Ultramicro Determination of Plasma Testosterone by Electron-Capture Detection of Testosterone Diheptafluorobutyrate

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1. A new highly sensitive and accurate ultramicro method for the estimation of testosterone in human peripheral plasma is described. The method uses paper- and thin-layer-chromatographic separation of plasma testosterone, which is determined as testosterone diheptafluorobutyrate by electron-capture detection after gas-liquid chromatography. 2. The average difference between duplicates is $\pm 2\%$ (range 1–5%) with as little as 2.5 ml. of human male peripheral plasma. With 10 ml. of plasma the method is sensitive enough for the accurate determination of testosterone in human female plasma. The high order of accuracy is achieved by the use of a radioactive label and an internal standard for gas chromatography, and by obtaining several gas chromatograms from the same plasma sample. 3. As little as 40 μg . of peripheral plasma testosterone can be detected. The method is 20 times as sensitive as electron-capture techniques with the monochloroacetate derivative. 4. The method is simpler and quicker than double-isotope-derivative methods, and slightly more sensitive. The advantages of the method, which is specific for testosterone, are its high sensitivity and accuracy, which are achieved with relative convenience.

The concentrations of steroid hormones in systemic blood require extremely sensitive methods for their determination. A recent study involving the search for small variations in the peripheral plasma concentrations of testosterone (17 β -hydroxyandrost-4-en-3-one) in the human male demanded the use of an accurate, highly sensitive and specific method. In the past only very laborious double-isotope-derivative techniques (Hudson, Coghlan, Dulmanis, Wintour & Ekkel, 1963; Riondel *et al.* 1963) have approached anything like the required sensitivity. A recent attempt to develop a simpler method for testosterone in plasma (Brownie, van der Molen, Nishizawa & Eik-Nes, 1964) and measurement by gas-liquid chromatography with electron-capture detection of testosterone monochloroacetate, though reasonably successful for some determinations, was not sensitive enough to be able to observe small variations, about 5%, in human male plasma concentrations. Despite the considerably higher electron-affinity of testosterone heptafluorobutyrate esters compared with the monochloroacetates first observed by Clark & Wotiz (1963), the only electron-capture methods described to date with these fluorinated derivatives are those of Exley (1966, 1967*a,b*), Exley & Chamberlain (1966*a,b*), Exley &

Feder (1967) and Vermeulen (1967). No full and detailed method has been published for plasma testosterone in which these derivatives are used. The lack of development of highly sensitive techniques with these esters appears to have been due to differences in the quality of the commercially available esterification reagent, and to confusion because testosterone 17-monoheptafluorobutyrate and testosterone diheptafluorobutyrate (produced by esterification of the 3-enol) could be produced by different esterification conditions (Scheme 1). Nakawaga, McNiven, Forchielli, Vermeulen & Dorfman (1966) reported the formation of a low yield of the diheptafluorobutyrate and stated that they could not recommend its use for quantitative measurement because of its instability and poor esterification yield. These difficulties were overcome by Exley (1966), who prepared the pure heptafluorobutyric anhydride reagent, and Exley & Chamberlain (1967), who prepared almost quantitative yields of testosterone diheptafluorobutyrate. The diheptafluorobutyrate is five times as electron-absorptive as testosterone 17-monoheptafluorobutyrate, and is possibly one of the most sensitive of all the electron-capturing derivatives yet devised for steroids. Unfortunately the compound is unstable on silica gel and alumina and therefore not

Scheme 1. Formation of the mono- and di-heptafluorobutyrate esters of testosterone by different concentrations of heptafluorobutyric anhydride (HFBA).

readily separable from other esterified compounds. A method involving this derivative must therefore purify the plasma sample sufficiently before esterification to enable immediate gas-liquid chromatography to be done after heptafluorobutyration. Despite this instability the high sensitivity afforded by testosterone diheptafluorobutyrate made it worth while to develop a method using it.

The present paper describes a new highly sensitive and accurate method for the determination of millimicrogram and sub-millimicrogram amounts of plasma testosterone that uses the high electron-absorptivity of testosterone diheptafluorobutyrate for determination by electron-capture detection after gas-liquid chromatography.

MATERIALS AND METHODS

Solvents and reagents. The solvents and reagents used were of analytical-grade quality whenever this grade was available. All solvents were distilled before use. Ether (peroxide-free) was washed with saturated FeSO_4 solution and water and then distilled. Ethanol was refluxed over CaH_2 for 1 hr. before distillation. Acetone (British Drug Houses Ltd., Poole, Dorset) was redistilled twice before use. Benzene was Mallinckrodt Nanograde (Camlab, Cambridge). Water was distilled after deionization. Heptafluorobutyric anhydride was prepared by distilling heptafluorobutyric acid (Minnesota Mining and Manufacturing Co., St Paul,

Minn., U.S.A.) and collecting the fraction distilling at 120°. The freshly distilled acid was then refluxed with a large excess of P_2O_5 for 1 hr. before distillation; the heptafluorobutyric anhydride boiling at 108–110° was then collected. The anhydride was stable for some months when stored at 5°.

All the steroids were obtained from the Medical Research Council reference collection. Testosterone diheptafluorobutyrate and progesterone heptafluorobutyrate standards were prepared by the method of Exley & Chamberlain (1967). These standards were not crystallized after preparation, but the products were checked by flame-ionization gas-liquid chromatography, which indicated that there was no free testosterone after esterification. A small by-product (4% by weight) was also formed, but this did not interfere with quantitative determinations. This by-product was consistently formed in the same percentage ratio and gave a very small additional peak well separated from the major peak on the gas chromatograms.

Although unstable on thin-layer media, testosterone diheptafluorobutyrate and progesterone heptafluorobutyrate were stable in acetone solution for some months even at high dilution. Catalytic breakdown of these heptafluorobutyrate esters did not occur on the stainless-steel gauzes for a period of up to 2 hr.

[1,2- $^3\text{H}_2$]Testosterone (specific radioactivity 17 c/m-mole) and [1,2- $^3\text{H}_2$]progesterone (1 c/m-mole), purchased from The Radiochemical Centre, Amersham, Bucks., were found by thin-layer chromatography to be 99% radiochemically pure and were used without further purification. The phosphor solution used for scintillation counting was made up by adding 2,5-diphenyloxazole (3.0 g.) and

1,4-bis-(5-phenyloxazol-2-yl)benzene (0.3 g.) to reagent-grade toluene (11.).

Paper and thin-layer chromatography. Paper chromatography was performed on Whatman no. 2 paper in the system *n*-hexane-methanol-water (20:13:7, by vol.). Paper chromatograms were equilibrated at 25° overnight and run at the same temperature for at least 4 hr.

Thin-layer silica gel GF₂₅₄ and HF₂₅₄ (Merck according to Stahl, Darmstadt, Germany) was washed with boiling methanol before use. The washed silica gel was dried at 100° for 24 hr. Thin-layer chromatography was performed with both media, which were spread in sheets approx. 250 μ thick. After being spread, the coated plates were heated at 100° for 30 min. and then stored in a desiccator until required. The developing system benzene-ethyl acetate (1:1, v/v) was used with the GF₂₅₄ silica gel and benzene-ethyl acetate (2:1, v/v) with the HF₂₅₄ material. Thin-layer and paper chromatograms were inspected under u.v. light (λ 250 m μ).

Precautions. All glassware had to be kept scrupulously clean for the later stages of the method (the second thin-layer and esterification steps), and all the precautions of a sensitive ultramicro analytical technique were taken. Solvents and thin-layer media had to be checked for gas-chromatographic peaks before use, and dust and dirt in the laboratory had to be kept to a minimum.

Apparatus. General glassware was first thoroughly cleaned by a chromic acid-H₂SO₄ mixture, washed with distilled methanol and water and finally rinsed with double-distilled acetone before use. After use the esterification tubes were rinsed with water and with double-distilled acetone, since putting the tubes through the chromic acid again merely introduced more contamination. Tubes (B10; Quickfit & Quartz Ltd., Stone, Staffs.) used for the thin-layer and esterification stages were 'siliconized' to prevent adsorption on the glass surfaces. The tubes were immersed in a solution of dichlorodimethylsilane in toluene (5%, v/v) and then rinsed twice with toluene before a final rinse with methanol and heating in an oven at 110° for 30 min.

Radioactive counting was performed on a Packard Tri-Carb liquid-scintillation spectrometer (model 3314). The efficiency for ³H was 33%.

Electron-capture gas-liquid chromatography. The gas-liquid chromatography apparatus with electron-capture detection used was described by Exley (1967a). An F & M model 402 performed equally well. In general a negative 50 v pulse of 0.5 μ sec. duration was applied across a 200 mc parallel-plate detector (of the type described by Lovelock, 1963) every 200 μ sec. A solid-injection system, a modification of that described by Menini & Norymberski (1965), was used. Solutions containing the material to be chromatographed were pipetted into wells made in polytetrafluoroethylene plates and allowed to soak into $\frac{1}{4}$ in. stainless-steel gauzes, which were injected into the chromatographic column by a polytetrafluoroethylene tap that allowed the gauze to enter but excluded air.

All-glass columns (150 cm. $\times$ 0.4 cm.) were used. They were packed with silicone-treated Gas-Chrom P (100-120 mesh) coated with 1% (w/w) SE-30 as described by Brooks & Hananeh (1963). The carrier gas used was argon containing 5% methane, with a flow rate of 50 ml./min. The detector and column temperature was 180° with a flash heater at 210°.

Standard analytical procedure

A flow sheet of the method is shown in Scheme 2.

Plasma extraction. Plasma obtained by centrifugation of heparinized blood (immediately after collection) was stored at -15° until required. The testosterone in such plasma was stable after several months' storage.

A solution of [1,2-³H₂]testosterone (approx. 25000 disintegrations/min.) in ethanol was evaporated to dryness in a 50 ml. glass tube, and 2.5 ml. of plasma and 7.5 ml. of aq. 0.9% sodium chloride were added. Each series included duplicate plasmas, and a water blank containing only [1,2-³H₂]testosterone was added to the series on numerous occasions. The contribution of the radioactive testosterone was such that it was never detectable by electron capture. The sample was then further diluted by *N*-NaOH (1 ml.) and extracted in small separation funnels with ether (3 $\times$ 20 ml.). The pooled ether extract was washed with water (2 $\times$ 5 ml.), filtered over Na₂SO₄ and evaporated to dryness on a water bath.

First thin-layer chromatogram. The residue was transferred to a 10 ml. centrifuge tube by ethyl acetate (0.5 ml.), which was then removed with a stream of N₂ at 40°, the extract being concentrated in the tip of the tube to a volume of about 50 μ l. Thin-layer chromatography was carried out on a GF₂₅₄ plate prepared as described above. An ascending chromatogram was run in the solvent system benzene-ethyl acetate (1:1, v/v). A standard of about 2.5 μ g. of epitestosterone was run on a separate lane. Epitestosterone was used instead of testosterone to prevent any contamination from standard testosterone used in this manner. Epitestosterone had the same *R_F* as testosterone in the thin-layer- and paper-chromatographic systems used, and any contamination of this was readily separable on the 1% SE-30 gas-chromatographic column.

At this stage of purification testosterone in plasma ran more slowly than the epitestosterone standard; consequently the area of silica gel that was scraped off was from a line 1 mm. behind the standard to a line 1 cm. nearer the origin than the standard. The zone (1.5 cm. wide) was scraped into a small sintered-glass funnel (porosity no. 3) and eluted with aq. 95% (v/v) methanol (3 $\times$ 1 ml.) into a small B10 tube. The methanol was removed with a stream of N₂.

Paper chromatogram. The dried residue was transferred to a paper-chromatographic strip (30 cm. $\times$ 7 cm.) by ethyl acetate (3 $\times$ 50 μ l.) and run in the system *n*-hexane-methanol-water (20:13:7, by vol.). Epitestosterone standard was run on a separate lane. After overnight equilibration the system was run for 4 hr. The paper was allowed to dry at room temperature after development, and the epitestosterone standard was detected under u.v. light. The appropriate section (2 cm. wide) of the sample lane was cut up into fine pieces and the sample was eluted by aq. 95% (v/v) methanol (3 ml.). The methanol was then filtered through cotton wool, the filtrate collected in a B10 tube and the solvent removed with a stream of N₂.

Second thin-layer chromatogram. The dried residue was transferred by ethyl acetate (3 $\times$ 50 μ l.) to a HF₂₅₄ thin-layer-chromatographic plate. After development, the appropriate sample zone (1.5 cm.) was scraped off into a narrow B7 (Quickfit & Quartz) tube, and water (0.2 ml.) and Nanograde benzene (0.5 ml.) were added. The tube was vigorously shaken and then centrifuged, and as much as possible of the benzene supernatant was transferred to a

Peripheral plasma (2.5 ml.) + approx. 10000 counts/min. of $[1,2-^3\text{H}_2]$ testosterone + saline + NaOH

Scheme 2. Outline of the present method for the measurement of testosterone in plasma.

scrupulously clean B10 tube. The silica gel was then extracted by a further 0.5 ml. of benzene and the process repeated. The solvent of the combined benzene extract was removed with a stream of N_2 .

Heptafluorobutyrylation. The dried residue was esterified by heptafluorobutyric anhydride. Nanograde benzene (5 $\mu\text{l.}$) and heptafluorobutyric anhydride (5 $\mu\text{l.}$) were quickly added to the tube, which was then tightly stoppered. Samples were esterified at 70° for 30 min. by heating in an aluminium block. Excess of reagents was quickly removed by dry N_2 at 40° , and 0.2 ml. of double-distilled acetone containing a progesterone heptafluorobutyrate internal standard (300 $\text{m}\mu\text{g./ml.}$) was immediately added. The tube

was shaken after the stopper had been rinsed with acetone.

Assay of radioactivity. Portions (2 $\times$ 0.05 ml.) from the 0.2 ml. of acetone solution were pipetted into a scintillation phial (20 ml.) and evaporated to dryness under N_2 . Phosphor solution (10 ml.) was added to the phials and the portions were assayed for ^3H by a Packard Tri-Carb liquid-scintillation spectrometer.

Determination by gas-liquid chromatography. Gas-liquid chromatography followed by electron-capture detection of the testosterone diheptafluorobutyrate was performed as described by Exley (1967a) under the conditions mentioned above. Inclusion of the internal standard (progesterone heptafluorobutyrate), which behaved similarly to testo-

Fig. 1. Calibration graph for testosterone. The calibration graph shows the ratio (r) of the peak height of testosterone diheptafluorobutyrate (H_1) to that of 15.0 mμg. of the internal standard (progesterone heptafluorobutyrate) (H_2), plotted against known amounts of testosterone used for esterification. The conditions used were: pulse duration 0.5 μsec., pulse repetition 200 μsec., pulse voltage 50 V, temperature of column and detector 180°, temperature of flash heater 210°. The column was 1% SE-30 and the carrier gas was argon + 5% methane with a flow rate of 50 ml./min.

sterone diheptafluorobutyrate but was readily separable from it on the gas chromatogram, allowed a correction to be made for any losses in transference to the column and for any losses on the column. Since the amount of material adsorbed on the steel gauzes was not necessarily quantitative, the amount actually injected on the column was not known, but inclusion of the internal standard enabled a ratio of the peak heights of the steroid esters to be obtained.

The amount of testosterone in the sample was calculated with the aid of a factor obtained from a calibration curve by using standards. A calibration curve was drawn by plotting the ratio (r) of the peak height of testosterone diheptafluorobutyrate (H_1) to that of 15.0 mμg. of progesterone heptafluorobutyrate (H_2) against known amounts of testosterone (0.1–3.0 mμg.) used for the esterification (Fig. 1). A calculation with the calibration curve showed that a ratio ($H_1/H_2=r$) of 1.0 was obtained when the amount of progesterone heptafluorobutyrate was 6.0 times as great as that of testosterone diheptafluorobutyrate; thus, since a 60.0 mμg. internal standard was added to each sample, the amount of testosterone in millimicrograms in the tube was 10 r mμg.

The ^3H assay performed immediately before the gas-liquid chromatography enabled a correction to be made for all losses up to that point. Correcting for a radioactive recovery of y counts/min. from an original addition of x counts/min., and use of 2.5 ml. of plasma:

$$\text{Concn. (mμg./100 ml. of plasma)} = 400xr/y$$

Portions (25–40 μl.) of the acetone sample solution were applied to the stainless-steel gauzes, and three or more

chromatograms were run for each sample. The final result was obtained by calculating the mean r of these chromatograms.

RESULTS

Accuracy. Although assessment of the accuracy by recovery methods is not necessary with a method that is internally corrected for recovered tritium, the sensitivity of the method depends on the overall accuracy. The average overall recovery of the method, assessed as ^3H counts/min. obtained immediately before gas chromatography against ^3H counts/min. originally added, was 30% ($n=40$), with a range of 20–40%, the biggest loss occurring across the paper-chromatographic step.

Duplicate amounts of 10 and 20 mμg. of authentic testosterone gave an average result that suggested that 9.7 and 19.7 mμg. respectively had been added, i.e. an average recovery of 97.7%. The accuracy of each single determination was assessed by calculating the standard deviation about the mean (100%) of three or more r values. The average accuracy of these individual determinations when several chromatograms were run was $\pm 2.3\%$ ($n=30$).

Precision. Quadruplicate determinations of a sample were made and the coefficient of variation was $\pm 3\%$. Sixteen pairs of duplicate determinations (Table 1) gave a mean duplicate difference of $\pm 2\%$ (range 1–3.3%). An estimate of the precision s of the method was calculated in a manner similar to that of Brown, Bulbrook & Greenwood (1957), i.e. estimate of $s.d. = s = \sqrt{(\epsilon d^2/2N)}$ from the differences between duplicate determinations. The s value from the 16 duplicate determinations (Table 1) was 28 mμg./100 ml. of plasma for an average of 870 mμg./100 ml. of plasma, i.e. a coefficient of variation of $\pm 3.2\%$.

Sensitivity. The qualitative sensitivity of the method, the least amount of material from plasma that could be measured distinguishable from zero, was 100 mμg., assessed as testosterone diheptafluorobutyrate (mol. wt. 680); this is equivalent to 40 mμg. of testosterone (mol. wt. 288). The quantitative sensitivity can be estimated from this figure (Brown & Blair, 1960), and calculations show that, for a method using 2.5 ml. of plasma whose recovery is 30%, a plasma concentration of 80 mμg./100 ml. is necessary to obtain an accuracy of $\pm 5\%$. Thus, with 10 ml. of plasma, a concentration of 20 mμg./100 ml. can be estimated with this accuracy; therefore the method is satisfactory for the determination of testosterone concentrations in human female plasma, provided that the amount of plasma is increased from 2.5 ml. to 10 ml.

Calculation of the qualitative sensitivity by the method of Brown & Blair (1960), defined as $ts/\sqrt{N}$, where t is Student's parameter and s the precision

Table 1. Precision between duplicate estimates of testosterone in plasma by the diheptafluorobutyrate method

Mean results	Mean of duplicates $\pm$ s.d. ($\mu\text{g.}/100\text{ml. of plasma}$)	s.d. of duplicates (%)	Mean results	Mean of duplicates $\pm$ s.d. ($\mu\text{g.}/100\text{ml. of plasma}$)	s.d. of duplicates (%)
269	*263 $\pm$ 7	2.7	1002	991 $\pm$ 11	1.1
256			980		
256	*264 $\pm$ 8	3.0	780	772 $\pm$ 8	1.0
271			764		
1069	1036 $\pm$ 34	3.3	1049	1018 $\pm$ 30	3.0
1002			988		
900	900 $\pm$ 10	1.1	914	925 $\pm$ 10	1.1
890			935		
888	914 $\pm$ 26	2.9	910	900 $\pm$ 10	1.1
940			890		
1089	1110 $\pm$ 27	1.9	1130	1094 $\pm$ 36	3.0
1131			1058		
824	838 $\pm$ 14	1.7	901	885 $\pm$ 15	1.7
853			870		
986	918 $\pm$ 22	2.4	1112	1127 $\pm$ 15	1.4
940			1142		

* 2.5 ml. of rat plasma.

for N duplicate determinations, gave a value of $12\mu\text{g.}/100\text{ml. of plasma}$ ($P 0.05$), and this means that $100\mu\text{g.}$ of plasma testosterone was just distinguishable from zero. Calculated in this way the qualitative sensitivity was therefore less than half of that actually found in practice. The reason for this discrepancy is most probably that s was calculated from a range of duplicates whose mean was of the order of $900\mu\text{g.}/100\text{ml.}$, and, since s usually increases with concentration (Brown & Blair, 1960), a lower value of s would be expected for lower concentrations.

Specificity. The behaviour of all the steroids that might possibly be present in plasma when chromatographed in the thin-layer benzene-ethyl acetate (1:1, v/v) system was studied by Brownie *et al.* (1964). An area corresponding to $R_{\text{testosterone}}$ (the present method used epitestosterone, which has an R_F value identical with that of testosterone in this system) of 0.75–1.2 was eluted from this thin-layer chromatogram; and, as Brownie *et al.* (1964) showed, the steroids most probably present in this zone were epitestosterone, testosterone, pregnanediol and 20α - and 20β -hydroxypregn-4-en-3-one. Paper chromatography in the hexane-methanol-water system separated all these except testosterone and epitestosterone. The last thin-layer-chromatographic system was necessary to remove contamination from the paper, which severely interfered with electron-capture detection of testosterone diheptafluorobutyrate. Gas-liquid chromatography on 1% SE-30 at 180° of testosterone and epitestosterone diheptafluorobutyrate

separated the epimers. Thus the chromatographic separations used in the method allowed a specific determination of testosterone from plasma to be made, provided that there was no contamination from reagents and solvents (see the Discussion section).

The specificity of the method in its present form was checked by acetylating the sample after the second thin-layer chromatogram and then running the testosterone acetate in a further thin-layer-chromatographic system before esterification. Estimation of testosterone acetate 3-enol heptafluorobutyrate by an appropriate calibration graph against progesterone heptafluorobutyrate gave exactly the same result as that obtained by the standard method without acetylation.

Typical gas-chromatographic recordings of standard testosterone diheptafluorobutyrate and standard progesterone heptafluorobutyrate, and of plasma samples processed by the method, are shown in Fig. 2. Although many of the chromatograms were reasonably clean, showing a few insignificant contaminating peaks, quite a number of the chromatograms unfortunately possessed larger extraneous peaks that were probably due to contamination from solvents or thin-layer media. No extraneous peaks, however, ever appeared in the vital regions of the testosterone diheptafluorobutyrate and the internal-standard retention-time areas. Blanks with water, processed through the full method, produced gas chromatograms that showed no deflexion in the testosterone diheptafluorobutyrate retention-time area, thus proving that

Fig. 2. Gas-chromatographic tracings obtained from standards and plasma samples. The conditions for gas chromatography were as described in Fig. 1. (a) Testosterone diheptafluorobutyrate standard (4.0 $\mu\text{g.}$), peak A, and progesterone heptafluorobutyrate (internal standard, 10.0 $\mu\text{g.}$), peak B. (b) Testosterone diheptafluorobutyrate peak produced from 40 $\mu\text{l.}$ portion of a sample of testosterone from human peripheral plasma (900 $\mu\text{g.}/100\text{ml.}$). Peak A is 4.0 $\mu\text{g.}$ of testosterone diheptafluorobutyrate and peak B is 2.0 $\mu\text{g.}$ of progesterone heptafluorobutyrate. (c) Testosterone diheptafluorobutyrate peak produced from a 40 $\mu\text{l.}$ portion of a sample of testosterone from human peripheral plasma (700 $\mu\text{g.}/100\text{ml.}$). Peak A is 1.5 $\mu\text{g.}$ of testosterone diheptafluorobutyrate and peak B is 6.0 $\mu\text{g.}$ of progesterone heptafluorobutyrate. (d) A small testosterone diheptafluorobutyrate peak produced from a 40 $\mu\text{l.}$ portion of a sample from human peripheral plasma (1000 $\mu\text{g.}/100\text{ml.}$) of accidental low recovery (3%). Peak A is 0.1 $\mu\text{g.}$ of testosterone diheptafluorobutyrate and peak B is 6.0 $\mu\text{g.}$ of progesterone heptafluorobutyrate. The peak is almost like a blank over the testosterone diheptafluorobutyrate retention-time area, as expected. (e) Contaminated gas chromatogram obtained from human peripheral plasma by using a 25 $\mu\text{l.}$ portion of the sample. Peak A is 2.0 $\mu\text{g.}$ of testosterone diheptafluorobutyrate and peak B is 10.0 $\mu\text{g.}$ of progesterone heptafluorobutyrate. The peak heights are, however, still accurately calculable despite the extraneous dirt on the chromatogram.

there was no interference in this vital region. Progesterone heptafluorobutyrate produced a small noticeable side peak (corresponding to 4% known impurity), but this did not interfere with the quantitative assessment.

DISCUSSION

The present method allows very accurate assays to be made. The average accuracy between duplicates is $\pm 2\%$ (range 1–5%) when as little as 2.5 ml. of plasma is used. This accuracy is achieved by the use of an internal standard for gas chromatography, by an accurate estimate of the radioactive label and by obtaining several chromatograms for each individual sample in order to assess r accurately. The method is more than twice as accurate as most gas-liquid-chromatographic methods, where the average accuracy is $\pm 5\%$.

Most of the results given in Table 1 were obtained from two subjects with specially chosen high values (the average concentration in males is 700 $\mu\text{g.}$ of testosterone/100 ml. of plasma). Values of up to 1.1 $\mu\text{g.}/100\text{ml.}$ of plasma, however, have been previously reported for young virile males.

The molar response of testosterone diheptafluorobutyrate to electron capture is 4.52×10^3 coulombs/mole (Exley & Chamberlain, 1967). Exley (1967a) detected 10 $\mu\text{g.}$ of pure testosterone diheptafluorobutyrate with high-sensitivity settings on the gas-chromatographic apparatus. The minimum detectable in a biological sample by the present method is 100 $\mu\text{g.}$, i.e. ten times the amount of the pure standard. The sensitivity settings were, however, not as high as those for the pure standard. Although the method has been used exclusively for male plasma, it is sensitive enough for female plasma provided that the amount of plasma used is increased from 2.5 ml. to 10 ml. The method is 20 times as sensitive as the testosterone monochloroacetate electron-capturing technique of Brownie *et al.* (1964) and five times as sensitive as a method using testosterone 17-monoheptafluorobutyrate, and is probably the most sensitive method yet devised for this hormone in plasma. The method is steroid-specific for testosterone, but contamination from solvents and thin-layer media may ruin the chromatogram, and indeed the main disadvantage is contamination from solvents, glassware and thin-layer media. The high sensitivity and accuracy for human male plasma compensates for the extra care needed to avoid contamination.

The method is simpler and quicker than double-isotope-derivative techniques and this improvement has been achieved without any decrease in specificity. The advantages of the method are, therefore, its high accuracy, its sensitivity and its

specificity coupled with its relative ease of performance.

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REFERENCES

- Brooks, C. J. W. & Hanaineh, L. (1963). *Biochem. J.* **87**, 151.
 Brown, J. B. & Blair, H. A. F. (1960). *J. Endocrin.* **20**, 331.
 Brown, J. B., Bulbrook, R. D. & Greenwood, F. C. (1957). *J. Endocrin.* **16**, 41.
 Brownie, A. C., van der Molen, H. J., Nishizawa, E. E. & Eik-Nes, K. B. (1964). *J. clin. Endocrin.* **24**, 1091.
 Clark, S. J. & Wotiz, H. H. (1963). *Steroids*, **2**, 535.
 Exley, D. (1966). *Excerpta med. int. Congr. Ser.* no. 101, p. 11.
 Exley, D. (1967a). *Mem. Soc. Endocrin.* no. 16, p. 117.
 Exley, D. (1967b). *Symp. Gas Chromatography, Paris.* (in the Press).
 Exley, D. & Chamberlain, J. (1966a). *Excerpta med. int. Congr. Ser.* no. 111, p. 116.
 Exley, D. & Chamberlain, J. (1966b). *Symp. Gas Chromatography, Rome.* New York: Academic Press Inc. (in the Press).
 Exley, D. & Chamberlain, J. (1967). *Steroids*, **10**, 509.
 Exley, D. & Feder, H. H. (1967). *Acta endocr., Kbh.*, Suppl. 111, p. 100.
 Hudson, B., Coghlan, J. P., Dulmanis, A., Wintour, M. & Ekkel, J. (1963). *Aust. J. exp. Biol. med. Sci.* **41**, 235.
 Lovelock, J. E. (1963). *Analyt. Chem.* **35**, 474.
 Menini, E. & Norymberski, J. K. (1965). *Biochem. J.* **95**, 1.
 Nakawaga, K., McNiven, N. L., Forchielli, E., Vermeulen, A. & Dorfman, R. I. (1966). *Steroids*, **7**, 329.
 Riondel, A., Tait, J. F., Gut, M., Tait, S. A. S., Joachim, E. & Little, B. (1963). *J. clin. Endocrin.* **23**, 620.
 Vermeulen, A. (1967). *Symp. Gas Chromatography, Paris.* (in the Press).