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THE EVALUATION OF A GAS–LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF PLASMA TESTOSTERONE USING NICKEL-63 ELECTRON CAPTURE DETECTION

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SUMMARY

A method is described for the determination of testosterone in human peripheral venous plasma. The procedure involves addition of a labelled internal standard, mild saponification with sodium hydroxide, extraction with diethyl ether and preliminary purification on thin-layer chromatography. After formation of the heptafluorobutyrate derivative, the extract is rechromatographed on silica gel, followed by gas–liquid chromatography using a solid injection technique, Nickel-63 electron capture detection and electronic digital integration. The percentage error associated with each part of the procedure has been estimated and the total theoretical random error determined for each assay. In addition, the practical errors have been determined by replicate analyses. The method has been applied to the determination of testosterone in plasma from 41 healthy males (mean 528 $\pm$ 261 ng/100 ml plasma) and 20 healthy females (mean 40 $\pm$ 14 ng/100 ml plasma).

INTRODUCTION

Methods for the determination of testosterone (17β-hydroxyandrost-4-ene-3-one) in human peripheral venous blood have involved the use of fluorimetry, double isotope dilution, and gas–liquid chromatography with either flame ionisation or electron capture detection[1]. In general, the gas–liquid chromatographic methods are less time-consuming, and the selectivity and potential sensitivity of the electron capture detector appears most suitable for the accurate determination of testosterone in the systemic blood of healthy women. The present report is concerned with a theoretical and practical evaluation of a method involving the formation of testosterone heptafluorobutyrate followed by thin-layer chromatography, gas–liquid chromatography, and Nickel-63 electron capture detection.

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The high electron affinity of steroid heptafluorobutyrate was first reported by CLARK AND WOTIZ[2] in 1963, while NAKAGAWA *et al.*[3] investigated the properties of several halo-alkyl derivatives of testosterone and concluded that the heptafluorobutyrate was one of the most sensitive and stable. At the same time other reports by EXLEY[4] and VERMEULEN[5] outlined methods involving heptafluorobutyrate derivative formation for the determination of testosterone in small samples of human peripheral plasma, and VAN DER MOLEN *et al.*[6] have suggested modifying the method of BROWNIE *et al.*[7] by replacing formation of the monochloroacetate with the more sensitive heptafluorobutyrate.

A study of the formation of testosterone heptafluorobutyrate and the stability of this derivative during thin-layer chromatography, has been the subject of a preliminary report (WYMAN AND COLLINS)[8].

The operation and design of an electron capture detector was first reported in 1960[9], and subsequently modified to operate with a pulsed collection voltage[10]. However, the use of tritium foil as a β-emitter impeded its extended application to steroid analysis as a significant loss of tritium may occur at temperatures above 200°. The choice of Nickel-63 [11] as a radioactive source, and a modification of the detector arrangement[12] has enabled the use of temperatures up to 350°. This allows higher column temperatures to be employed, and the detector may readily be cleaned by raising the temperature over-night. The use of such a detector for the determination of steroid derivatives and an investigation into the operating conditions has previously been reported[8].

MATERIALS

Solvents and reagents

Diethyl ether (peroxide free) was redistilled immediately before use. Benzene, ethyl acetate, methanol, acetone, hexane and tetrahydrofuran (all analar grade) were redistilled and stored over granular anhydrous sodium sulphate.

Heptafluorobutyric anhydride was obtained from K and K Laboratories, Inc., Jamaica, N.Y.

Eastman chromagram sheets 6060 (silica gel with fluorescent indicator) were supplied by Distillation Products Industries, Division of Eastman Kodak Co., Rochester, N.Y.

The liquid phase for gas–liquid chromatography Xe-60 (cyanoethyl methyl silicone) and the support—Gas Chrom Q— were obtained from Applied Science Laboratories, Mc. State College, Pennsylvania.

Standards

Testosterone-4-^{14}C s.a. 55.2 mC/mmole and testosterone-7α-^{3}H s.a. 1.63 C/mmole were obtained from the Radiochemical Centre, Amersham, Bucks., Great Britain.

A sample of testosterone heptafluorobutyrate was kindly supplied by Dr. H. J. VAN DER MOLEN, University of Utrecht, The Netherlands. Subsequent batches of testosterone heptafluorobutyrate were prepared in the department by the method of VAN DER MOLEN *et al.*[6].

METHOD

A flow sheet of the method is shown in Fig. 1.

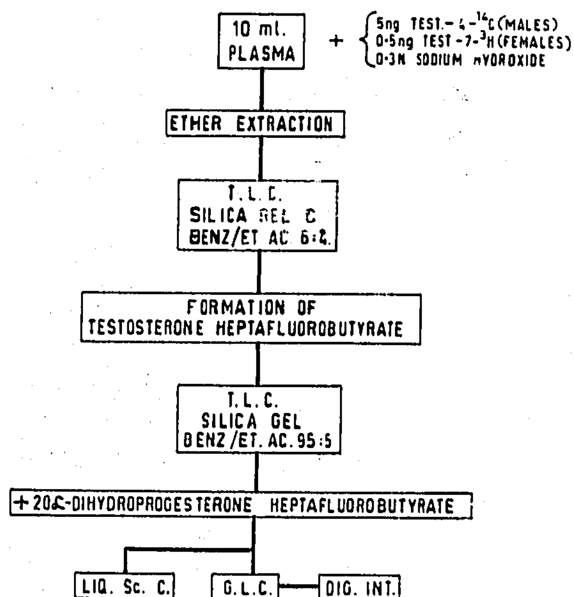


Fig. 1. Flow diagram of method.

Extraction

Peripheral venous blood (15–20 ml) is withdrawn by a non-greased syringe, transferred to a lithium heparin bottle, and centrifuged. The plasma is removed, and the volume measured. The sample may be processed immediately or stored at $-15°$.

An internal standard of 5 ng testosterone-4-^{14}C is added to plasma from male subjects, and 0.5 ng testosterone-7α-^{3}H to plasma from female subjects; an equal aliquot is taken for liquid scintillation counting. All standards are added from a 100 μl syringe with a Hamilton PB600 attachment (Hamilton Co. Inc., U.S.A.), which delivers 1/50 of the total syringe volume with each depression. After the addition of an equal volume of $^{1}/_{3}$ N sodium hydroxide, the mixture is gently shaken, and extracted with 2 × 100 ml diethyl ether. The ether is washed twice with 20 ml distilled water, and evaporated to dryness. The extract is then transferred with 3 × 1.0 ml methanol–chloroform (1:1, v/v) to a conical test tube, and taken to dryness under vacuum (using a Buchler rotatory evapo-mix).

Thin-layer chromatography

Glass plates 20 × 20 cm are coated with Silica Gel G, activated and stored in a desiccator until use. The extracts are transferred to the plate with a glass capillary using 3 × 0.05 ml methanol–chloroform (1:1, v/v). The first solvent system is benzene–ethyl acetate (6:4, v/v), the solvent front being allowed to run 15 cm. The plates are then removed and thoroughly dried. The testosterone-4-^{14}C in extracts from male plasma may be located by autoradiography (Kodirex non-screen X-ray film, overnight), and the testosterone-7α-^{3}H in female plasma by extrapolation from side and central standards.

The silica gel containing testosterone is loosened with a microspatula; aspirated on to a filter disc (1cm diam., No. 3 grade), and eluted with 3 × 0.05 ml ethanol under reduced pressure. The eluate is then evaporated under vacuum and thoroughly dried in a vacuum desiccator.

Derivative formation

A reaction mixture containing 1.0 ml dry hexane, 40 µl dry tetrahydrofuran and 20 µl heptafluorobutyric anhydride is prepared with each batch of samples, and 100 µl of the mixture added to each tube. The tubes are heated at 50° for 30 min, and the reagent subsequently removed under vacuum in a desiccator.

Thin-layer chromatography of derivatives

The extracts are transferred to an Eastman chromogram sheet with 3 × 0.5 ml dry acetone, and developed in benzene–ethyl acetate (95:5, v/v). Both solvents were dried over granular sodium sulphate before use. Testosterone heptafluorobutyrate is located by autoradiography or extrapolation from side and central standards. The silica gel containing the testosterone heptafluorobutyrate is aspirated onto a filter disc, and the extract eluted with 3 × 0.5 ml dry acetone, which is evaporated under vacuum in a desiccator.

Addition of second internal standard

100 µl from a standard solution of 20α-dihydroprogesterone heptafluorobutyrate is added to each tube so that $^1/_{20}$ of an extract of male plasma (5 µl) and $^1/_{10}$ of an extract of female plasma (10 µl) will contain 2 ng of the 20α-dihydroprogesterone heptafluorobutyrate for gas chromatography.

Gas–liquid chromatography

The gas–liquid chromatograph was a Pye 104 model 84 with a Nickel-63 electron

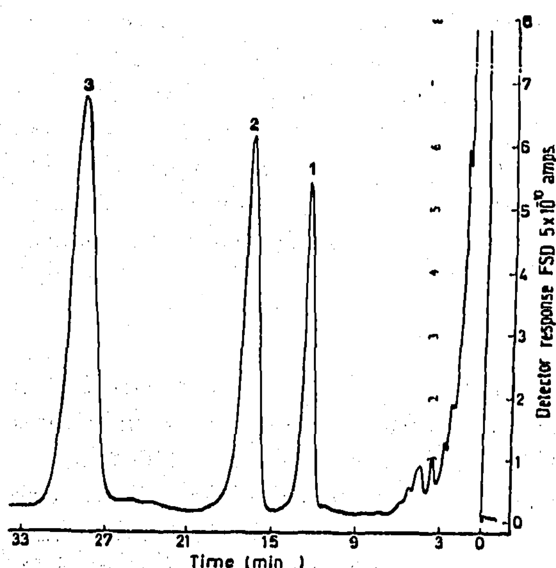
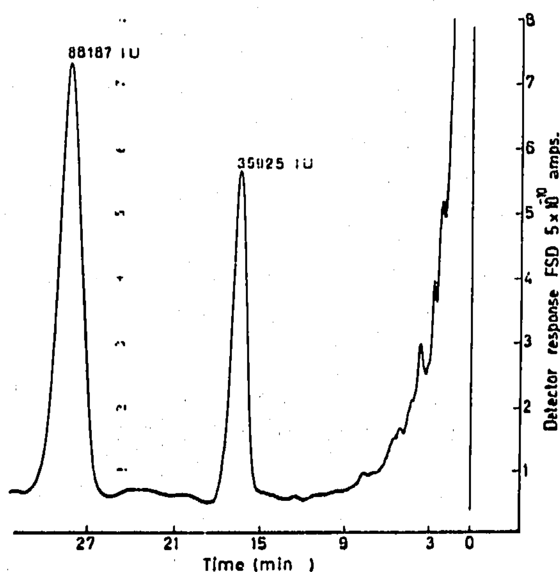


Fig. 2. Detector response following gas–liquid chromatography of 1.0 ng epitestosterone heptafluorobutyrate (peak 1), 1.0 ng testosterone heptafluorobutyrate (peak 2), and 2 ng 20α-dihydroprogesterone heptafluorobutyrate (peak 3).

capture detector. Glass columns 144 cm by 4 mm I.D. were packed with 3% Xe-60 on Gas Chrom Q, and operated at 215°. The carrier gas was 5% methane in argon at 50 ml/min. The detector was maintained at 225°, and every 150 μsec a 47 V pulse of 0.75 μsec duration was applied. These conditions gave a standing current of 1.7×10^{-9} A; the minimum level of testosterone heptafluorobutyrate detectable (a signal of twice background) was 0.005 ng, and the detector gave a linear response to 1.0 ng. The attenuation setting used for analysis produced a full scale deflection for 5×10^{-10} A. The samples are transferred to a solid injection syringe (SS60 3½" needle, Hamilton Co. Inc., U.S.A.) and applied directly to the top of the column. The detector response was displayed on a chart recorder, and the peak heights and areas (by triangulation) were compared with the results from a digital integrator (Infotronics, Model CRS-10HB). The appropriate operating conditions for digital integration were selected for the typical response from an electron capture detector to steroid heptafluorobutyrates in biological samples. An automatic base-line drift corrector was used to overcome the difficulties arising from sloping base lines. Representative traces of standards and extracts are shown in Figs. 2, 3 and 4.



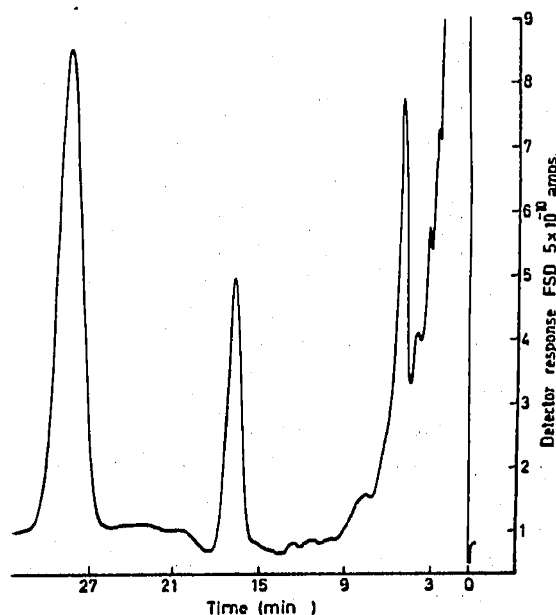


Fig. 3. Detector response following gas–liquid chromatography for 1/20th of the final extract from 10 ml of male plasma with the second internal standard.

Fig. 4. Detector response following gas–liquid chromatography for $^1/_{10}$th of the final extract from 10 ml of female plasma with the second internal standard.

Liquid scintillation counting

The remaining sample from each tube ($^{10}/_{20}$ from male plasma and $^9/_{10}$ from female plasma) is transferred to a potassium-free counting vial with 3×0.5 ml of methanol and dried. 8 ml of toluene containing 3.0 g/l of 2,5-diphenyl-oxazole (PPO) are added to the vials containing carbon-14 and 10 ml to the vials containing tritium. A liquid scintillation counting system—Nuclear Chicago Model 6860 (Mark 1)—was used, and each sample stabilised at 2° for 2 h, and a counting time selected to give a coefficient of variation of less than 1.5%. The counting efficiencies were determined

for each sample from calibration curves for an external standard channels ratio method using a ^{133}Ba source.

Calculation of results

A desk-top computer, the Olivetti Programma 101, was used to calculate the results according to the following formula:

$$S = \left[\left[\frac{C_s}{E_s} \cdot \frac{E_x}{\alpha C_x} \cdot \frac{I_s}{I_x} \cdot \frac{\beta T_x}{T_s} \cdot \frac{W_1}{W_2} \cdot A\right] - M\right] \cdot \frac{100}{V} \qquad (1)$$

where:

C_s = counts/min of labelled standard,
E_s = counting efficiency for the labelled standard,
C_x = counts/min of the labelled standard in the extract,
E_x = counting efficiency for the labelled standard in the extract,
α = the aliquot taken for liquid scintillation counting,
I_s = detector response of the second internal standard,
I_x = detector response of the second internal standard in the extract,
β = the aliquot taken for gas–liquid chromatography,
T_x = detector response of unknown testosterone heptafluorobutyrate,
T_s = detector response of testosterone heptafluorobutyrate standard,
W_1 = molecular weight of testosterone,
W_2 = molecular weight of testosterone heptafluorobutyrate,
A = mass in ng of testosterone heptafluorobutyrate standard,
M = mass in ng of internal standard,
V = volume in ml of plasma,
S = ng of testosterone per 100 ml plasma.

Results

The method has been applied to the determination of testosterone in peripheral venous plasma taken from 20 healthy females (age 21–33 years) and from 41 healthy

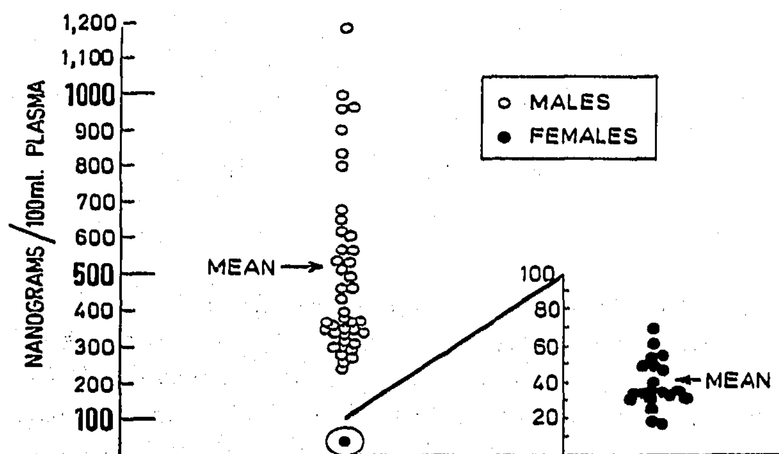


Fig. 5. Testosterone levels in peripheral venous plasma from 41 healthy males and 20 healthy females.

males (age 19–45 years). These values are shown in Fig. 5; the mean value for women was 40 ± 14 ng per 100 ml of plasma (range 18–71) and for men 528 ± 261 ng per 100 ml plasma (range 238–1195). In addition testosterone levels have been determined in plasma from women with idiopathic hirsutism, and in plasma from hypogonadal males before treatment. Some of these results have been used to assess the theoretical error involved in the determination of endogenous testosterone at values which lie between the normal female and male ranges (Fig. 6 and Table II).

Theoretical assessment of error in individual samples

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 × 100)/S) was then determined from equation (2) below, derived from the usual laws for the combination of additive and multiplicative independent normal errors.

$$e_S^2 = \left[\left[\frac{(e_{C_s}^2+e_{E_s}^2+e_{E_x}^2+e_{C_x}^2+e_s^2+e_{I_x}^2+e_{T_s}^2+e_{T_x}^2+e_A^2)(\Delta^2)+M^2\cdot e_M^2}{\gamma^2}\right] + e_V^2\right] \qquad (2)$$

where:

$$\Delta = \frac{C_s}{E_s}\cdot\frac{E_x}{\alpha C_x}\cdot\frac{I_s}{I_x}\cdot\frac{\beta T_x}{T_s}\cdot\frac{W_1}{W_2}\cdot A$$

$$\gamma = \Delta - M$$

and

e_{C_s} = the overall percentage error on C_s, and similarly for the other terms.

As α, β, W_1, and W_2 are constants, they do not 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. If the first aliquot from the PB600—100 µl syringe assembly is discarded, the subsequent pipetting error was found to be 1.25%, and as 20,000 counts were recorded for both carbon-14 and tritiated standards, the counting error was about 0.7%, assuming Poisson statistics.

These two errors were then combined to give:

$$e = (e_1^2 + e_2^2)^{\frac{1}{2}} = (1.25^2 + 0.7^2)^{\frac{1}{2}} = 1.4\%$$

There is a negligible pipetting error for C_x as the extract is washed into a counting vial. From 75 extracts a minimum of 3,000 counts and a maximum of 12,000 counts were recorded, which gave a range in percentage counting error of 0.9%–1.8%; the mean of 1.4% was used as the error in C_x for the calculation of total error in individual samples.

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% using an external standard channels ratio technique.

A, M, V. There are only three pipetting errors (all taken to be 1%) associated

with A, as the weighing error is negligible. This results in an overall error of 1.5%. The amount of labelled standard M added to the sample is determined from the liquid scintillation counting of an aliquot and the specific activity (in μC/mg) quoted by the Radiochemical Centre. As the error in the activity is 3% and that on the specific activity 1%, these combined with a 1% pipetting error give an overall error of 3.6%. The error on V was taken as a maximum of 5%.

I_s, T_s, I_x, T_x. I_s and T_s each have a 1% pipetting error in addition to the error in measuring detector response, while for I_x and T_x it is more complex, as there is a 1% pipetting error involved in adding the internal standard to the extract and a 1% pipetting error involved in removing both simultaneously; T_x is considered to have no other error besides that of the detector response, and I_x is considered to have two additional 1% pipetting errors.

The inherent error in the measurement of detector response has been evaluated using the three methods below.

(a) *Peak height*. Several factors contributing to the overall error in peak height have been considered. These included the base line variation, the selection of the base line position under the peak, the limitations in using a ruler, and the finite thickness of the trace. The resulting percentage error was calculated for the range of peak heights shown in Table I.

(b) *Peak area*. The error in peak area is compiled from that in peak height and from that involved in the determination of the peak width at half height. The error in peak width includes similar measurements as discussed above, and it is also influenced by the peak height. This is particularly important for small peaks where the uncertainty in the peak height is large and could lead to the measurement of the peak width at an incorrect position.

Under our conditions, the peak width for testosterone heptafluorobutyrate was about 4 mm, and the average error in the width was estimated to be 10% for all peak heights. The 20α-dihydroprogesterone heptafluorobutyrate gave a peak width of approximately 6 mm, and the average error in width ranged from 10% at a height of 10–30 mm to 6% for peak heights of 100 mm and greater.

These errors were then combined to give the overall error in area:

$$e^2_{area} = (e^2_{height} + e^2_{width})^{\frac{1}{2}}$$

and the values are shown in Table I.

(c) *Digital integration*. After attenuation the detector signal is amplified by the

TABLE I

PERCENTAGE ERRORS IN HEIGHT, WIDTH AND AREAS FOR TESTOSTERONE HEPTAFLUOROBUTYRATE AND 20α-DIHYDROPROGESTERONE HEPTAFLUOROBUTYRATE

Peak height (mm)	10	20	30	40	50	60	70	80	90	100	150	200
% Error (heights)	21.2	10.6	7.1	5.3	4.2	3.6	3.0	2.7	2.4	2.1	1.4	1.1
% Error (width) w = 4 mm	10	10	10	10	10	10	10	10	10	10	10	10
% Error (width) w = 6 mm	10	10	10	9	9	8	8	7	7	6	6	6
% Area error w = 4 mm	23.4	14.6	12.3	11.3	10.8	10.6	10.4	10.4	10.3	10.2	10.1	10.1
% Area error w = 6 mm	23.4	14.6	12.3	10.4	9.9	8.7	8.5	7.5	7.4	6.4	6.2	6.1

integrator and converted to pulses which are counted by a decade scaler. The count-rate corresponding to a full scale deflection of 1 mV is 60,000 counts/min. The integrator recognises the start of a peak from a combination of three factors—the rate, size, and duration of the signal increase. The electronic error in the integration of an ideal peak is quoted as 0.5%, but for biological extracts at high sensitivity the overall error is probably much greater. As it is difficult to assess the error in the integration units representing a single peak, the mean percentage error of 5.5% obtained from the integration of replicate standard samples at different levels (0.1 → 0.75 ng) was taken for all peaks.

These three methods of evaluating detector response have been used to determine the overall percentage error in the testosterone levels from 200 plasma samples; representative values are shown in Table II, and there is good agreement between the

TABLE II

VARIATION OF THEORETICAL PERCENTAGE ERROR WITH CONCENTRATION OF TESTOSTERONE FOR THREE METHODS OF DETERMINING DETECTOR RESPONSE

*Sample**	*ng/100 ml plasma*					
	Area	*% Error*	*Height*	*% Error*	*Integral units*	*% Error*
F	19.7 ± 5.0	23.3	21.8 ± 2.4	11.2	25.6 ± 4.1	16.2
F	33.4 ± 7.2	21.5	33.6 ± 3.1	9.2	31.7 ± 4.8	15.2
F	36.8 ± 7.8	21.1	36.6 ± 3.2	8.9	40.5 ± 5.9	14.6
F	36.9 ± 8.0	21.6	40.9 ± 3.7	9.2	42.4 ± 6.9	14.6
HF	107.7 ± 21.2	19.7	115.4 ± 10.1	8.7	101.0 ± 13.9	13.8
M	285.1 ± 75.2	26.4	294.9 ± 39.4	13.4	247.6 ± 41.6	16.8
M	312.1 ± 76.3	24.5	333.6 ± 40.0	10.8	434.6 ± 67.6	15.6
M	483.0 ± 109.0	22.6	498.1 ± 48.6	9.8	502.0 ± 77.4	15.4
M	693.0 ± 130.7	18.8	643.4 ± 53.5	8.3	657.1 ± 87.7	13.4
M	873.7 ± 175.9	20.1	931.7 ± 79.4	8.5	949.4 ± 133.8	14.1

* F = female, HF = hirsute female, M = male.

testosterone levels found by the three methods. The overall percentage error appears to be lowest for the peak height method; however, this is not the optimum way of calculating the concentration in this method, as a constant peak width is assumed for each biological sample, and this has not been found in practice.

In Fig. 6, the variation of overall percentage error is plotted against the concentration in ng/100 ml for the two levels of labelled standard (5 ng and 0.5 ng) added respectively. The percentage errors for the testosterone values are hypothetical below 18 ng/100 ml plasma, and were determined from representative values for recovery, standards, and peak areas for testosterone heptafluorobutyrate.

The shape of these curves are an inherent feature of equation (2), and result in errors that are virtually independent over the entire male range of testosterone levels and the majority of the female range, only increasing rapidly at the lower limit of each range. Those points lying well above the peak area curve in Fig. 6b are the result of abnormally low recoveries of the labelled standard, which led to a greater error in the measurement of the sample detector response.

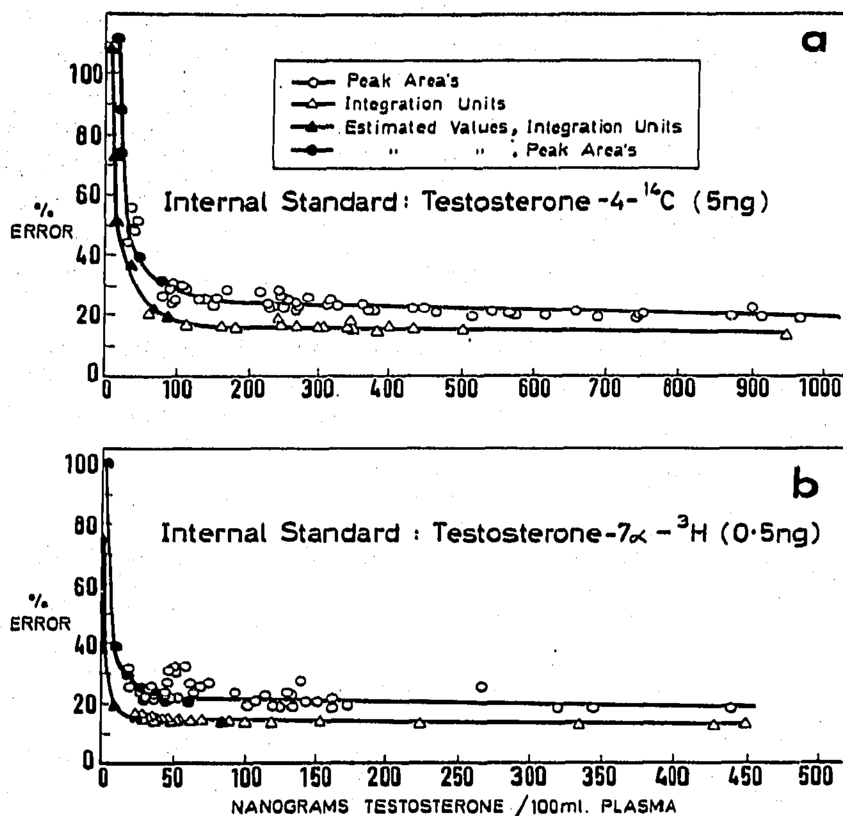


Fig. 6. The variation in theoretical random percentage error with plasma testosterone concentration.

Practical errors

After determining the theoretical error on individual samples, the practical errors were determined from recovery of known amounts of testosterone from water, and from replicate analyses on pools of male and female plasma.

Accuracy

The accuracy of the method was assessed by replicate recoveries of authentic testosterone added to 10 ml of distilled water. The results were calculated from peak areas, and expressed as ng/100 ml of water (Table III). The mean recovery from ten determinations at the 50 ng level was 94.8 ± 5.4%, while at the 4 ng level ten determinations gave a mean of 89 ± 32%.

Precision

The precision of the method was assessed from serial determinations on plasma pools taken from a 35 year old male blood donor and a 22 year old female donor. The detector response was measured by peak areas, and the results expressed as ng per 100 ml plasma. The mean value from the male donor was 258 ± 30 ng/100 ml plasma with a coefficient of variation of 11.6%, and the mean value from the female donor was 34.6 ± 5.45 ng with a coefficient of variation of 15.7% (Table III).

TABLE III

REPLICATE RECOVERY EXPERIMENTS AND ANALYSIS OF POOLED PLASMA

	Water, blank, ^{14}C STD.	*Water, recovery, 50 ng*	*Male, plasma, pool*	*Water, blank, ^{3}H STD.*	*Water, recovery, 4 ng*	*Female, plasma, pool*
	−20	488	310	7.1	50	32
	−21	456	250	18.4	27	29
	−10	494	252	9.1	42	45
	−19	481	223	8.6	30	37
	+16	525	247	8.6	28	43
	−28	454	303	10.6	25	31
	+ 9	484	268	5.6	25	31
	−37	472	240	8.6	27	31
	− 3	425	233	15.6	40	36
	−23	456			62	31
Mean ± S.D.	−13 ± 17	474 ± 27	258 ± 30	10.2 ± 4.1	35.6 ± 12.6	34.6 ± 5.45
Coefficient of variation		5.8	11.6		35.4	15.7

Sensitivity

The sensitivity of the method has been evaluated by relating the endogenous level of testosterone with the theoretical percentage error associated with its measurement. As can be seen from Fig. 6, if a 50% error is taken as that which defines the lower limit of sensitivity, then the lower limits (using peak areas) are 30 ng/100 ml plasma, when 5 ng of testosterone-4-^{14}C is taken as labelled internal standard, or 7.5 ng/100 ml plasma when 0.5 ng of testosterone-7α-^{3}H is taken. If a digital integrator is used these values may be reduced to 15 ng/100 ml plasma, and 2.5 ng/100 ml plasma.

TABLE IV

THE CHROMATOGRAPHIC MOBILITIES OF STEROIDS WITH SIMILAR PROPERTIES TO TESTOSTERONE

Steroid	*Thin-layer chromatography*		*Gas–liquid chromatography, 3% Xe-60*
	Rt (Benzene–ethyl acetate, 6:4)*	*Rth** (After heptafluorobutyrate formation) (Benzene–ethyl acetate, 95:5)*	*R_{ph}*** (After heptafluorobutyrate formation)*
19-Nortestosterone	0.82	0.95	0.53
Epitestosterone	0.91	0.87	0.45
Testosterone	1.00	1.00	0.60
20α-Dihydroprogesterone	1.09	1.06	1.00
Aetiocholanolone	1.09	1.72	0.25
17α-Hydroxyprogesterone	1.18	—	—
20β-Dihydroprogesterone	1.20	0.99	0.82
Dehydroepiandrosterone	1.36	1.68	0.28
Androsterone	1.43	1.67	0.22

* Rt = mobility relative to testosterone.
** Rth = mobility relative to testosterone heptafluorobutyrate.
*** R_{ph} = retention time relative to 20α-dihydroprogesterone heptafluorobutyrate.

Specificity

At the present time, the specificity can only be inferred, as it is difficult to assess the purity of each sample. The good separation of testosterone and its derivative from steroids possessing similar chromatographic properties is shown in Table IV. In addition, the low values for the water blanks (Table III) suggest that solvents and reagents do not interfere with the assay. Further evidence for specificity may be deduced from the fact that the range of values in healthy men and women are similar to those reported using other techniques[1].

DISCUSSION

The plasma testosterone levels from 41 healthy males were in the range 238–1195 ng/100 ml plasma, which is similar to that reported by other workers[1]. However, as 50% of the values were below 400 ng/100 ml plasma, the mean value of 528 $\pm$ 261 ng/100 ml plasma is slightly lower than that obtained by other methods. The plasma testosterone levels from 20 healthy females were within a narrow range, 18–71 ng per 100 ml plasma, with a mean value of 40 $\pm$ 14 ng/100 ml plasma. This value and range is similar to those reported by VAN DER MOLEN *et al.*[6] using electron capture detection, and by LOBOTSKY *et al.* (1964)[13] using the lengthy double isotope dilution derivative method of RIONDEL *et al.*[14].

The method is similar in principle to that described by BROWNIE *et al.* in 1964[7]; however, the use of the more sensitive heptafluorobutyrate derivative and the thermally stable Nickel-63 detector followed by digital integration of the response has led to more precise measurements on testosterone levels in female plasma. Furthermore, this method is less time-consuming than those based upon the principle of double isotope dilution, and can be performed with similar accuracy and precision. Recovery experiments (4 and 50 ng) suggest that the present technique slightly underestimates the testosterone level, but both recoveries are within the standard deviation of the expected value. The precision of nine determinations upon a pool of male plasma was 11.6%, and 15.7% for ten determinations upon a pool of female plasma. From Fig. 6 the theoretical errors for the corresponding testosterone levels are 24.5% and 24.0%; this difference in practical and calculated error is reasonable, as all the samples from the pool were assayed simultaneously under optimum conditions which would result in a smaller practical random error.

Another advantage of this method and the use of equation (1) to calculate the results, is that the theoretical error (and, by inference, the practical error) is essentially constant over the testosterone levels of interest. All the terms in equation (2) influence the magnitude of the error for a particular testosterone level; this is well illustrated by the difference in the errors for peak areas and integrator units. In all cases, the use of an integrator, which implies smaller errors on the terms concerning detector response, has led to a significant decrease in the overall error. In addition to this general effect, one factor in equation (1)—the quantity of labelled internal standard added—greatly influences the overall error, as in equation (2) the magnitude of this term appears as well as the error associated with it. This effect is illustrated in Figs. 6a and 6b. In Fig. 6a, 5 ng of testosterone-4-^{14}C was added as the labelled standard, and the theoretical error begins to increase rapidly for testosterone levels under 75 ng/100 ml. However, with 0.5 ng of testosterone-7α-^{3}H as labelled standard, the error begins to increase rapidly

for testosterone levels of less than 25 ng/100 ml plasma. Thus, by reducing tenfold the amount of labelled standard added, it is possible to achieve results in the normal female range with the same accuracy as is obtainable for values in the normal male range. As it is difficult to locate tritium accurately on thin-layer chromatograms, the method would be improved by the availibility of testosterone-4-^{14}C of much higher specific activity.

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