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FURTHER STUDIES ON THE GAS–LIQUID CHROMATOGRAPHIC DETERMINATION OF C_{19}-STEROIDS IN HUMAN PLASMA USING NICKEL-63 ELECTRON CAPTURE DETECTION

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SUMMARY

Gas–liquid chromatographic methods are described and evaluated for the determination of testosterone, androstenedione and dehydroepiandrosterone in the same sample of peripheral plasma from healthy women. In addition, testosterone glucuroniside has been determined in the same sample after enzymatic hydrolysis and testosterone sulphate and dehydroepiandrosterone sulphate after solvolysis with ethyl acetate. The procedures involve addition of labelled internal standards, mild saponification with sodium hydroxide, extraction with diethyl ether and preliminary purification on thin-layer chromatography. After formation of derivatives with heptafluorobutyric anhydride, the extracts are rechromatographed on silica gel or cellulose, followed by gas–liquid chromatography and nickel-63 electron capture detection. The total theoretical random error has been determined for each individual assay, and the practical errors have been determined by replicate analyses. The method has been applied to the determination of testosterone, androstenedione and dehydroepiandrosterone in plasma from 24 healthy women. The mean values expressed per 100 ml plasma were: testosterone 58.5 ± 30.4 ng, androstenedione 92.8 ± 81.3 ng and dehydroepiandrosterone 655 ± 510 ng. The conjugated derivatives were determined in ten samples. The mean values expressed as testosterone or dehydroepiandrosterone per 100 ml plasma were: testosterone glucuroniside 92.8 ± 81.3 ng, testosterone sulphate 18.5 ± 38.8 ng and dehydroepiandrosterone sulphate 32.6 ± 13.8 μg. In addition, serial analyses of these compounds and calculation of the random error associated with each determination have revealed marked differences in concentration throughout the menstrual cycles of two healthy women.

INTRODUCTION

The recent development and application of microanalytical techniques for the determination of testosterone (17β-hydroxyandrost-4-en-3-one) have indicated that many factors influence the level of this compound in the systemic blood of women.

In 1962 MAHESH AND GREENBLATT[1] reported that administered dehydroepiandrosterone (3β-hydroxyandrost-5-en-17-one) and androstenedione (androst-4-ene-3,17-dione) could be converted to testosterone, and further work by VANDE WIELE *et al.*[2] upon the secretion and interconversion rates of C_{19}-steroids indicated the importance of peripheral metabolism and the complexity of transformations. Subsequently HORTON AND TAIT[3] calculated the metabolic clearance rate and blood production rate of androstenedione and testosterone, and deduced that approximately 60% of the plasma testosterone in women originated from the peripheral conversion of androstenedione. Other studies by HADD AND RHAMY[4] demonstrated the presence of testosterone glucuroniside in human plasma, and VAN DER MOLEN *et al.*[5] estimated the level in healthy subjects. Subsequently, testosterone sulphate was also identified in human plasma and the level determined in normal subjects and patients with gonadal and adrenal disorders (SAEZ *et al.*[6]).

These studies suggested that it might be of considerable interest to determine several C_{19}-steroids and their conjugates in the same plasma sample and there have been reports upon the use of double isotope derivative methods (KIRSCHNER *et al.*[7]; SAROFF *et al.*[8]) and gas-chromatographic methods using either flame ionisation detection (GOLDFIEN *et al.*[9]; AAKVAAG AND FYLLING[10]) or electron capture detection (VAN DER MOLEN *et al.*[5]) for the measurement of two or more of these compounds.

The present work is concerned with the gas–liquid chromatographic determination of testosterone, androstenedione, dehydroepiandrosterone, and their conjugated derivatives testosterone-17-monosulphate, testosterone-17-monoglucuroniside, and dehydroepiandrosterone-3-monosulphate in the same plasma sample. The levels and the random theoretical error associated with their measurement were assessed in a group of healthy women, and in serial samples collected throughout two menstrual cycles.

MATERIALS

Solvents were of reagent grade and distilled before use. Heptafluorobutyric anhydride (K. & K. Laboratories Inc., Jamaica, N.Y.) was distilled over anhydrous calcium chloride and stored in a desiccator. β-Glucuronidase, a preparation from beef liver (1 ml/5000 units), was obtained from General Diagnostics Dept., William R. Warner and Co. Ltd., Eastleigh, Hants.

Eastman silica gel sheets 6060 were supplied by Distillation Products Industries Division of Eastman Kodak Co., Rochester, N.Y., and pre-coated cellulose plates NR 1440 LS/254 of Carl Schleicher and Schuell were obtained from Anderman & Co., Ltd., Battlebridge House, 87/95, Tooley St., London.

The radioactive steroids testosterone-7α-^{3}H (s.a. 1.63 C/m*M*), androstenedione-4-^{14}C (s.a. 56.6 mC/m*M*), dehydroepiandrosterone-4-^{14}C (s.a. 57.1 mC/m*M*), and dehydroepiandrosterone-7α-^{3}H-sulphate (K-salt s.a. 605 mC/ m*M*) were purchased from the Radiochemical Centre, Amersham, Bucks. Testosterone-7α-^{3}H-β-D-glucuroniside (s.a. 20 C/m*M*) and testosterone-7α-^{3}H-sulphate (ammonium salt, s.a. 10 C/m*M*) were obtained from New England Nuclear Corp., Boston, Mass.

Steroid heptafluorobutyrates

The authentic heptafluorobutyrate derivatives of testosterone, dehydroepian-

drosterone and pregnenolone were prepared according to the method described by VAN DER MOLEN *et al.*[11], and recrystallised from 70 % aq. methanol.

The formation of 3-enol heptafluorobutyrate derivatives was first described by EXLEY AND CHAMBERLAIN[12]. Their method was slightly modified to obtain crystals of androstenedione 3-enol heptafluorobutyrate. 100 mg of androstenedione was dissolved in the minimum volume of dried acetone, 200 μl of heptafluorobutyric anhydride was added, and heated at 60° for 1 h. The contents were then evaporated to dryness and crystallised from 70 % aqueous methanol.

The melting point of the derivative was 119° and the infrared spectrum in chloroform solution showed a strong ester carbonyl absorption at 1785 cm^{-1}, and a steroid carbonyl band at 1735 cm^{-1}.

Gas–liquid chromatography

A Pye 104 Model 84 gas chromatograph fitted with a nickel-63 electron capture detector was used. The advantages and operating variables of such a detector for the determination of steroid derivatives have previously been discussed (WYMAN AND COLLINS[13]).

Glass columns 144 cm by 4 mm I.D. (for testosterone) and 274 cm by 4 mm I.D. (for dehydroepiandrosterone and androstenedione) were packed with 3 % XE-60 (cyanoethyl methyl silicone) on Gas-Chrom Q support (obtained from Applied Science Laboratories, N.C. State College, Pa.). The column temperature was maintained at 220° and that of the detector at 230°. The carrier gas (5 % methane in argon) flow was 50 ml/min. A pulsed voltage of 47 V of 0.75 μsec duration was applied every 150 μsec. The samples were injected onto the column with a Hamilton solid injection syringe (SS60 3½ in. needle, Hamilton Co. Inc., U.S.A.).

Liquid scintillation counting

A Nuclear Chicago Model 6860 (Mark 1) liquid scintillation counting system was used for the determination of radioactivity. The scintillation fluid contained 3 g of 2,5-diphenyl oxazole (PPO) in 1 l of toluene. For the counting of samples labelled with ^{14}C 8 ml were used, and 10 ml for samples containing tritium. The counting efficiencies for each sample were determined by reference to a calibration curve, plotted from quenched standards by a channels ratio method using an external ^{133}Ba source.

The activity of the steroid sulphates and glucuroniside was determined in a similar manner; the scintillation fluid was 10 ml of NE 240 (Nuclear Enterprises Ltd., Edinburgh) to which 4 % Cab-o-sil (Packard Instrument Company Inc., Ill.) was added to form a thixotropic gel.

METHOD

Peripheral venous blood (15–20 ml) was withdrawn with a non-greased syringe, transferred to a lithium heparin bottle and centrifuged. The plasma was separated and stored at —15° until analysed.

Extraction of free steroids

The plasma volume was measured, and radioactive internal standards of testosterone-7α-^{3}H (0.5 ng), androstenedione-4-^{14}C (5.0 ng) and dehydroepiandrosterone-

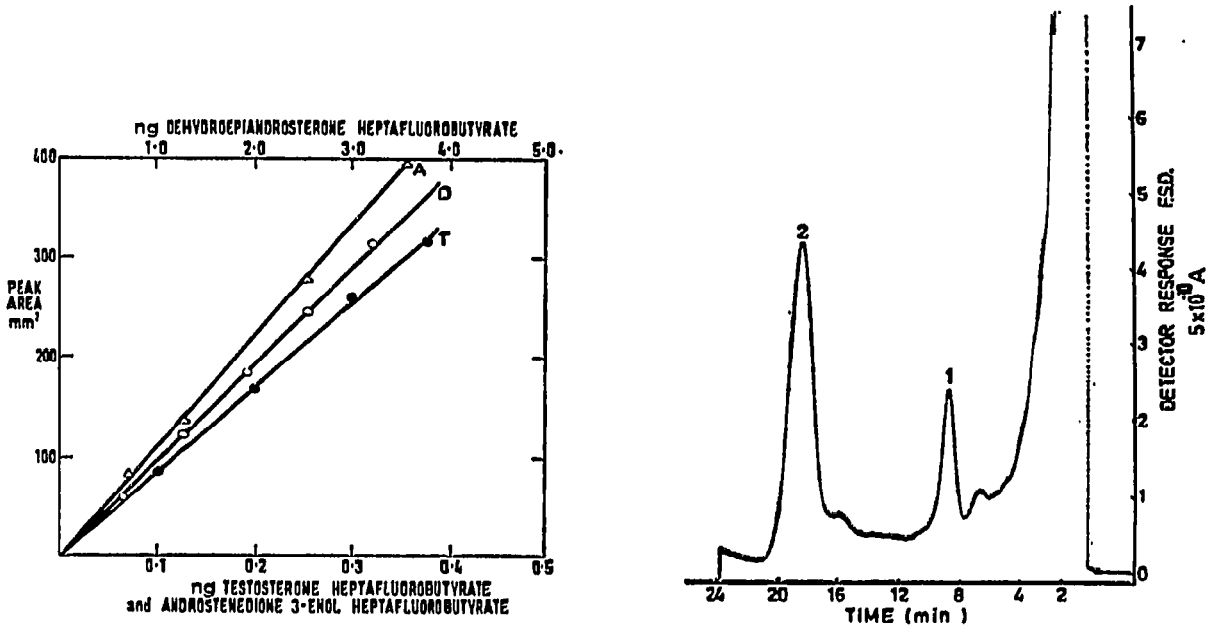

Fig. 1. Linearity and range of detector response. A = Androstenedione-3-enol heptafluorobutyrate; D = dehydroepiandrosterone monoheptafluorobutyrate; T = testosterone monoheptafluorobutyrate.

Fig. 2. Gas–liquid chromatographic response from 10 ml plasma (1/10 extract). Peak 1 = 3.2 ng dehydroepiandrosterone heptafluorobutyrate of which *ca.* 0.1 ng is labelled internal standard. Peak 2 = 0.3 ng testosterone heptafluorobutyrate.

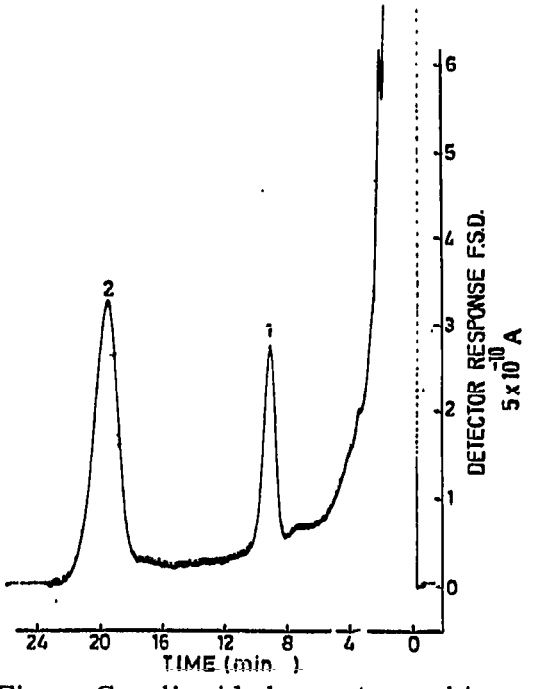

Fig. 3. Gas–liquid chromatographic response from 10 ml plasma (1/10 extract). Peak 1 = 0.16 ng androstenedione-3-enol heptafluorobutyrate of which *ca.* 0.05 ng is labelled internal standard. Peak 2 = 0.24 ng testosterone heptafluorobutyrate.

4-^{14}C (5.0 ng) were added with a 100 μl Hamilton syringe and PB 600 attachment. Ten milliliters of a 0.3 *N* sodium hydroxide solution were added and the plasma was extracted with 3 × 50 ml of diethyl ether. The ether extract was washed twice with distilled water and evaporated to dryness. The washings were collected along with the aqueous fraction from hydrolysis of the conjugated steroids.

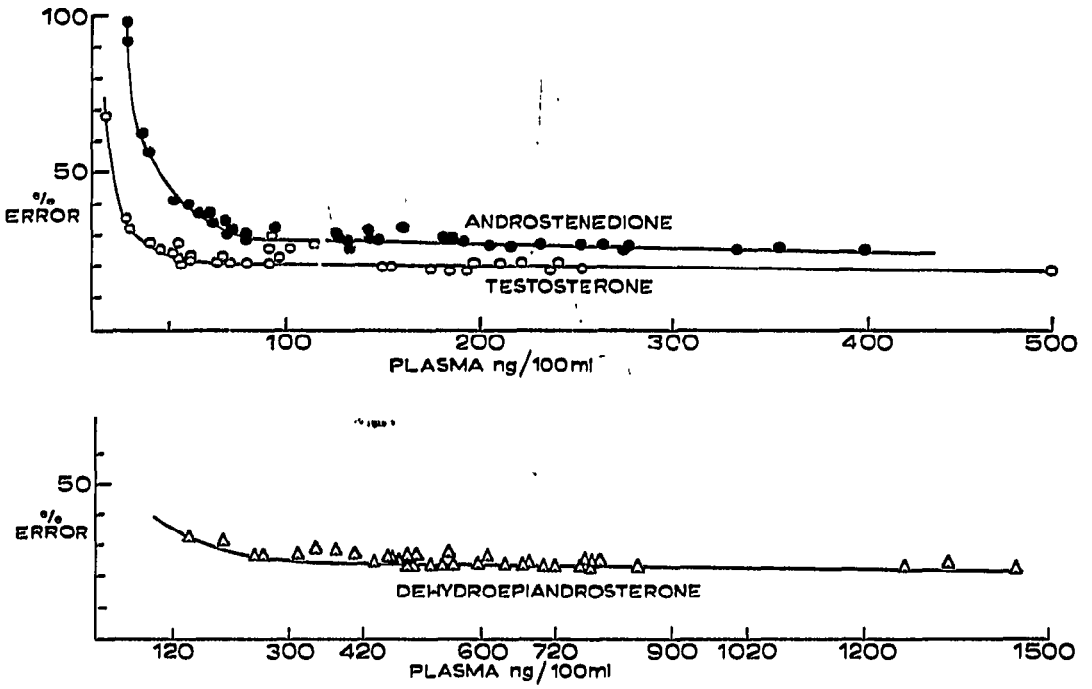

Fig. 4. The variation in theoretical random percentage error with the concentration of androstenedione, testosterone and dehydroepiandrosterone in plasma.

Thin-layer chromatography

Glass plates 20 × 20 cm, coated with Silica Gel G (DSF-5) were pre-washed with methanol, and used for thin-layer chromatography of the dried extracts. These were quantitatively transferred onto the plates as single spots and developed in the system chloroform–acetone (98:2, v/v). The area corresponding to testosterone was located from side and central standards, which were seen under ultraviolet light. Dehydroepiandrosterone and androstenedione were located by autoradiography (overnight exposure of the plates to Kodirex X-ray film).

The corresponding areas of steroids were aspirated on to a filter disc (No. 3 grade) and eluted with ethanol (3 × 0.5 ml). The eluates were evaporated and thoroughly dried in a desiccator.

Formation of heptafluorobutyrate derivatives

The heptafluorobutyrate derivatives of testosterone and dehydroepiandrosterone were prepared according to the method of COLLINS *et al.*[14]

Androstenedione-3-enol heptafluorobutyrate was formed by heating the sample with 10 μl of acetone and 10 μl of heptafluorobutyric anhydride at 60° in a water bath for 1 h. The reagent was removed by evaporation in a heated vacuum desiccator, at 60°.

Thin-layer chromatography of heptafluorobutyrate derivatives

Testosterone heptafluorobutyrate was chromatographed as described by COLLINS *et al.*[14]. The extracts containing dehydroepiandrosterone heptafluorobutyrate were applied to an Eastman Chromagram sheet No. 6060, and developed in the system hexane–ether (8:2, v/v). The compounds were located by autoradiography, eluted with dry acetone and the solvent evaporated under vacuum in a desiccator.

TABLE I

PERCENT RECOVERY OF HEPTAFLUOROBUTYRATE DERIVATIVES AFTER THIN-LAYER CHROMATOGRAPHY

	No. of analyses	*Liquid scintillation counting (Mean recovery ± S.D.)*	*Gas–liquid chromatography (Mean recovery ± S.D.)*
Testosterone	10	70.8 ± 10.4	60.4 ± 10.8
Androstenedione	10	32.8 ± 6.6	29.2 ± 4.6
Dehydroepiandrosterone	10	87.0 ± 6.0	80.3 ± 12.5

The 3-enol heptafluorobutyrate of androstenedione was purified on a cellulose plate developed in the system isooctane–petroleum ether (95:5, v/v). The derivative was located by autoradiography, eluted and dried as above.

Addition of internal standards

One hundred microliters of 20α-dihydroprogesterone heptafluorobutyrate were added to testosterone heptafluorobutyrate, and one tenth of the sample was removed for gas chromatography. The rest of the sample was transferred into a counting vial, and the radioactivity determined.

For dehydroepiandrosterone heptafluorobutyrate and androstenedione-3-enol heptafluorobutyrate, 100 μl of a standard solution of pregnenolone or testosterone heptafluorobutyrate were used to dissolve the extract. Of the sample 1/10 or 1/20 was analysed by gas–liquid chromatography and the rest counted for radioactivity.

Calculation of results

The detector response was assessed from the measurement of peak areas, and the amount of steroid present in the plasma sample (S, expressed as ng/100 ml plasma) was calculated according to the following formula:

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

where C_s and C_x are the counts per minute of labelled standard and labelled standard in the extract and E_s and E_x the counting efficiencies; α is the aliquot taken for liquid scintillation counting and β the aliquot taken for gas chromatography; I_s and I_x are the detector responses of the second internal standard and the second internal standard in the extract; T_x and T_s are the detector responses of the unknown steroid heptafluorobutyrate and steroid heptafluorobutyrate standard; W_1 is the molecular weight of steroid and W_2 the molecular weight of steroid heptafluorobutyrate; A is

the mass in ng of steroid heptafluorobutyrate standard; V is the volume in ml of plasma and M the mass of labelled standard in ng.

Hydrolyses of conjugated steroids

After ether extraction the aqueous fraction was hydrolysed with β-glucuronidase and the liberated testosterone re-extracted with diethyl ether. The aqueous residue was solvolysed with ethyl acetate for the analysis of testosterone sulphate and dehydroepiandrosterone sulphate.

β-Glucuronidase hydrolysis

Testosterone-7α-^{3}H-β-D-glucuroniside (2 ng) was added to the aqueous fraction and heated over a water bath to remove any remaining ether. The pH was adjusted to 4.5 with 0.1 N hydrochloric acid, and 2 ml of β-glucuronidase, 1 ml of acetate buffer (pH 4.5) and 5 mg of benzyl penicillin were added and incubated overnight at 37°. The pH was re-adjusted to 10.5 and extracted with 3 × 50 ml diethyl ether. The ether extract was washed twice with distilled water and evaporated to dryness. The thin-layer chromatography, formation of testosterone heptafluorobutyrate and analysis by gas–liquid chromatography are the same as described for testosterone.

TABLE II

RECOVERY OF RADIOACTIVITY AFTER HYDROLYSIS OF CONJUGATES

	Testosterone-7α-^{3}H-glucuroniside	*Testosterone-7α-^{3}H-sulphate*		*Dehydroepiandrosterone-7α-^{3}H-sulphate*	
Amount added	2.2 ng 21,700 d.p.m.	0.4 ng 25,800 d.p.m.		40 ng 108,660 d.p.m.	
Hydrolysis	β-glucuronidase	solvolysis	acid	solvolysis	acid
Recovery, mean ± S.D. (10 replicates)	79.8 ± 7.3	84.0 ± 11.0	51.6 ± 12.7	66.5 ± 3.0	32.1 ± 1.9

Solvolysis of sulphates

Radioactive internal standards of testosterone-7α-^{3}H-sulphate (0.5 ng) and dehydroepiandrosterone-7α-^{3}H-sulphate (30 ng) were added to the aqueous fraction after the extraction of testosterone glucuroniside as free testosterone. Solvolysis of the sulphates was performed according to the method of BURSTEIN AND LIEBERMAN[15]. The ethyl acetate extract was filtered, separated from the aqueous layer, and evaporated to dryness under vacuum in a rotatory evaporator. The procedure for analysis was similar to that described for testosterone and dehydroepiandrosterone.

RESULTS

The quantitative determination of steroids in biological samples by gas–liquid chromatography depends upon the linear response of the detector, stability of the derivative, and the accuracy of each step in the procedure.

Linearity of the detector response

The linearity of the detector response for all three steroids is shown in Fig. 1. The molar responses of testosterone monoheptafluorobutyrate and androstenedione-3-enolheptafluorobutyrate were similar, and approximately tenfold higher than dehydroepiandrosterone heptafluorobutyrate.

Fig. 2 and 3 show representative traces of dehydroepiandrosterone heptafluorobutyrate and androstenedione-3-enol heptafluorobutyrate from plasma extracts with testosterone heptafluorobutyrate as an internal standard. These separations were obtained on a 274 cm column of 3 % XE-60 at 225°. Traces illustrating the determination of testosterone in human plasma have been shown previously (COLLINS *et al.*[14]).

Stability of heptafluorobutyrate derivatives

The percentage recoveries of testosterone-4-^{14}C (10.0 ng), androstenedione-4-^{14}C (5.0 ng) and dehydroepiandrosterone-4-^{14}C (10.0 ng) after formation of the derivative and thin-layer chromatography are shown in Table I. The amount recovered was determined by gas–liquid chromatography and liquid scintillation counting. The recoveries calculated by both methods are essentially the same, suggesting that virtually no hydrolysis occurred during thin-layer chromatography and elution. The low recoveries of androstenedione reflect incomplete derivative formation, which depends on the enolisation of the 3-keto group.

Recoveries

In each instance the final result was corrected in terms of a labelled internal standard, and a second internal standard (unlabelled) added prior to gas chromatography. In general, the recoveries of labelled testosterone and dehydroepiandrosterone were 20–30 %, whereas the recovery of androstenedione ranged from 10–15 %.

The determination of the conjugated derivatives requires an additional step of hydrolysis, and this reduced the over-all recoveries to 10–15 %. The percentage recoveries of radioactivity after β-glucuronidase hydrolysis, solvolysis and acid hydrolysis of the conjugated derivatives of known radioactivity are given in Table II. The liberated steroids were extracted with ether and the radioactivity determined after one thin-layer chromatogram. The enzymatic hydrolysis of testosterone-7α-^{3}H-β-D-glucuroniside gave an 80 % recovery of testosterone, whereas solvolysis, which is evidently better than acid hydrolysis for testosterone-7α-^{3}H-sulphate and dehydroepiandrosterone-7α-^{3}H-sulphate, gave an 84 % recovery of testosterone and a 66 % recovery of dehydroepiandrosterone.

Specificity

The specificity of the method depends upon the elimination of non-steroidal impurities and the adequate separation of steroids with similar chromatographic properties which may be present in plasma. The chromatographic mobilities of several steroids in the systems employed are shown in Table III. Furthermore, evidence for lack of interference from solvents and reagents was obtained by the analysis of distilled water (Table IV). Indirect evidence of specificity is obtained from the comparison of results with those obtained by other methods.

Accuracy

The accuracy of the method was estimated by replicate analyses of known amounts of testosterone (4 ng) dehydroepiandrosterone (70 ng) and androstenedione (15 ng) added to 10 ml of distilled water. The mean recoveries were: testosterone 89 ± 32 %, dehydroepiandrosterone 85 ± 10.5 %, and androstenedione 70 ± 37 % (Table IV).

TABLE III

CHROMATOGRAPHIC MOBILITIES OF STEROIDS

Systems: 1 = Free steroids, $CHCl_3$–acetone (98:2).
2 = Heptafluorobutyrates, silica gel, benzene–ethyl acetate (95:5).
3 = Heptafluorobutyrates, silica gel, hexane–ether (8:2).
4 = Heptafluorobutyrates, cellulose, isooctane–petroleum ether (40–60°) (95:5).

R_T = Mobility in relation to testosterone, R_{TH} = mobility in relation to testosterone heptafluorobutyrate, R_{PH} = relative retention time/20α-dihydroprogesterone heptafluorobutyrate.

Steroid	*Thin-layer chromatography*				*Gas–liquid chromatography*
	R_T	R_{TH}			R_{PH}
	1	*2*	*3*	*4*	*3% XE-60 at 200°*
Testosterone	1.00	1.00	1.00	1.00	0.54
Epitestosterone	0.75	0.92	0.86	1.00	0.40
17α-Hydroxyprogesterone	0.88	—	—	—	—
Dehydroepiandrosterone	1.16	1.34	2.90	1.36	0.24
Androstenedione	1.34	2.63	6.50	1.56	0.24
Actiocholanolone	0.71	1.52	—	—	0.22
Progesterone	1.84	—	—	—	—
20α-Dihydroprogesterone	0.98	1.00	1.26	1.00	1.00
20β-Dihydroprogesterone	1.01	0.91	0.97	0.76	0.84
19-Nortestosterone	0.88	0.94	0.60	—	0.49
Pregnenolone	1.20	1.76	3.16	—	0.36
Androsterone	0.86	—	streak	—	0.20
Cholesterol	1.44	2.59	6.2	—	0.34
Androstene-3β,17β-diol	—	—	—	—	0.13

Precision

Replicate analyses were performed upon a pool female plasma (Table IV). The mean value for testosterone was 34.5 ± 5.45 ng/100 ml plasma (coefficient of variation 15.7 %). Androstenedione had a mean value of 123 ± 23 ng/100 ml plasma (coefficient of variation 18.7 %) while the mean value for dehydroepiandrosterone was 442 ± 35 ng/100 ml plasma (coefficient of variation 7.2 %).

Sensitivity

The limit of detection (a signal of twice background level) was 0.005 ng for testosterone heptafluorobutyrate and androstenedione-3-enol heptafluorobutyrate. The corresponding value for dehydroepiandrosterone heptafluorobutyrate was 0.05 ng.

The overall sensitivity of the method was evaluated in terms of the total random theoretical percentage error associated with this procedure for the determination of endogenous steroids in individual plasma samples. This involved an assessment of the random errors on each term in eqn. (1). The overall percentage error on S (e_S = (standard deviation × 100)/S) was then determined from eqn. (2) below, derived

from the usual laws for the combination of additive and multiplicative independent normal errors (COLLINS *et al.*[14]).

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

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.

Fig. 4 illustrates the total error involved in the measurement of varying amounts of testosterone, androstenedione and dehydroepiandrosterone in peripheral venous plasma. The errors increased rapidly below 7.5 ng/100 ml plasma for testosterone, 34 ng/100 ml plasma for androstenedione and 120 ng/100 ml plasma for dehydroepiandrosterone.

Levels in plasma from healthy females

The method has been applied to determine the range of plasma concentrations of the six C_{19} steroids and conjugates in the peripheral venous blood of healthy females (20–35 years). The results are summarised in Table V, in which the values are all expressed as ng/100 ml plasma, with the exception of dehydroepiandrosterone sulphate, which is given in μg/100 ml plasma.

Levels in plasma during the menstrual cycle

The steroid levels were determined in serial plasma samples removed at the same time on different days of the cycle from two normally menstruating women. The results are summarised in Table VI, and illustrated in Figs. 5 and 6. The occurrence of ovulation in both cycles was assessed by a rise in basal body temperature, and by the determination of progesterone[16] and 5β-pregnane-3α,20α-diol glucuroniside[17] in the same plasma samples.

DISCUSSION

The method has been evaluated in terms of the total random theoretical error associated with the determination of each steroid in individual plasma samples and also by replicate analyses and recovery experiments. The total random theoretical percentage error for testosterone over the range of values found in healthy women was 24 %, for androstenedione 25 %, and for dehydroepiandrosterone 29 %. This probably represents the maximal theoretical error within which the practical error must fall and this is exemplified by the fact that the precision of the method on replicate analyses was 15.7 %, 18.7 % and 7.2 % for testosterone, androstenedione and dehydroepiandrosterone, respectively. The differences between the calculated and practical errors are reasonable, in that the practical estimates were obtained from simultaneous determinations upon a pool of female plasma.

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TABLE IV

REPLICATE RECOVERY EXPERIMENTS AND ANALYSIS OF POOLED PLASMA

	Water blanks[a]			*Water recoveries*			*Plasma pool*		
	T	*D*	*A*	*T*	*D*	*A*	*T*	*D*	*A*
Amount added (ng)	—	—	—	4	70	15	—	—	—
Mean	10.2	10.2	2.0	35.6	596	105	34.6	442	123
S.D.	± 4.1	±12.4	±6.6	±12.6	± 63	± 39	± 5.45	± 33	± 23
Accuracy (%)	—	—	—	89 ± 32	85 ± 10	70 ± 37	—	—	—
Coefficient of variation (%)	—	—	—	—	—	—	15.7	7.2	18.7

[a] T = testosterone; D = dehydroepiandrosterone; A = androstenedione. All the values are expressed as ng/100 ml plasma.

The plasma concentrations for the six compounds were determined in a group of healthy women. The testosterone levels were within the range 58.5 ± 30.4 ng/100 ml plasma, which is slightly higher than that previously reported using the same method (COLLINS *et al.*[14]). A wide variation in the levels of dehydroepiandrosterone (109–2500 ng/100 ml plasma) was observed, which is in agreement with the results of other workers. Several reports have appeared upon the determination of androstenedione, and the mean value of 140 ng/100 ml plasma obtained from the present study is in good agreement, but such a wide range of concentrations (24-418 ng/100 ml) has only been observed by VAN DER MOLEN *et al.*[5] (100–410 ng/100 ml).

The physiological significance of testosterone glucuroniside and testosterone sulphate is not known, but their concentrations in plasma might be associated with the regulation of free plasma testosterone levels. Furthermore, HADD AND BLICKENSTAFF[18] have indicated that testosterone glucuroniside may have some androgenic

TABLE V

FREE AND CONJUGATED STEROIDS IN PERIPHERAL VENOUS PLASMA OF NORMAL WOMEN

	No. of samples analysed	*Range*	*Mean ± S.D.*
		ng/100 ml plasma	
Testosterone	24	19.8–176.0	58.5 ± 30.4
Dehydroepiandrosterone	24	109.0–2500.0	655.0 ± 510.0
Androstenedione	21	24.0–418.0	140.0 ± 100.0
Testosterone glucuroniside[a]	10	14.0–295.0	92.8 ± 81.3
Testosterone sulphate[a]	10	1.0–119	18.5 ± 38.8
		µg/100 ml plasma	
Dehydroepiandrosterone sulphate[a]	10	21.9–67.7	32.6 ± 13.8

[a] Expressed as testosterone or dehydroepiandrosterone.

TABLE VI

FREE AND CONJUGATED STEROIDS THROUGHOUT TWO MENSTRUAL CYCLES

	Range	*Mean*	*±S.D.*
Steroids in 24-day cycle	*ng/100 ml plasma*		
Testosterone	9.8–163.0	52.2	54.0
Testosterone glucuroniside	14.8–65.1	36.7	17.3
Testosterone sulphate	28.2–126.6	59.0	33.1
Androstenedione	51.0–258.9	139.0	70.0
Dehydroepiandrosterone	504.0–782.0	632.0	105.0
	µg/100 ml plasma		
Dehydroepiandrosterone sulphate	6.03–60.7	16.95	17.30
Steroids in 28-day cycle	*ng/100 ml plasma*		
Testosterone	5.5–46.2	16.7	14.5
Testosterone glucuroniside	1.3–32.8	8.6	12.2
Testosterone sulphate	7.6–72.4	26.0	21.8
Androstenedione	26.8–92.4	52.4	23.2
Dehydroepiandrosterone	207.0–710.0	431.0	152.2
	µg/100 ml plasma		
Dehydroepiandrosterone sulphate	8.04–28.48	17.38	8.99

properties, and SAEZ *et al.*[6] have reported a high plasma level of testosterone sulphate in patients with ovarian and adrenal tumours. In the present study the level of testosterone glucuroniside varied widely (14–295 ng/100 ml plasma) with a mean value of 92.8. The results are comparable with those of BURGER *et al.*[10] and VAN DER MOLEN *et al.*[5] and in any individual plasma sample the testosterone glucuroniside level was usually higher than the free testosterone. The values for testosterone sulphate were usually low or undetectable, and the mean value for ten random samples was 18.5 ng/100 ml plasma.

A mean value of 32.6 ± 13.8 μg/100 ml plasma was obtained for dehydroepiandrosterone sulphate after correction for losses during solvolysis. A wide range of values was observed but the mean was similar to that reported by LAMB *et al.*[20].

The finding of androstenedione, testosterone and dehydroepiandrosterone sul-

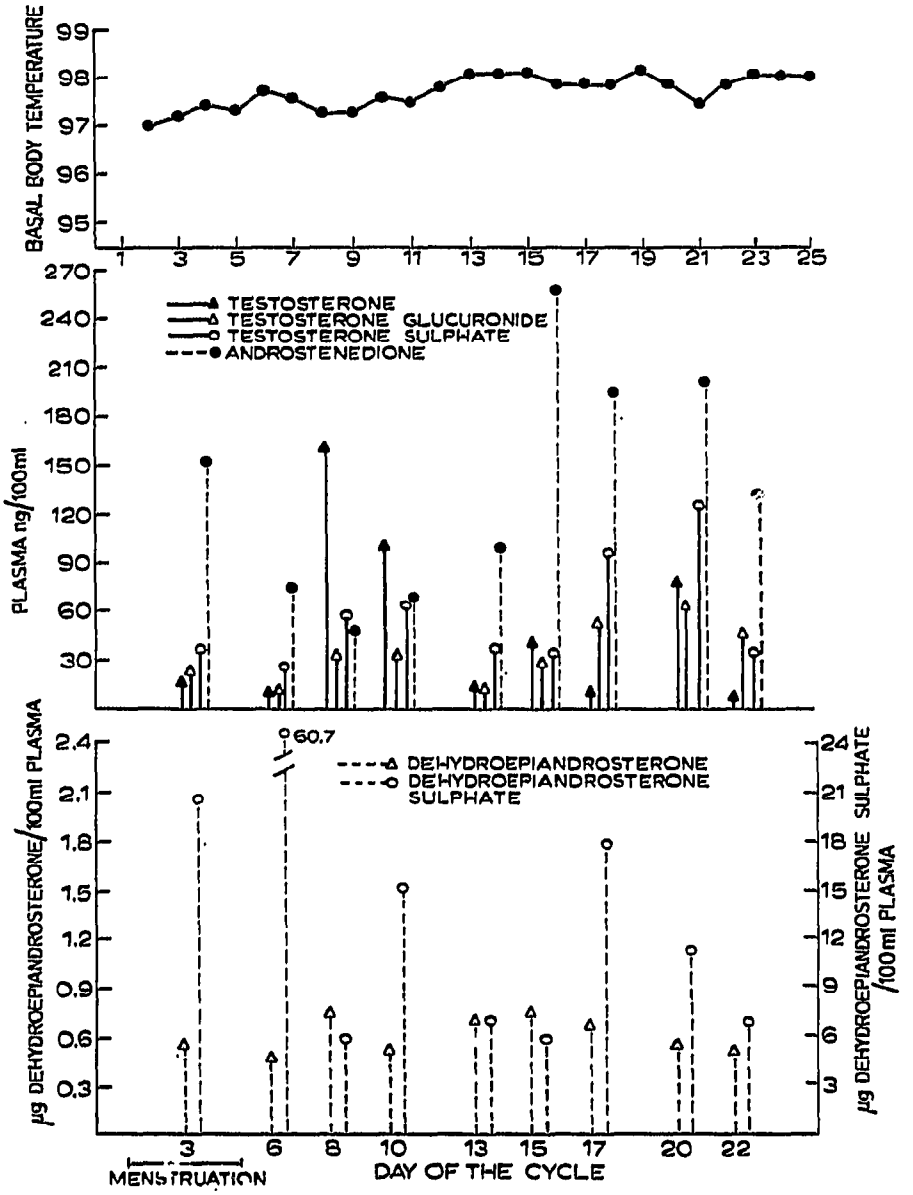

Fig. 5. Steroid levels in serial plasma samples from a 24-day cycle of a healty menstruating woman.

phate in ovarian venous blood (HORTON *et al.*[21] and AAKVAAG AND FYLLING[10]) suggested that serial analysis of these compounds may show fluctuations throughout the menstrual cycle. In this connection, preliminary studies by LOBOTSKY *et al.*[22] revealed higher levels of testosterone in peripheral plasma at the time of ovulation and in the luteal phase, and HORTON[23] has reported a similar rise in the level of androstenedione during the second half of the cycle. These findings are consistent with the incubation studies of SAVARD *et al.*[24], who observed elevated incorporation of labelled acetate into dehydroepiandrosterone, androstenedione and testosterone, by ovarian stromal tissue removed during the luteal phase of the cycle.

In the present study the levels of all six compounds were different in the two cycles investigated. The values of all compounds during the 28-day cycle (Table VI

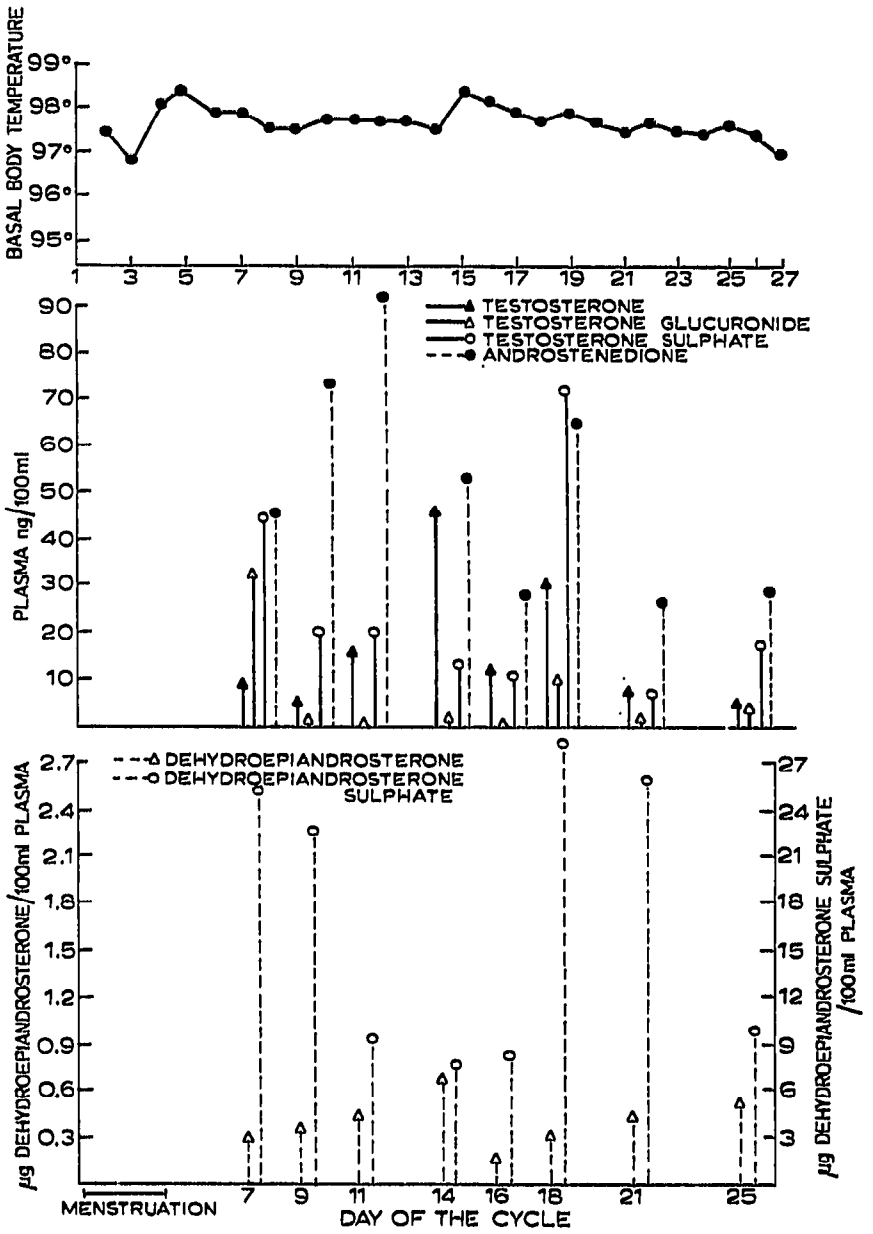

Fig. 6. Steroid levels in serial plasma samples from a 28-day cycle of a healthy menstruating woman.

and Fig. 6) were very low, whereas those from the 24-day cycle were well within the range for healthy women. In addition there was considerable variation in the level of most of the compounds in serial plasma samples collected throughout both cycles. It is premature to draw any definite conclusions from the study of only two cycles, but there does appear to be a biphasic pattern in the peripheral plasma levels of testosterone, androstenedione and dehydroepiandrosterone sulphate. The level of dehydroepiandrosterone was relatively constant but the relationship between testosterone, testosterone glucuroniside and testosterone sulphate appears to be more complex and requires further study. The range of values observed in the group of healthy women and the variations observed throughout the two menstrual cycles emphasise the need for serial analyses in these investigations.

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