.

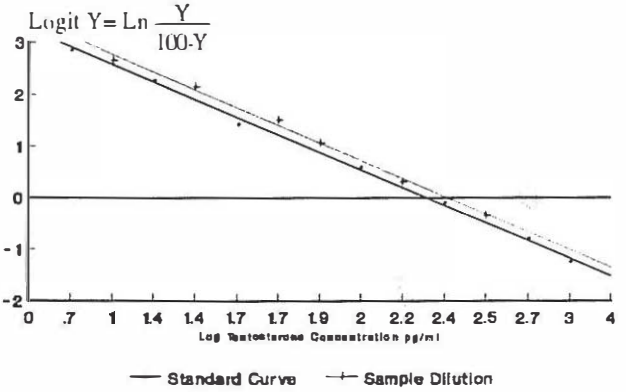

Fig. 13. Logit-log transformation of standard curve and sample dilution using anti-T-11B-CME-BSA (test of parallelism).

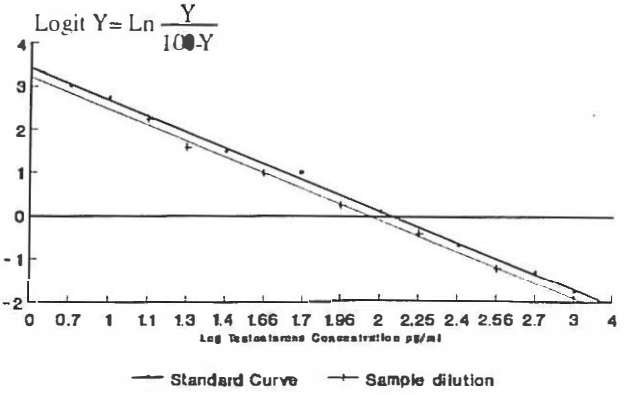

Fig. 14. Logit-log transformation of standard curve and sample dilution anti-T-3-O-CMO-BSA (test of parallelism).

and T-3O-CMO (AN2&3), respectively. Figures 13 and 14 indicate the parallelism between the dose-response and sample dilution curve for both the antibodies used finally in this study. The slope of standard curve and sample dilutions are calculated to be -1.61 and -1.8 using anti-T-11B-CME and -2.2 using anti-3O-CMO (AN2), respectively. As can be seen the two slopes in each case are in a good agreement with each other. Finally, in Table V are presented data obtained on determining testosterone in normal and pathological samples. The normal level of testosterone measured by this method was between 0.2 to 0.9 ng/ml for normal female samples using anti-T-3O-CMO-BSA and 4-9.5 ng/ml for normal male using T-11B-CME-BSA. The results obtained for testosterone content in prediagnosed samples are also shown in this table. The correlation coefficient (r) is calculated as shown in Figure 15. The values obtained (r = 0.96) is indicative of high correlation between the values obtained using present method and those using kits imported from abroad.

DISCUSSION

RIA of testosterone has been described way back in 1974. Since then a large numberof assay systems have been reported. Various kit preparations are available for use in this country. Most hospitals and clinics preferably use ready made preparations imported from abroad. We have made an attempt to develop a very simple and equally efficient and highly specific assay system. The antibodies used in this study are highly specific, therefore chromatographic separation was excluded. The results obtained in this study using a C11 derivative of testosterone agrees with the general rule that keeping the antigenic determinants of a molecule intact is essential for obtaining a better antibody. In this way since T-3O-CMO-BSA is derivatized at C3 position and a bulky group such as BSA molecule hinders the space around ring A in the steroid molecule hence the antibody obtained showed cross reaction with 5 DHT. However, choosing a carbon atom at the center of molecule (i.e., C11) that too with a B orientation will avoid any sort of hindrance caused by bulky molecule (i.e., BSA). Similarly using an ether linkage which is a less bulky group will improve the antibody quality. However, as the case in our preparation, use of a C11 position has an adverse effect on D ring of steroid molecule and 2% cross reaction with androstenedione is observed which can limit the use of antibody. especially in the female samples obtained during pregnancy or other reason causing androstenedione excess. Our attempt to measure testosterone directly in serum failed, since binding protein interference does not allow for this strategy. We have also tried two of the known blockers such as danazol and estradiol in order to block the binding protein inteference (unpublished data). These do not show any cross reaction with the antisera but were not able to displace testosterone from SHBG even at a high concentration (i.e., 400 ng/vial). On the other hand, many authors have expressed their concern over direct assays of a fast binding steroid such as

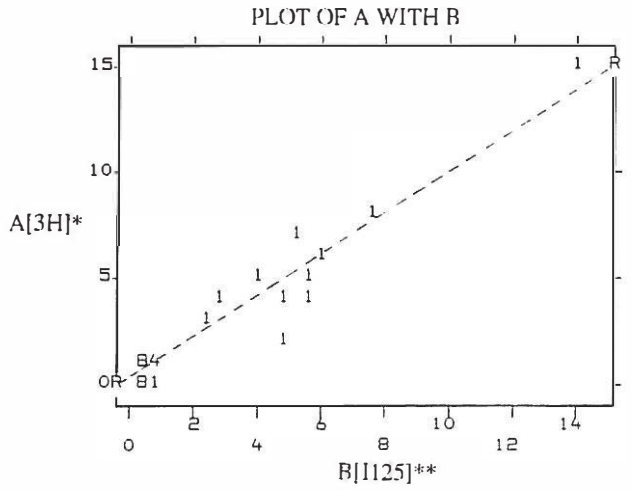

Fig. 15. Correlation coefficient of testosterone measurement following our procedure * and kits imported from abroad** The results are obtained using SPSS program.

32 caes plotted. Regression statistics of A on B:
Correlation .96391 R Squared S.E. of Est .86177 Sig. .0000
Intercept (S.E.) .02793 (.19108) Slope (S.E.) .98475 (.04966)

testosterone. Therefore, we preferred to include the extraction step to be on the safe side. The use of a beta-emitting tracer will also improve the assay performance and the cost effectiveness in such a way that one can prepare the tracer solution from a stock preparation of 3H testosterone and the stock tracer may be stored years together without any loss of activity. The antibody used is raised in rabbits and is of polyclonal nature. This could be produced in large quantities locally. The diluted antibody is stable at 4°C for at least 60 days. These reagents when kept at -20°C in concentrated form are satble for more than 2 years without any loss of activities. All other reagents and buffer systems used in this study are also stable at 4°C for more than a year. The assay can be accurately carried out for a large number of samples (i.e., 200 or more) in one working day. Since we have fully validated the assay we can porpose that the present method may replace the ready made kits imported from abroad. This can further clear the way for preparing RIA kits for determination of other sex hormones.

REFERENCES

1. Yallow RS, Berson SA: Assay of plasma insulin in human subjects by immunological method. Nature(London) 184: 1648-9, 1959.
2. Browine AC, Van der Molen HJ, Nishizawa EE, Eik-Nes KB: Determination of testosterone in human peripheral blood using gas liquid chromatography with electron capture detection. J Clin Endocrinol Metab 24 1091-102, 1964.
3. Collins PW, Sisterto JM, Kaulapis EN, Mansfield MD, Sommerville IF: The evaluation of a gas liquid chromatographic method for determination of plasma testosterone using a nickel-63 electrone capture detedtion. J Chromat 37: 33-45, 1968.
4. Satyawaroop PG, Lopezde Laosa E, Gourpide E: High pressure liquid chromatographic separation of C18 and C19 steroids. Steroids 30: 139-45, 1977.
5. Darny KJ Jr, Wirg TY, Ewirg LL: Simultaneous measurement of fourtesticular4-3 ketosteroids by isocratic high performance liquid chromatography with online ultraviolet absorbance detection. J Chromat 257: 81-90, 1983.
6. Furuyama S, Mayes DM, Nugent CA: A radioimmunoassay for plasma testosterone. Steroids 16: 415-28, 1970.
7. Grove PK, Odell ED: Specificities of antisera to sex steroid. The effect of substitution and stereochemistry. J Steroid Biochem 8: 121-6, 1977.
8. Exley D, Woodhead B: The specificity of antisera raised by estradiol-17B-3-hemisuccinyl-bovineserum albumin. Steroids 27: 813-20, 1976.
9. Hosoda H, Tadano K, Miyairi S, Nambara T: Preparation and antigenic properties of testosterone-4-BSA conjugates. J Steroid Biochem 10: 513-7, 1979.
10. Rao PN, Moor PH Jr, Peterson DM, Tcholakion RK: synthesis of new steroid haptens for radioimmunoassay. Part V, 19-O-corboxymethyl ether derivative of testosterone. A highly specific antiserum for immunoassay of testosterone for both male and female plasma without chromatography J Steroid Biochem 9: 539-45, 1978.
11. HosodaH, Sakai Y, Yshida H, Miyairi S., Ishii K, Nambara T: The preparation of steroid N-hydroxysucciniimide ester and their reactivities with BSA. Chem Pharma Bull 27: 742-6, 1979.
12. Erlanger BF, Borek F, Beiser SM, Liebermans S: Steroid-protein conjugates I. Prepration and characterization of conjugates of bovine serum albumin with testosterone and with cortisol. J Biol Chem 228: 713-27, 1957.
13. Vaitukaitis JL, Robbins JB, Nieschlag E, Ross GT: A method for producing specific antisera with small doses of immunogen. J Clin Endocorinol Metab 33: 988-91, 1971.
14. Abraham GE: Radioimmunoassay of steroids in biological materials. Acta Endocrinol (Copenhagen) 183: 1-42, 1974.