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RADIOIMMUNOASSAY OF PLASMA TESTOSTERONE

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SUMMARY

A simple method for the determination of testosterone in human peripheral venous plasma is described. Various factors affecting the method blank, accuracy, sensitivity, specificity and precision are investigated. A compromise procedure is evaluated in terms of theoretical and practical errors. The method involves addition of a labelled internal standard, extraction with diethyl ether and chromatography on Sephadex LH 20. Standards and unknowns are equilibrated with antiserum to testosterone-3-oxime-bovine serum albumin and the unbound steroid removed with dextran-coated charcoal. The mean total random theoretical percentage error is calculated to be 13 per cent, and the coefficients of variation on replicate analyses of male and female plasma ranged from 8–16 per cent. The concentration (mean ± S.D.) of testosterone in plasma from a group of healthy men (aged 20–40 yr) was 573 ± 191 ng/100 ml and in samples collected from healthy women during the menstrual cycle 53 ± 10 ng/100 ml. These values are compared with those obtained by omitting the chromatographic step, by using an antiserum to testosterone-17β-hemisuccinate-bovine serum albumin, and by using a gas–liquid chromatographic procedure with Nickel-63 electron capture detection.

INTRODUCTION

RECENTLY, methods based upon the principles of radioimmunoassay have been applied to the determination of steroid hormones. Thus, the production and properties of antisera to testosterone–protein complexes have been reported by Midgley and Niswender[1]. In addition, a method involving the use of antiserum to testosterone-3-oxime-bovine serum albumin (BSA) has been described in detail for the determination of testosterone in peripheral venous plasma from healthy men and women[2]. In this procedure, small volumes of plasma were extracted with hexane : ether (8 : 2 v/v) and the extracts purified on micro columns of alumina. After equilibration of eluates and standards with antiserum, those steroids bound to antibody were separated by precipitation with ammonium sulphate.

The present report will describe and evaluate a similar procedure for the determination of plasma testosterone using antisera to testosterone-3-oxime-BSA. Diethyl ether has been used to extract the steroids from plasma, the effect of chromatography on Sephadex LH 20 has been examined and the optimal conditions for equilibration with antiserum investigated. Dextran-coated charcoal was used to remove steroids not bound to antibody. Specificity has been checked by analysing the same plasma samples with an antiserum to testosterone-17β-hemisuccinate-BSA and by a gas–liquid chromatographic procedure using $Nickel_{63}$ electron capture detection[3].

EXPERIMENTAL

Solvents and reagents

Benzene, diethyl ether (peroxide free), methanol and acetone were all Analar grade and used without further purification. Water was glass distilled and deionized before use. Azobenzene was obtained from B.D.H. Chemicals Ltd.,

Poole, Dorset, England. Sephadex LH 20 was obtained from Pharmacia, Uppsala, Sweden.

Buffers

Tris-buffer was prepared by dissolving 12·1 g of Tris-(hydroxymethyl)-methylamine in 1 l of deionized distilled water. The pH was adjusted to 8·5 by addition of concentrated hydrochloric acid.

Solutions containing 0·1 and 0·5 per cent gelatine in buffer were prepared by dissolving gelatine in the buffer at 50°C and then cooling. All buffer solutions were stored at 4°C.

Standards

Authentic neutral steroids were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. and recrystallised in appropriate solvents before use. Testosterone glucuroniside and epitestosterone were donated from the MRC Steroid Reference Collection, Westfield College, London, N.W.3. All steroids were serially diluted in acetone to a concentration of 1 pg/μl.

[1, 2, 6, 7-^{3}H]testosterone, S.A. 100 Ci/mmol, was obtained from the Radiochemical Centre, Amersham, Bucks., England. Solutions were prepared containing 10 μCi/ml in benzene/ethanol (95–5 v/v). Subsequently, 100 μl were removed, thoroughly dried and redissolved in 10 ml of Tris-buffer containing 0·1 per cent gelatine. One hundred μl containing approx. 20,000 d.p.m. is used in the assay system. An internal standard to correct for experimental losses is prepared by diluting 100 μl of original solution in 10 ml of acetone such that 10 μl contains 2000 d.p.m. and 2·5 pg of testosterone.

Antiserum

Testosterone-3-oxime-BSA and testosterone-17β-hemisuccinate-BSA were prepared according to the methods of Erlanger *et al.*[4]. Both contained 27 mol of steroid per mol of protein. For injection 2 mg of antigen were dissolved in 0·5 ml of saline, emulsified with an equal volume of Freunds complete adjuvant and injected subcutaneously into a rabbit at multiple sites. This procedure was repeated once a week for a further 3 weeks, then once a fortnight for the following month and subsequently once a month. From two rabbits so treated with each antigen an antiserum was developed that could be used at a dilution of 1:5,000, 10 weeks after the first injection. The antisera were found suitable for use without purification. Both antisera are stored in and diluted prior to use with Tris-buffer containing 0·1 per cent gelatine.

Dextran-coated charcoal

Norit A charcoal obtained from the Sigma Chemical Co. was washed with deionized distilled water until all fine particles had been removed and was then thoroughly dried. Dextran T 70 from Pharmacia was dissolved in deionized distilled water to a concentration of 100 mg/ml 1·25 g of Norit A charcoal and 1·25 ml of the dextran T 70 solution were then added to 500 ml of 0·1 M Tris-buffer. The solution was stored at 4°C. During use the charcoal was maintained in suspension by a magnetic stirrer.

Radioactivity measurement

All samples were added to disposable glass vials containing 12 ml of scintillant, prepared by dissolving 3·0 g of 2,5-diphenyl-oxazole (PPO) in 1 l of toluene and adding 500 ml of Triton X-100 (Koch-Light Laboratories Ltd.). An automatic liquid scintillation counting system—Nuclear Chicago Model 6860 (Mark I)—was used. Each sample was stabilised for a minimum of 75 min at 2°C and then counted for a total of 10,000 counts. The counting efficiencies were determined for each sample from calibration curves for an external standard channels ratio method using a ^{133}Ba source.

METHOD

Peripheral venous blood is withdrawn by a disposable syringe, transferred to a lithium heparin tube, and centrifuged. The plasma is removed and processed immediately or stored at −15°C. A flow sheet of the method is shown in Fig. 1. This is an adaptation and slight modification of the procedure developed by Hotchkiss *et al.*[5] for the determination of plasma oestrogens.

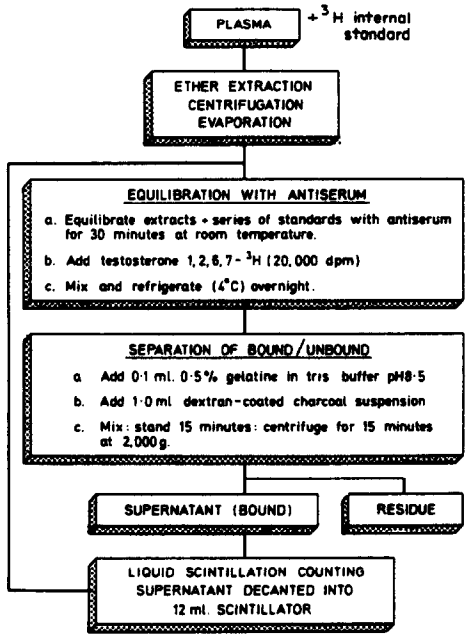

Fig. 1. Flow sheet of method for the determination of plasma testosterone.

The internal standard of tritiated testosterone is added to each plasma sample and to two vials for liquid scintillation counting. The plasma volumes required for each antiserum and the aliquots subsequently taken for assay are shown in Table 1. The plasma is extracted twice with 15 ml of diethyl ether using a vortex mixer. The ether layers are removed with a Pasteur pipette and the pooled extracts dried at 40°C under nitrogen. The extracts are either dried in a pointed test tube or in a counting vial, depending on whether or not a chromatographic step is to be included.

Table 1. Volumes of plasma and aliquots removed for radioimmunoassay

Antigen	Men		Women	
	Volume of plasma (ml)	Aliquot for RIA (%)	Volume of plasma (ml)	Aliquot for RIA(%)
T-17-Hemisuccinate-BSA	2·0	10	10·0	20
T-3-Oxime-BSA	0·2	10	2·0	10

Column chromatography

The dried extract is dissolved in a few drops of benzene: methanol (95:5 v/v), coloured yellow with a small amount of azobenzene. The extract is transferred to a column of Sephadex LH 20, prepared in the benzene-methanol mixture and occupying 80 per cent of a disposable 9-in. Pasteur pipette (Harshaw Chemicals Ltd., P.O. Box 4, Daventry, Northants, England). Elution is performed with the same solvent mixture and a 2 ml fraction immediately following the azobenzene is collected in a counting vial. The eluate is dried under nitrogen at 40°C and redissolved in 1·0 ml of acetone. Appropriate aliquots according to Table 1 are removed for assay. The remainder is subjected to liquid scintillation counting to determine the recovery.

Assay procedure

The assay is performed in disposable glass tubes (10 × 75 mm). A series of standards (10, 20, 50, 100 and 150 pg using the antiserum to testosterone-3-BSA and 250, 500, 750, 1000, 1500 and 3000 pg using the antiserum to testosterone-17-BSA) are prepared in triplicate and dried under nitrogen in a dry block heater at 40°C. The aliquots from plasma extracts are prepared in duplicate and dried in a similar manner. One hundred μl of antiserum, at a dilution such that in the absence of unlabelled steroid 50–60 per cent binding is obtained (from 1:1,000 to 1 in 5,000), is added to each tube. The contents of each tube are mixed on a vortex mixer and left to equilibrate for 30 min at room temperature. Then 20,000 d.p.m. of tritiated testosterone (100 μl) is added. The solutions are mixed on a vortex mixer and incubated from 0·5–24 h depending on the sensitivity required. In addition, two tubes designated 00 and 0 are prepared in triplicate with the standard curve. The 00 tubes contain antiserum [^{3}H]testosterone and 1 ml of Tris-buffer; the 0 tubes antiserum [^{3}H]testosterone and 1 ml dextran-coated charcoal. These combinations enable the percentage of labelled steroid bound to antibody and its subsequent displacement by non-labelled steroid to be determined. The antiserum and [^{3}H]testosterone are added with a Biopette (Schwarz Bio Research, Orangeburg, New York 10962, U.S.A.) which has a disposable nylon tip.

Separation of antibody-bound and free steroids

The separation is performed at 4°C. One hundred μl of Tris-buffer containing 0·5 per cent gelatine is added to each tube with a Biopette. Then 1 ml of dextran-coated charcoal (maintained in suspension by a magnetic stirrer) is added with an automatic pipetting syringe (F. Froud and Sons, 126 Dalston Lane, London, E.8, England) to all tubes except the 00. The contents of each tube are mixed on a vortex mixer, allowed to stand for 15 min and centrifuged (4°C, 2,000 g, 15 min). The dextran-coated charcoal adheres to the bottom of the tube and the

supernatant containing the bound fraction is decanted into a counting vial containing 12 ml of scintillation fluid.

Factors affecting binding and displacement

The optimum dilution of each antiserum is checked at regular intervals, and a typical effect of dilution on the percentage binding and displacement of antiserum to testosterone-3-BSA is shown in Fig. 2. In this instance, the largest number of counts together with the maximum displacement of [^{3}H]testosterone occurs with a dilution of 1:1,250. The effect of pH on percentage binding and displacement is shown in Fig. 3. The optimum pH of this antiserum in Tris-buffer would appear to be around 8·5. The effect of temperature on the same system is shown in Table 2. The results indicate that at 4°C the initial binding and subsequent displacement are greater than at 25°C. Finally, the effect of incubation time is shown in Table 3. The longer the time, the better the initial binding and greater the overall displacement but periods as short as 30 min may be used with some decrease in resolution at lower levels.

Standard curves

Standard curves for testosterone using the antiserum to testosterone-3-BSA are shown in Figs. 4 and 5. These cover the range 10–150 pg of testosterone and are plotted as percentage of tritiated testosterone bound and as d.p.m. The curves may also be plotted on logit paper which has the advantage that a computer may be used to fit the best line (Fig. 6).

A standard curve using the antiserum to testosterone-17-BSA and covering the range 250–3,000 pg of testosterone is shown in Fig. 7. The amount of tritiated testosterone bound to antibody, diluted 1:1,000, is expressed as d.p.m.

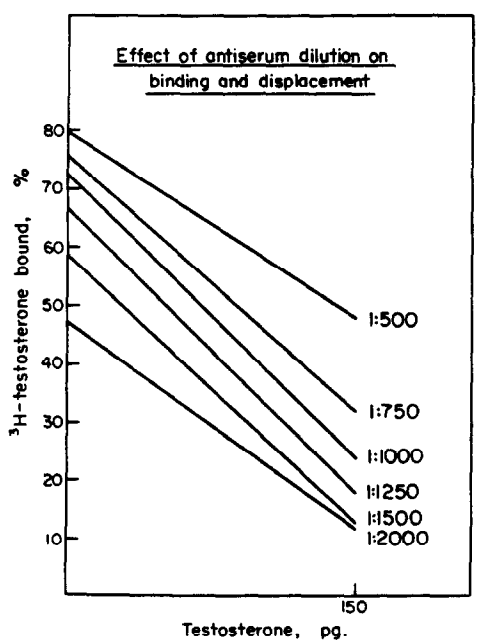

Fig. 2. The effect of progressive dilution of antiserum on the percentage of [^{3}H]-testosterone bound and the overall displacement by 150 pg of testosterone.

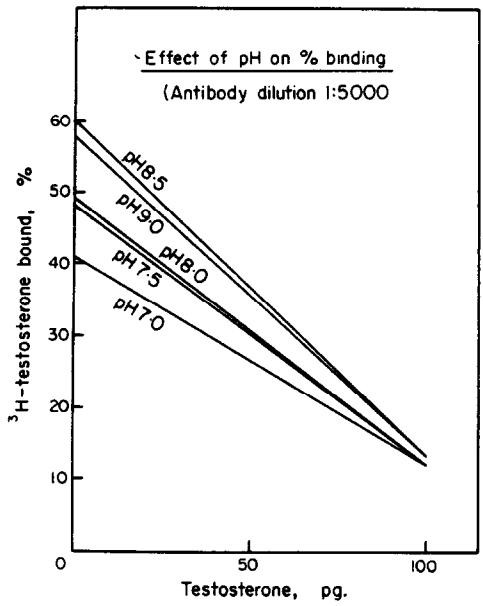

Fig. 3. The effect of pH on the percentage binding of [^{3}H]-testosterone and the overall displacement by 150 pg of testosterone.

Table 2. The effect of temperature on the percentage binding and displacement of [^{3}H]testosterone

Time (hr)	%[^{3}H]testosterone bound		Displacement of [^{3}H]T by 150 pg T (dpm).	
	4°C	25°C	4°C	25°C
0·5	39	39	6595	5903
1·0	50	40	8395	5927
2·0	53	43	9361	6287

Table 3. The effect of time on the percentage binding and displacement of [^{3}H]testosterone at 4°C

Time (hr)	%[^{3}H]testosterone bound	Displacement of [^{3}H]T by 150 pg T (dpm)
24	62	10,000
2	50	8,500
1	41	7,250
0·5	37	6,250

As the steroid is linked to the antigen at carbon 17, testosterone glucuronoside readily displaces [^{3}H]testosterone, and there is also some cross reaction with epitestosterone (17α-hydroxy-4-androsten-3-one).

Calculation of results

A desk-top computer, the Olivetti Programma 101, is used to calculate the

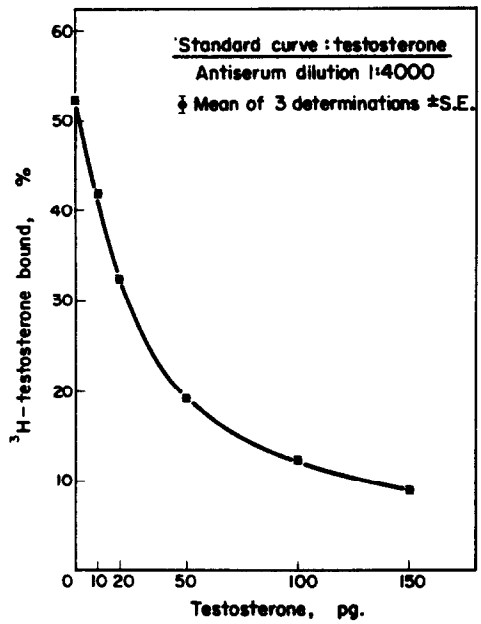

Fig. 4. Standard curve for testosterone using antiserum to testosterone-3-BSA.

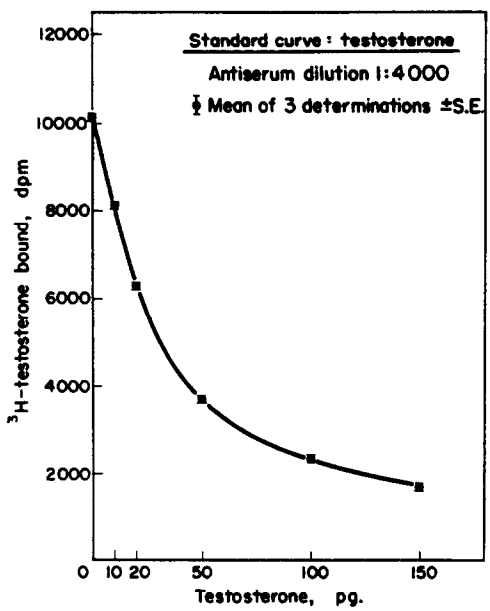

Fig. 5. Standard curve for testosterone, with the amount of [^{3}H]-testosterone bound plotted as d.p.m.

result (S, expressed as pg/ml plasma) according to the following formula:

$$S = \left[\alpha T_x \cdot \frac{C_s}{C_x} \cdot \frac{E_x}{\beta E_s} - M\right] \frac{1}{V} \tag{1}$$

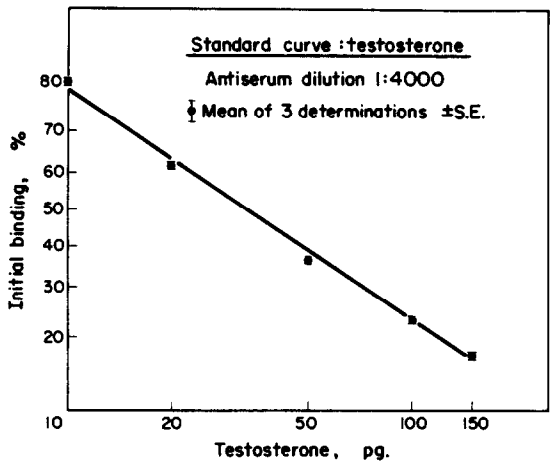

Fig. 6. Standard curve for testosterone plotted on logit paper.

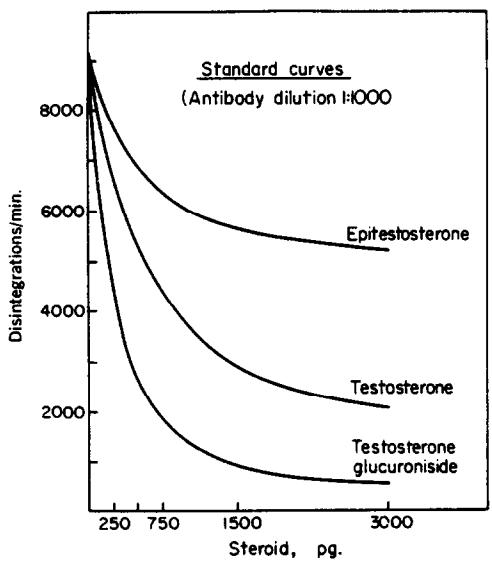

Fig. 7. Standard curves for testosterone and related steroids using an antiserum to testosterone-17-BSA.

where α is the aliquot taken for radioimmunoassay and T_x the reading from the standard curve; C_s is the c.p.m. in the standard and C_x the c.p.m. in an aliquot β of the unknown; E_x and E_s are the counting efficiencies of the unknown and standard and M the mass of internal standard. V is the volume in ml of plasma.

Theoretical assessment of error

In an attempt to determine the random error on individual samples, the random errors on each term in equation (1) were assessed. The overall percentage error on S (e_s = standard deviation $\times$ 100/S) was then determined from equation (2) below, derived from the usual laws for the combination of additive and multiplicative independent normal errors.

$$e_S^2 = \left[\frac{(eT_x^2 + eC_s^2 + eC_x^2 + eE_s^2 + eE_x^2)(\Delta^2) + M^2 \cdot eM^2}{\gamma^2}\right] + eV^2 \qquad (2)$$

where

$$\Delta = \alpha T_x \cdot \frac{C_s}{C_x} \cdot \frac{E_x}{\beta E_s}$$

$$\gamma = \Delta - M$$

and eT_x^2 = the overall percentage error on T_x, and similarly for the other terms.

As α and β are constant, they do not have any error, but contribute to the overall error in S because of the form of equation (2). The maximum error on the other terms in equation (1) were evaluated as follows:

C_s, C_x, E_s and E_x. There are two percentage errors in the term C_s, one from pipetting the standard into a counting vial, and the other from liquid scintillation counting. The pipetting error using a Biopette was found to be 2·0 per cent, and as 10,000 counts were recorded, the counting error was about 1·0 per cent assuming Poisson statistics. The two errors were then combined to give:

$$e = (e_1^2 = e_2^2)^{1/2} = (2{\cdot}0^2 + 1{\cdot}0^2)^{1/2} = 2{\cdot}2\%.$$

There is no pipetting error for C_x as the eluate from the chromatography column is collected in a counting vial. All samples were counted for 10,000 counts so the error is approximately 1·0 per cent.

The error in the counting efficiency E_s and E_x is dependent upon the method used for evaluating the degree of quenching in a sample. This error was estimated to be 3 per cent using an external standard channels ratio method.

M and V. The amount of labelled standard M added to the samples is determined from the liquid scintillation of an aliquot and the specific acitivty (d.p.m./pg) quoted by the Radiochemical Centre. As the error in the activity is 3 per cent and that on the specific activity 1 per cent, these combined with a 2 per cent pipetting error give an overall error of 3·7 per cent. The error on V was taken as a maximum of 2 per cent.

T_x. The overall error on T_x contains the errors involved in calculating d.p.m. in the aliquots removed for assay and in the subsequent preparation and reading from the standard curve. As each sample was counted for 10,000 counts and corrected for efficiency, the overall error on calculating d.p.m. in each aliquot was 3·2 per cent. The error in reading from standard curves depends upon the accuracy of preparing the curves and the position from which the reading is taken. When a standard curve based on the mean of triplicate determinations is used for the calculation of T_x, the overall error e_s on the determination of testosterone in both male and female plasma is approx. 5·1 per cent. On the other hand, due to the potential variation in standard curves prepared in triplicate at the same time (Fig. 8), the maximum error at these concentrations could be as high as 12·9 per cent.

Sensitivity

The sensitivity of the method has been assessed by relating the endogenous level of testosterone to the total random theoretical percentage error associated

with its measurement in individual samples according to equation (2). The mean number of d.p.m. from the aliquots removed for assay from each plasma extract was used to obtain the maximum and minimum readings from the standard curve (Fig. 8), and hence the percentage error on T_x. The mean value for e_S over the range of concentrations found in plasma from healthy men and women was 12·5 per cent. Using the plasma volumes and aliquots for assay as shown in Table 1 it is possible to measure as little as 5 ng/100 ml of testosterone in female plasma and 100 ng/100 ml in male plasma before the total error starts to rise.

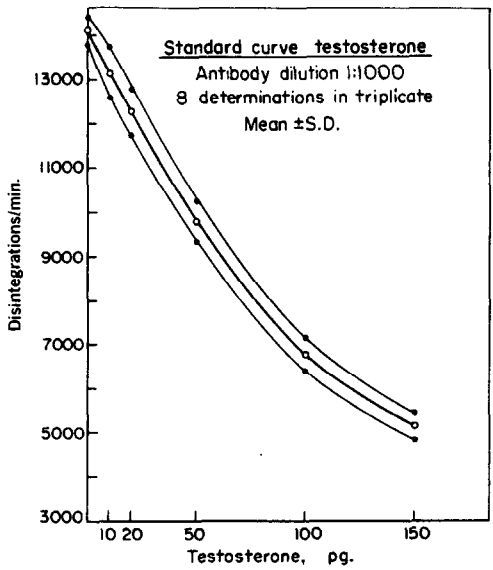

Fig. 8. The mean ± S.D. of 8 standard curves prepared in triplicate at the same time.

Practical errors

The practical, systematic errors were determined from replicate analyses of male and female plasma and by recovery experiments of known amounts of authentic testosterone added to deionized, distilled water.

Accuracy

Authentic testosterone in amounts similar to the mean values found in male and female plasma were added to deionized, distilled water and processed. The mean recoveries ± S.D. are shown in Table 4. The accuracy from water is invariably good and the coefficient of variation lower than in replicate analysis of plasma samples.

Table 4. Replicate analyses of authentic testosterone added to deionised, distilled water

Amount added	Amount calculated ng/100 ml	Coefficient of variation (%)
1250 pg/0·2 ml	690 ± 40	5·8
800 pg/2·0 ml	40 ± 2·4	6·0

Precision

The precision of the method as applied to plasma from both men and women was assessed from multiple determinations on plasma from male and female blood donors. The results are presented in Table 5. It may be seen that the coefficient of variation at these levels is between 8 and 16 per cent.

Table 5. Replicate analyses of plasma testosterone from male and female donors

	No. of determinations	ng/100 ml mean ± S.D.	Coefficient of variation %
Male plasma	8	467 ± 37	7·8
Female plasma (1)	8	34 ± 3·9	11·6
Female plasma (2)	10	28 ± 4·5	16·1

Specificity

The specificity of the method using antiserum to testosterone-3-BSA has been investigated by analysing the same plasma samples using the antiserum to testosterone-17-BSA and by gas–liquid chromatography with nickel$_{63}$ electron capture detection.

The antiserum to testosterone-17-BSA cross-reacts to almost the same extent with progesterone and androstenedione and to a lesser degree with 17α-hydroxyprogesterone. The cross-reaction with other C_{19} steroids is shown in Fig. 9.

The ability of 21 neutral steroids and oestradiol to displace [^{3}H]testosterone bound to the antiserum to testosterone-3-BSA has been investigated. Only those steroids with an hydroxyl group in the β position at carbon 17 reacted to any significant degree, as is illustrated in Fig. 10. Accordingly, the different characteristics of these two antisera have been used to check the efficacy of the chromatographic step and the overall specificity of the method. This involved analysing

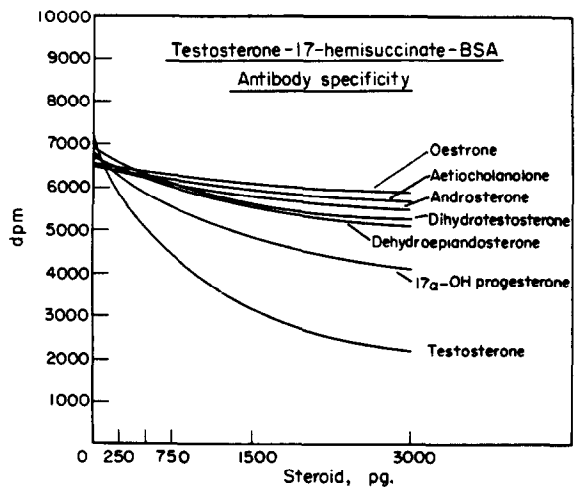

Fig. 9. Standard curves for various steroids related to testosterone using an antiserum to testosterone-17-BSA.

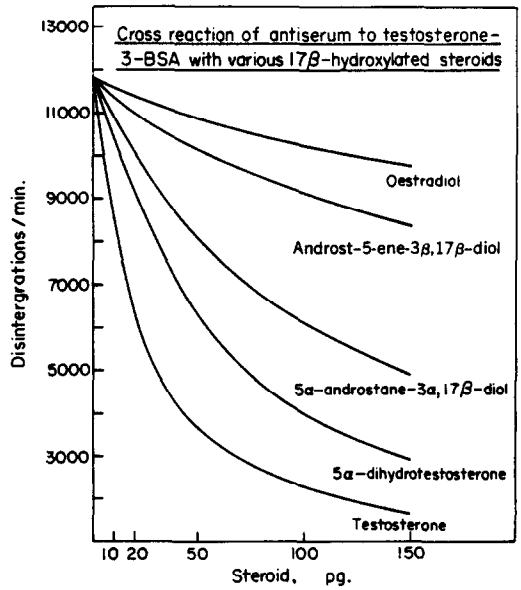

Fig. 10. Standard curves for various steroids with an hydroxyl group in the β position at carbon 17 using antiserum to testosterone-3-BSA.

plasma samples from men and women by both radioimmunological methods and comparing the results. These are shown in Table 6. It may be seen that there is good agreement with both male and female plasma.

The effect of chromatography on the results from female plasma using the antiserum to testosterone-3-BSA is shown in Table 7, which indicates only minimal reduction by inclusion of the chromatographic step. As both 5-androstene-3β,17β-diol and 5α-adrostane-3α,17β-diol are separated from testosterone by the Sephadex LH 20, this experiment would indicate only minimal amounts of 5α-dihydrotestosterone (17β-hydroxy-5α-androstan-3-one) in the plasma from healthy women.

A further check on the specificity of the method without chromatography was made by comparing the values obtained by radioimmunoassay with those obtained

Table 6. A comparison of plasma testosterone levels using different antisera after chromatography on Sephadex LH 20

	T-17-BSA ng/100 ml plasma	T-3-BSA ng/100 ml plasma
M1	320	314
M2	440	445
M3	560	519
M4	736	701
F1	87	83
F2	53	50
F3	42	38
F4	39	38

Table 7. The specificity of antiserum to testosterone-3-BSA. Effect of chromatography on Sephadex LH 20

Female plasma	Testosterone ng/100 ml plasma Ether extract	After chromatography
1	41	40
2	58	53
3	50	53
4	31	22
5	50	45
6	24	17
Mean ± S.D.	42 ± 13	38 ± 15

by a gas–liquid chromatographic procedure. Examples of this comparison using plasma from healthy men and women are shown in Fig. 11, where a 15 per cent error is plotted with each result. From a total of 20 plasmas from healthy individuals analysed by both methods, all ranges overlapped, suggesting a reasonable correlation.

Further studies have been made on plasma from patients with endocrine dysfunction and after stimulation and suppression tests. These are part of a long-term study but the results to date suggest that after adrenal or ovarian suppression, the correlation is within experimental error, but after testicular or ovarian stimulation with HCG an increased response may be obtained by radioimmunoassay.

Water blanks

Further evidence for specificity may be deduced from the low values for water blanks taken through the whole procedure. These values, as applied to the methods for plasma from men and women, are shown in Table 8.

Recoveries

The mean recoveries ± S.D. of tritiated internal standard from 50 male plas-

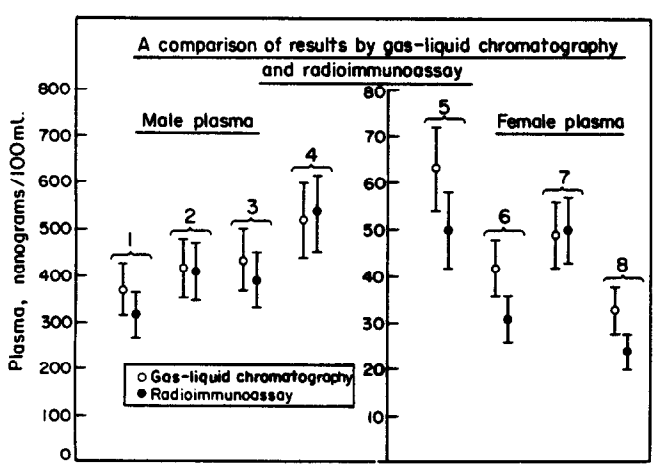

Fig. 11. The analyses of various plasma samples from healthy men and women by a radioimmunological and a gas–liquid chromatographic method.

Table 8. Method blanks (12 determinations) and plasma testosterone levels for groups of healthy men and women (ng/100 ml; mean ± S.D.)

Method	Water blanks*	Female plasma	Water blanks*	Male plasma
RIA (without chromatography)	0·7 ± 0·7	64 ± 14	9 ± 3	675 ± 233
RIA (with chromatography)	2·8 ± 0·9	53 ± 10	25 ± 6	573 ± 190
GLC	10 ± 4	40 ± 14	<0	528 ± 261

*Values quoted are calculated from positive readings on the standard curve.

mas was 95 per cent ± 6 and from 50 female plasmas 77 per cent ± 17. After chromatography on Sephadex LH 20 the mean recoveries from both sets of plasmas dropped by a further 20 per cent.

RESULTS

The method without chromatography and using the antiserum to testosterone-3-BSA, has been applied to the determination of testosterone in peripheral venous plasma from 12 healthy men and 10 healthy women at various phases of the menstrual cycle. The values, together with those previously obtained by a gas-liquid chromatographic procedure are shown in Table 8. It may be seen that the mean values in plasma from men and women are slightly higher by the radioimmunological procedure. However, after chromatography on Sephadex LH 20, these values fall to 573 ± 190 ng/100 ml plasma and 53 ± 10·3 ng/100 ml. plasma in men and women respectively.

DISCUSSION

Although the procedure is not specific for testosterone on theoretical grounds since 5α-dihydrotestosterone is not removed by the column and there is appreciable cross-reaction with this compound, the results suggest that if chromatography on Sephadex LH 20 precedes equilibration with antisera to testosterone-3-oxime-BSA, there is only slight overestimation of plasma testosterone.

The present method is similar in principle to that devised by Furuyama *et al.* [2]. However, the use of column chromatography on Sephadex LH 20 is preferred to alumina, as the blank values are consistently low, and there is complete separation of 5α-androstane 3α,17β-diol and 5-androstene-3β,17β-diol. In addition, the use of azobenzene enables the testosterone fraction to be located with reasonable accuracy. The efficacy of the column in removing interfering compounds was tested by reacting different aliquots from the same plasma samples with antisera to testosterone-3-BSA and testosterone-17-BSA. The similarity in values obtained by both procedures—bearing in mind a possible error of 15 per cent in each—and by gas–liquid chromatography indicates no gross overestimation. Further evidence on specificity is obtained from the observation that the mean and range of values obtained are similar to those reported

by other workers, using techniques based upon the principles of double isotope derivative formation, gas-liquid chromatography and competitive protein binding (see review by Sommerville and Collins [6]).

Investigations into factors affecting binding and displacement of steroid to antibody indicate that binding is best effected in an alkaline medium and at low temperatures. Both the initial binding of [^{3}H] steroid and subsequent displacement by non-labelled steroid are increased with time. The use of dextran-coated charcoal to separate unbound steroids after equilibration with antiserum and the inclusion of gelatine in the buffer was first reported by Hotchkiss *et al.*[5] for the determination of plasma oestrogens. Under these conditions, the antibody-bound fraction is subjected to liquid scintillation counting, the results are highly reproducible and the technique is very robust. The use of tritiated testosterone of high specific activity (100 Ci/mmol) enables precise measurements to be made with shorter counting times.

The recovery of authentic testosterone from distilled water indicates that the accuracy of the method is acceptable and the precision as assessed by replicate determination on pools of male and female plasma is from 8 to 10 per cent. The maximum theoretical error for the corresponding levels of testosterone would be approximately 12·5 per cent. The similarity in practical and calculated error is reassuring as practical, systematic errors include such factors as absorption of steroid to glassware, incomplete separation of antibody-bound and free steroids and varying amounts of 'blank material' in each sample.

If the chromatographic step is omitted, the mean testosterone values are higher in plasma from groups of men and women. Since a large percentage of the increase in level is probably due to other steroids in plasma with an hydroxyl group in the β position at carbon 17, and since these steroids have been shown to possess biological activity, the assay may be of value in determining the total 'androgen' content of plasma.

Attempts are in progress to improve the method either by complete separation of 5α-dihydrotestosterone on a column of hydroxyalkoxypropyl sephadex [7] or by production of more specific antisera. Furthermore, the assay may be shortened by using testosterone-3-oxime-tyrosine labelled with ^{125}I in place of [^{3}H]testosterone.

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DISCUSSION

Exley: I wonder if Dr. Collins could tell us a little about the specificity of the C-3 oxime product; I know that at C-17β it is terrible but the C-3 oxime antiserum which we have prepared has very little cross-reaction with anything except dihydrotestosterone with which it cross-reacts to about 30%.

Collins: Yes, I'm sorry. I had to miss the section on specificity owing to the time. All 17β-hydroxylated steroids which we have tested compete to some extent with [^{3}H]testosterone in the assay system. The maximum cross-reaction was with 5α-dihydrotestosterone which we found to be approximately 50%.

Munck: What's the capacity of your technician, in terms of assays per week?

Collins: At the moment we assay about 12 plasma samples per run, in the procedure which includes the chromatographic step. Under these conditions, it should be possible to obtain the results within 3 days of starting.

Munck: I see, so it's still of the same order of magnitude as Dr. van der Molen's method.

Collins: Yes.

Munck: A breakthrough is necessary.

Fazekas (A. G.): Dr. Collins, regarding the separation of dihydrotestosterone from testosterone, we did a number of assays with the Sephadex LH-20 column, and found that by increasing the length of the column described by Murphy to 40 cm, we could separate dihydrotestosterone from testosterone quite well using the heptane : chloroform : ethanol : water mixture.

Collins: Yes, we have managed to do the same thing, but the blank values were not so reliable. We are now trying more hydrophobic derivatives of Sephadex.

Rosner: Published values for dihydrotestosterone in plasma indicate that the concentration of testosterone is about 10 times greater than DHT in men and about 3 times greater in women. This could obviate the need for the separation of these two steroids in many assays.

Collins: This is perhaps true with respect to determinations on male plasma, where the overestimate may be of the order of 10%. However, determinations on female plasma may overestimate by as much as 20%. If viewed in the light that there is an error of 15% in the method anyway, these values may be acceptable for certain purposes.

Munck: But the day you find a syndrome in which there are high dihydrotestosterone concentrations, you'll have trouble.

Collins: Yes.