CHROM. 5192

MEASUREMENT OF PLASMA TESTOSTERONE AS THE IODOMETHYLDIMETHYLSILYL ETHER BY GAS–LIQUID CHROMATOGRAPHY

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SUMMARY

A method is described for the measurement of plasma testosterone as the iodomethyldimethylsilyl ether derivative, by gas–liquid chromatography and electron capture detection. As the derivative is stable to chromatography on alumina columns these are used in the preliminary purification of plasma extracts. Specificity, precision, accuracy are all satisfactory and amounts below 1 ng may be measured in 8 ml of plasma. Comparison of results has been made with those from competitive binding assays.

INTRODUCTION

A variety of derivatives has been used for the measurement of testosterone in plasma by gas–liquid chromatography (GLC) with electron capture detection; *e.g.* the monochloracetate[1], the heptafluorobutyrate[2] and the hexadecafluorononoate and eicosafluoroundecanoate[3].

EABORN *et al.*[4] introduced iodomethyldimethylsilyl ethers (IDMSE) for GLC of steroids. These derivatives are very sensitive to electron capture detection by the ^{63}Ni detector and are more stable than the corresponding trimethylsilyl ethers (TMSE). However, they are also more polar than the TMSE derivatives on selective phase columns (*e.g.* XE-60). This can be an advantage since interference by less polar materials is reduced and preliminary purification of plasma testosterone extracts can be kept to a minimum[3,5]. This paper describes a method for the quantitative determination of plasma testosterone as the IDMSE derivative.

The trivial names used in the text represent the following steroids: testosterone, androst-4-en-17β-ol-3-one; androsterone, 5α-androstan-3α-ol-17-one; aetiocholanolone, 5β-androstan-3α-ol-17-one; DHEA, androst-5-en-3β-ol-17-one; pregnenolone, pregn-5-en-3β-ol-20-one; dihydrotestosterone, 5α-androstan-17β-ol-3-one; 1,4-testosterone, androsta-1,4-dien-17β-ol-3-one; 20-OH progesterone, pregn-4-en-20ξ-ol-3-one.

MATERIALS

Two Pye 104 gas chromatographs (Models 74 and 84) each fitted with pulse operated ^{63}Ni electron capture detectors were used. Glass columns (length 92 and 153 cm, 0.4 cm I.D.) were fitted with Nilo K glass-to-metal connectors[4] and packed with either 1.5 % XE-60, 2 % SE-30 or mixed phases consisting of 1 % XE-60 + 0.8 % HiEff-8B (cyclohexanedimethanol succinate) + 1 % SE-30. These were coated by evaporation onto "Supasorb" (acid washed and silanised; BDH Ltd.) or "Diatomite CQ" (Pye-Unicam) of 100–120 mesh. The columns were conditioned at 250° (XE-60 and mixed phase) or 275° (SE-30) with nitrogen (Air Products Ltd.) for at least 48 h before use.

All chemicals were of "Analar" quality where available. Ethanol, acetone and cyclohexane were redistilled before use. Ether, isopentane, benzene and hexane were further purified by passing them through columns containing 50 g of activated alumina (Woelm-neutral, activity Grade 1).

Bromomethyldimethylchlorosilane and diethylamine were redistilled, the latter over KOH pellets, and then passed through columns containing 10 g of activated alumina. Both were stored in sealed tubes at 4°. [7-^{3}H]Testosterone (30.8 Ci/mM)[7,8] was diluted with toluene to give a working solution of approximately 500,000 d.p.m. per ml.

Scintillation counting was performed with a Packard Tricarb (Series 314E) counter (^{3}H efficiency 35 %) using glass counting vials containing 10 ml of PPO/POPOP phosphor prepared as follows: PPO (2,5-diphenyloxazole), 10 g; POPOP (1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene), 1 g; toluene to 2 l.

Non-radioactive steroids were obtained from Steraloids Ltd.

SGE 10 μl micro syringes for gas chromatography were fitted with 11.5 cm needles and obtained from Pye-Unicam Ltd.

Glass columns for alumina chromatography (20 × 1 cm I.D.) were fitted with a 50 ml reservoir at the top. The bottom was annealed to a 4 cm length capillary (0.2 cm I.D.) and plugged with silanised glass wool.

All glassware was treated before use with 2 % Decon 75 detergent, followed by chromic acid in 50 % H_2SO_4 overnight. After treatment with water, sodium metabisulphite solution and distilled water, the glassware was dried at 200°.

DEVELOPMENT OF METHOD

Extraction of plasma

Plasma was separated by centrifugation from heparinised blood immediately after collection and stored at —20°. To 2 ml (male) or 6–8 ml (female) of thawed plasma in a 30 ml separating funnel was added 25 μl of the [^{3}H]testosterone solution (approx. 12,500 d.p.m.). After shaking, the plasma was allowed to stand at room temperature for 15 min. The volume was then made up to 8 ml, if necessary, with distilled water, 0.6 ml 2 N NaOH added, and extraction carried out with 3 × 8 ml of ether. The pooled ether extract was washed with 1 × 2 ml 2 % acetic acid and 2 × 2 ml water, evaporated on a rotary flash evaporator and the residue re-dissolved in 5 ml 70 % ethanol in water. This solution was partitioned against 5 ml hexane in a small separating funnel and the 70 % ethanol (lower layer) evaporated to dryness.

1st Chromatography stage

The extract was transferred with 1 × 1 ml, 2 × 0.5 ml benzene washes to a column containing 5 g of alumina (Woelm neutral containing 9 % water) prepared in benzene. Chromatography was as follows: (1) 15 ml benzene — discarded; (2) 14 ml 1.5 % ethanol in benzene — discarded; (3) 10 ml 1.5 % ethanol in benzene — collected.

Fraction (3) which contained testosterone, DHEA and other steroids was evaporated to dryness in a 25 ml flask, 0.1 ml ethanol, 1 ml water and 5 ml isopentane added and the stoppered flask was well shaken. The contents were transferred to a 10 ml centrifuge tube and centrifuged for 2 min (1400 g) to ensure complete separation of the two phases. The isopentane (top layer) was then transferred with a Pasteur pipette to a 10 ml tapered C14 tube and blown to dryness with N_2 in a water bath at 30°. (It is important to ensure complete absence of water in the tube at this stage.)

Preparation of IDMSE derivatives

The reagent iodomethyldimethylchlorosilane was difficult to synthesize. Therefore the following procedure was used for preparing IDMSE derivatives[4].

Hexane (2 ml), bromomethyldimethylchlorosilane (0.2 ml) and diethylamine (0.1 ml) were mixed by means of a vortex vibrator. The contents were centrifuged for 2 min (1400 g) and 3–4 drops of the supernatant added to the steroid extract. The tube was stoppered and left at room temperature for 45 min to form the bromomethyldimethylsilyl ethers (BDMSE).

The reagent was then evaporated to dryness with N_2 at 37° and 3–4 drops of acetone, saturated with sodium iodide, were added. The stoppered tube was sealed with parafilm and incubated at 37° for a further 30 min. During this reaction the BDMSE derivatives were quantitatively converted to IDMSE derivatives by the process of ionic halide exchange. The reaction is summarised in Fig. 1.

Hexane (1 ml) was then added to precipitate out the excess of sodium iodide and, after centrifuging, the supernatant was transferred to a small tube. To this was

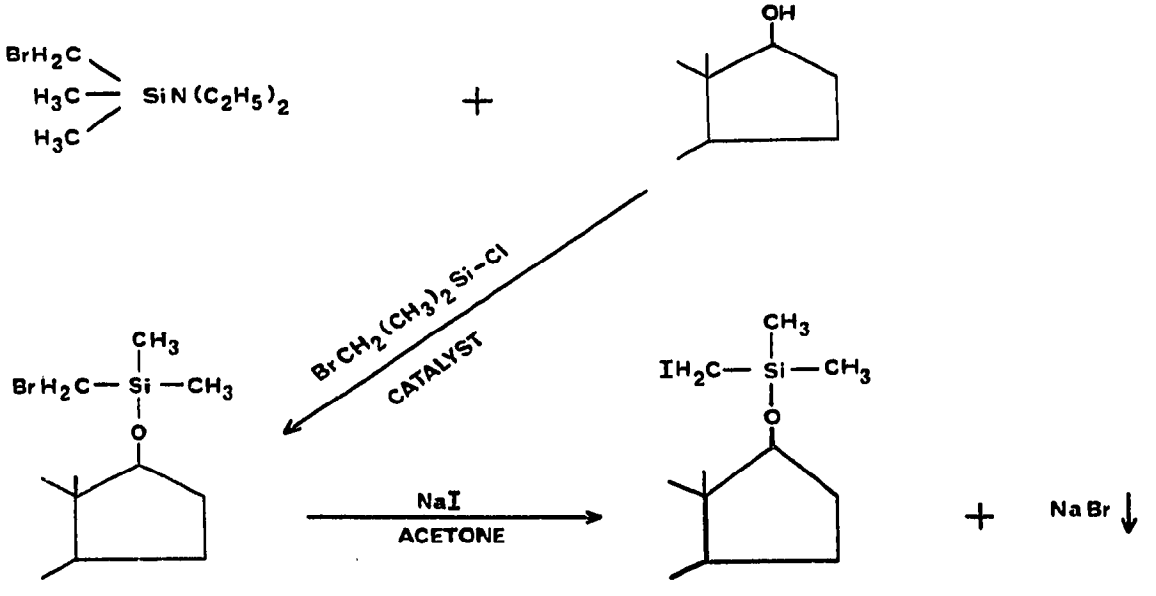


Fig. 1.

added a further 0.5 ml of hexane wash from the reaction tube. The contents were blown to dryness and redissolved in 0.2 ml hexane.

2nd Chromatography stage

The hexane extract was transferred to a column containing 6 g of alumina, as used in the first chromatography stage and prepared in cyclohexane. This was followed by 2 × 0.2 ml washes of cyclohexane from the reaction tube. Chromatography was as follows: (1) 15 ml cyclohexane pre-wash — discarded; (2) 30 ml 1 % acetone in cyclohexane pre-wash — discarded. This fraction would contain androsterone, DHEA, pregnenolone, aetiocholanolone and 5α-dihydrotestosterone IDMSE if present. (3) 3 ml acetone–cyclohexane–benzene (1:49:50) pre-wash — discarded; (4) 6 ml acetone–cyclohexane–benzene (1:49:50) — collected.

This last fraction contained testosterone IDMSE and also 20α- and 20β-hydroxyprogesterone if present. The amounts of the various solvents in the two chromatography stages must be pre-determined for each batch of alumina. A typical elution pattern is shown in Fig. 2.

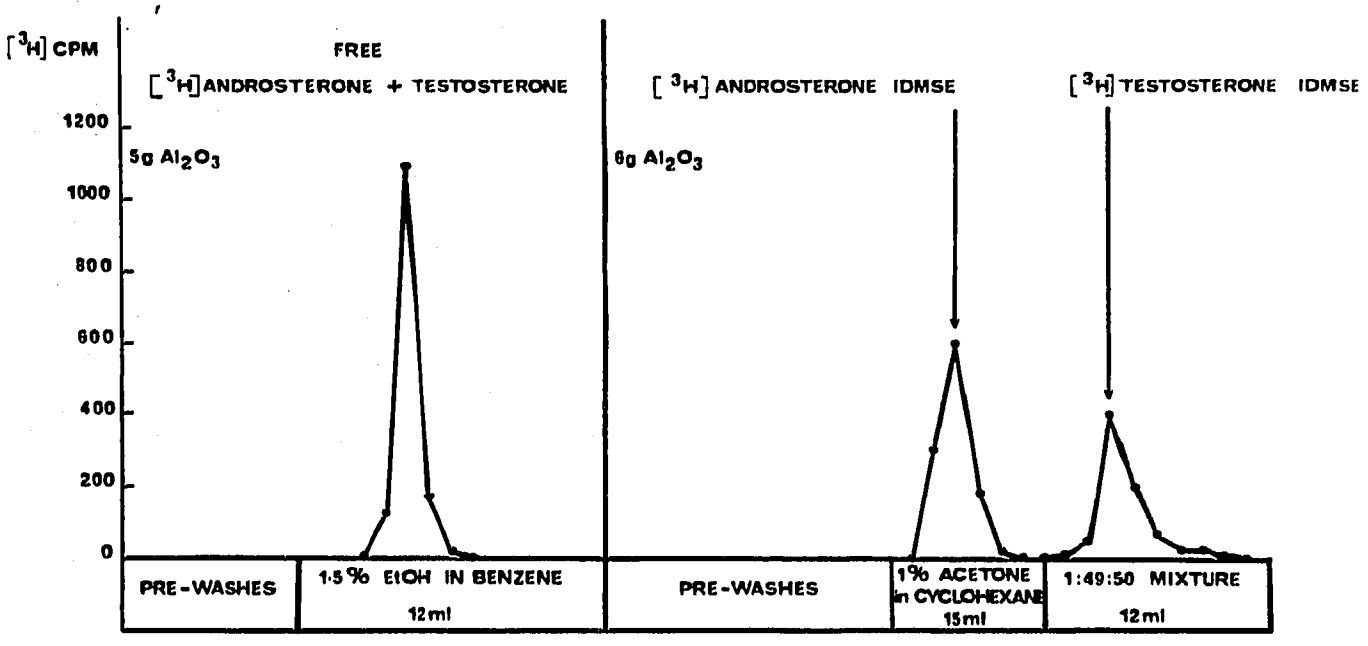


Fig. 2. Elution patterns of free[^{3}H]androsterone and [^{3}H]testosterone and their IDMSE derivatives from alumina columns.

Recoveries of [^{3}H]testosterone

The testosterone IDMSE fraction was evaporated to dryness in a stream of N_2 and immediately transferred with a Pasteur pipette into a Dreyers tapered agglutination tube (capacity 1 ml) with successive minimal washes of hexane. Internal standard for gas chromatography purposes (10 ng 1,4-testosterone IDMSE) was added and the contents were taken to dryness with N_2. Hexane (50 μl) was added and 10 μl removed for counting. Comparison of samples was made with 3 standards each containing 5 μl of [^{3}H]testosterone. No loss of counting efficiency due to quenching by the plasma extracts has been found.

Another 10 μl of extract was removed in a microsyringe for injection into the

gas chromatography column. The remainder was sealed into the Dreyers tube by means of a flame and stored at 4° for further use if necessary; IDMSE extracts keep several months under these conditions.

Gas–liquid chromatography

Normally the 153 cm 1.5 % XE-60 column was used, although for normal male plasma the shorter 92 cm column was satisfactory. Operating conditions were as follows: N_2 column flow, 100-120 ml/min; column temperature, 240°–245°; attenuation, 5×10^{-10} or 2×10^{-10} A; ^{63}Ni ECD oven, 300°; pulse interval, 500 μsec.

The column injector heaters were maintained at the same temperature as the column oven. When not in use the column temperature was lowered to 200°.

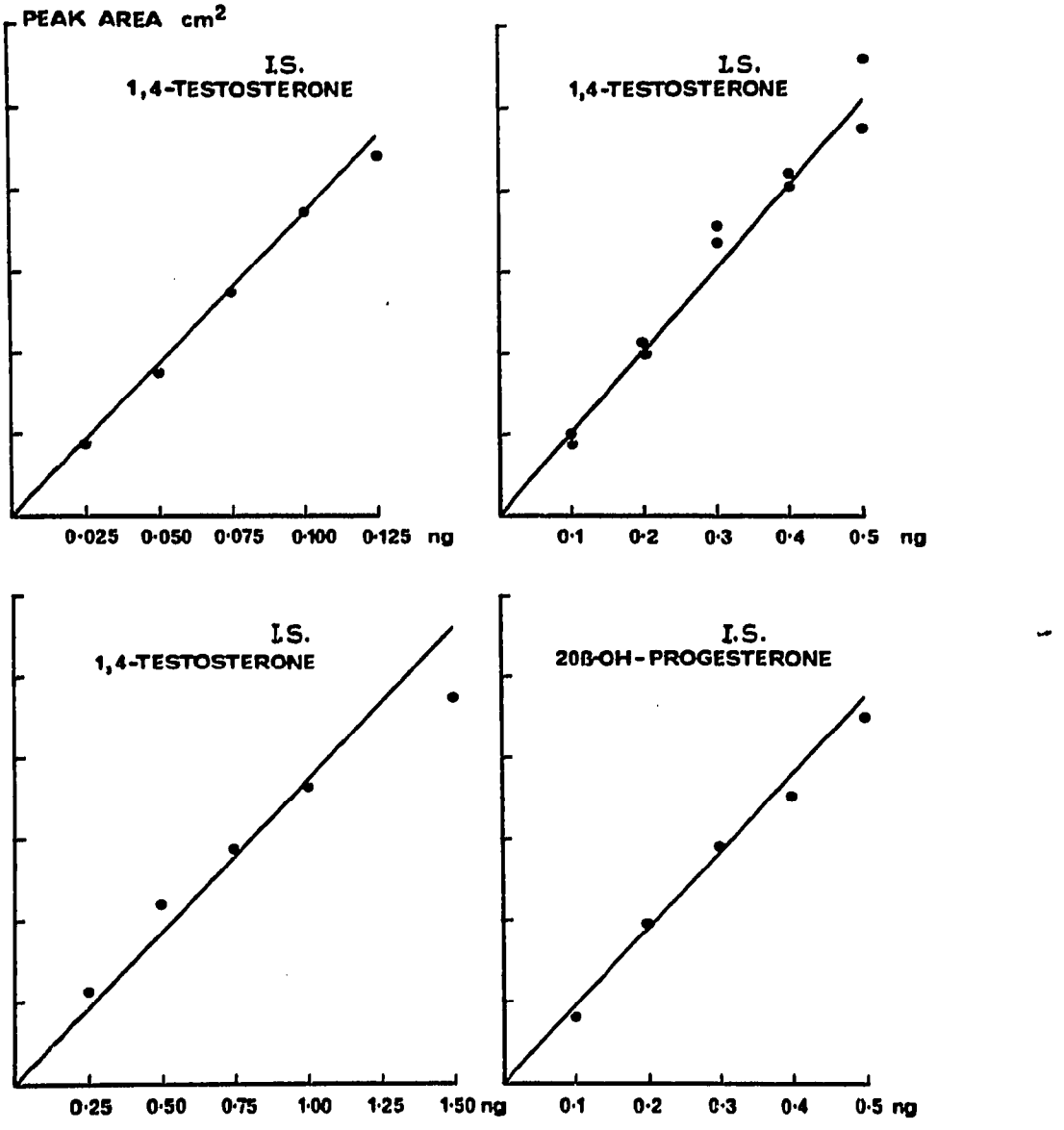


Fig. 3. Calibration curves of testosterone IDMSE using 1,4-testosterone and 20β-OH-progesterone IDMSE derivatives as internal standards.

Quantitation of testosterone peak

The areas of the testosterone and internal standard peaks were measured by triangulation as recommended by HORNING[9] and the testosterone area expressed as a ratio to that of the internal standard. Results in nanogram were read from prepared calibration curves which were checked daily and calculated (in ng/100 ml) as follows:

$$\text{Plasma testosterone} = \frac{\text{ng per sample} \times \text{ng internal standard} \times 100 \times 100}{\text{ml sample injected} \times \%\ [^3\text{H}]\text{testosterone recovery}}$$

Typical calibration curves are shown in Fig. 3 and a typical gas chromatogram of 1 ng of standard testosterone IDMSE is shown in Fig. 4.

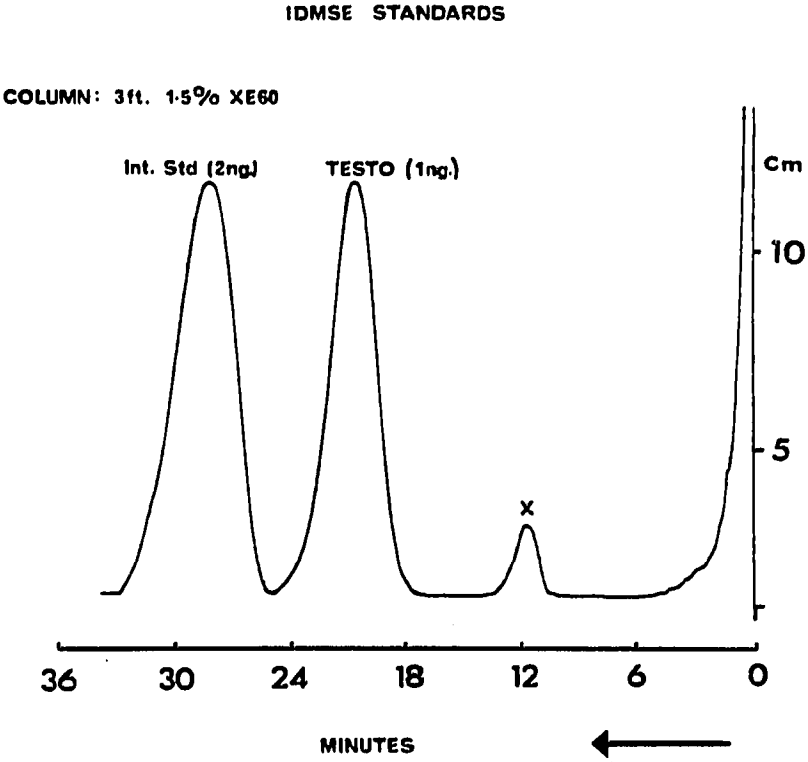


Fig. 4. Gas chromatogram of standard testosterone IDMSE (1 ng) and 1,4-testosterone IDMSE (I.S.). Peak X is impurity present in I.S. Attenuation, 5×10^{-10}.

Sensitivity of detector

Maximum sensitivity with the Pye-104 instrument was obtained during a detector pulse interval of 500 μsec. However, linearity was strictly limited to a range of 0.1–0.8 ng at this setting. Therefore, with concentrations above 0.8 ng it was advisable to inject less material into the column. A higher attenuation may be necessary for levels of testosterone below 0.1 ng.

A series of typical chromatograms of plasma extracts are given in Figs. 5a–d.

RESULTS

Recoveries of testosterone

The mean recovery of tritiated testosterone from 88 consecutive assays was 33.1 % (S.D. = ± 9.7). Recoveries of 5 ng and 10 ng of non-radioactive testosterone

from 8 ml of water (14 assays) processed through the method, and corrected for [^{3}H]testosterone losses, were 100.9 % (S.D. = ± 14.2 %).

Precision and sensitivity

The precision (*s* estimate of standard deviation) calculated[10] from differences between 14 duplicate assays of plasma containing 0–100 ng/100 ml (mean = 47.0) was 7.0. From this the lower limit of sensitivity was calculated to be 6.0 ng/100 ml.

A further 7 samples from a pool of female plasma gave a mean value of 44 ng/100 ml (S.D. = ± 4).

Specificity

At the levels of testosterone encountered in the analytical samples (0–0.8 ng) absolute proof of specificity was virtually impossible to obtain. The evidence obtained for the specificity of the method was as follows: (1) The plasma testosterone had the same retention time as standard testosterone IDMSE in both the selective phase (XE-60), non-selective phase (SE-30) and mixed phase columns; (2) There is no significant difference between results obtained using the XE-60 and the mixed phase columns as shown in Table I; (3) endogenous testosterone IDMSE and [^{3}H]testosterone IDMSE from a pooled plasma extract were eluted simultaneously from the second alumina column prior to gas chromatography (*cf.* DEVELOPMENT OF METHOD Section), as shown in Fig. 6; (4) No other steroid was found in the testosterone IDMSE fraction which gave the same retention time as testosterone IDMSE (see Table II).

TABLE I

PLASMA TESTOSTERONE; SELECTIVE AND MIXED PHASE (HYBRID) COLUMNS

The results are not significantly different.

Plasma extract measured (ml)	*Testosterone, 10^{-9} g per sample*	
	XE-60 column	*HYBRID column*
0.8	0.22	0.18
0.26	0.25	0.25
0.8	0.24	0.30
1.0	0.90	0.84
1.0	0.24	0.25
1.2	0.08	0.08
0.6	0.90	0.85
mean	0.404	0.393
S.D. ±	0.344	0.317

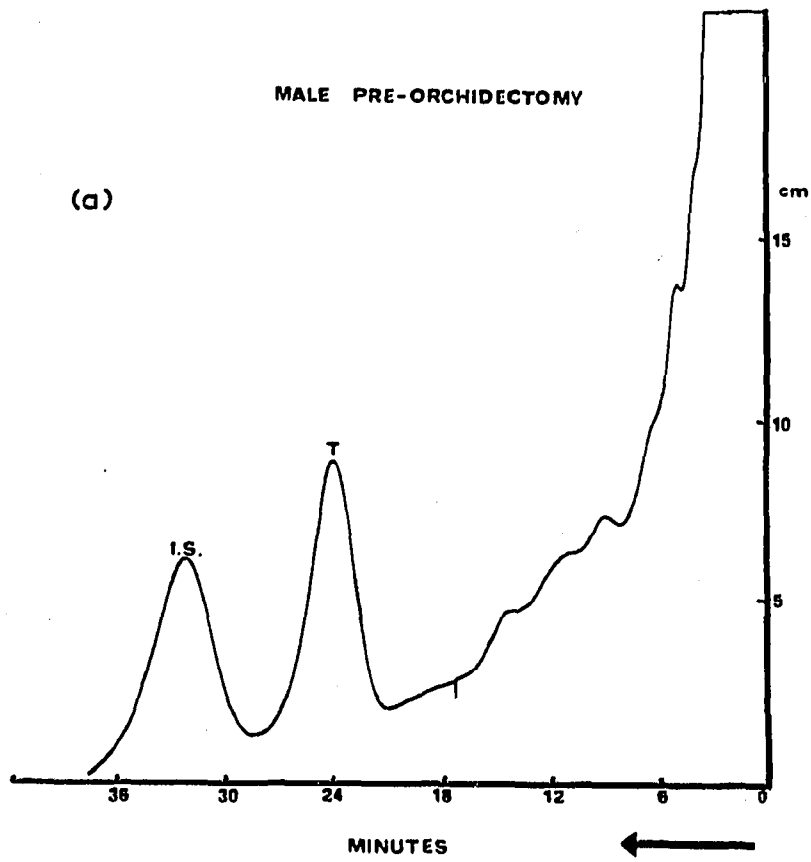
Comparison with results of competitive binding assays

Results have been compared with those obtained by competitive protein binding assays performed in other laboratories and these are presented in Table III.

The overall correlation coefficient is 0.95 but there are several discrepancies.

Blank values

The sensitivity of the method depends to a large extent on the values obtained for the blank. In the early stage of the development of the method water blanks gave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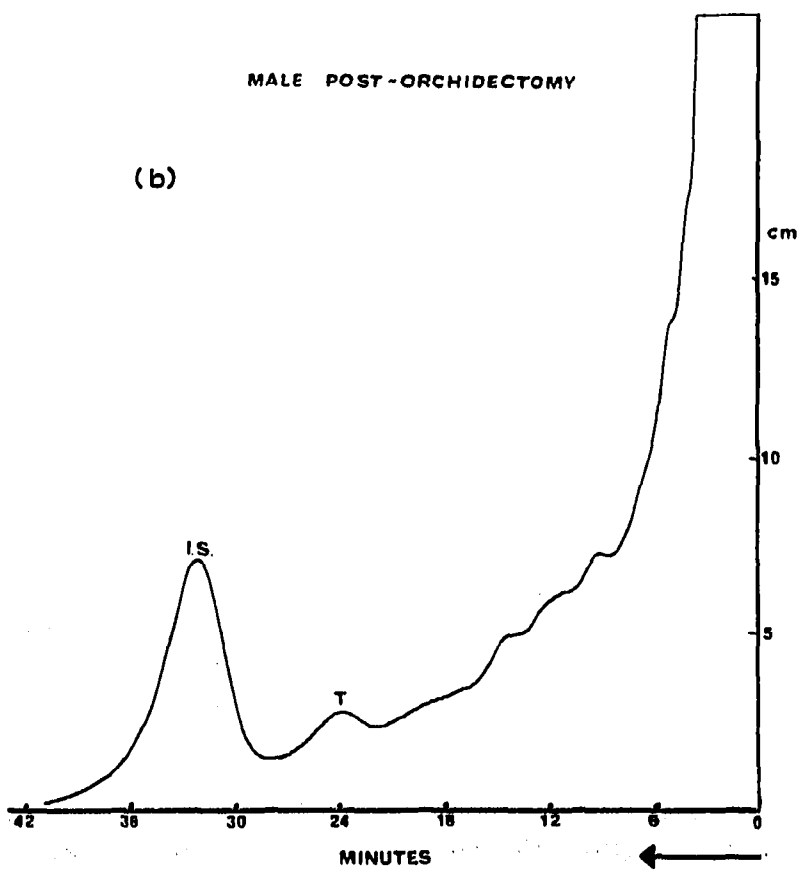
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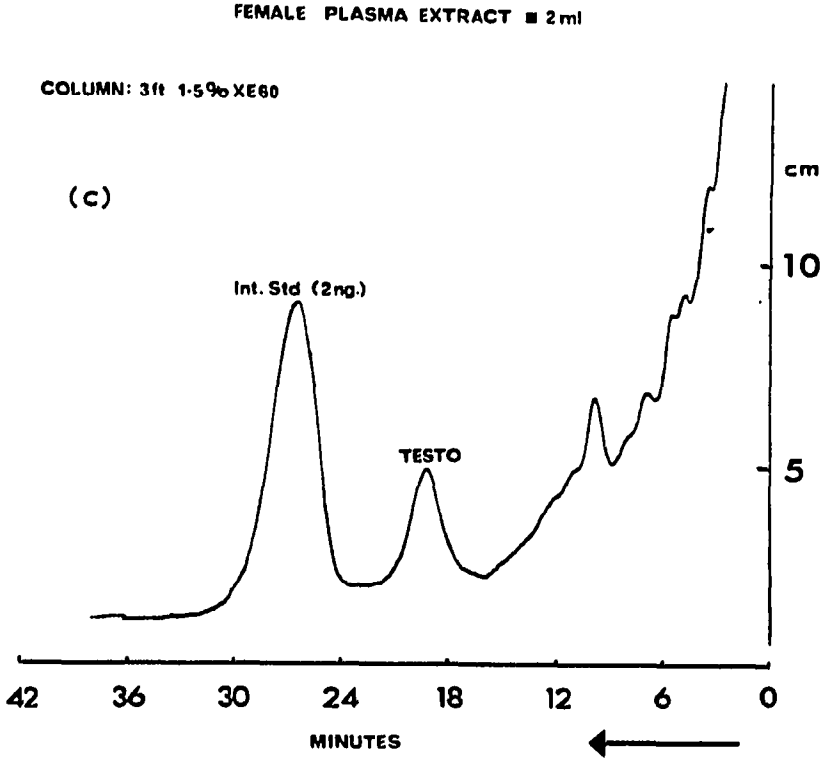

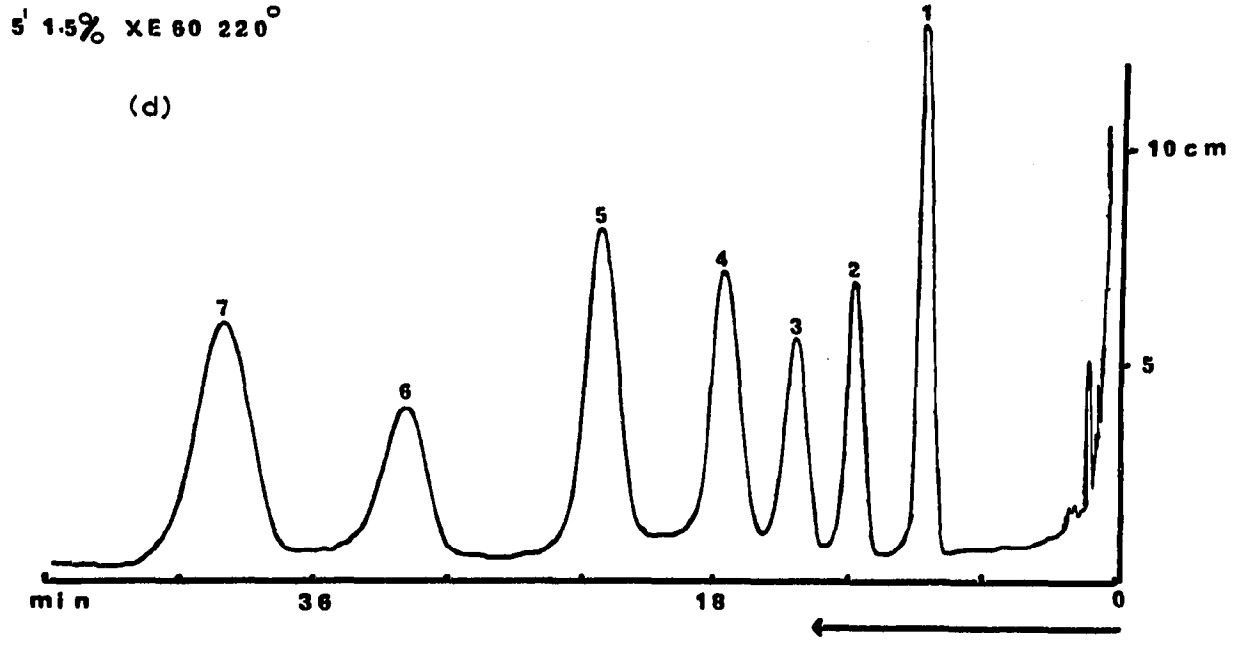

Fig. 5. (a) Male plasma, prostatic cancer pre-orchidectomy; amount injected ≡ 0.6 ml plasma; testosterone = 514 ng/100 ml; attenuation = 5×10^{-10}. (b) As (a); post-orchidectomy; testosterone = 38 ng/100 ml. (c) Female plasma; amount injected ≡ 1.6 ml plasma; testosterone = 44 ng/100 ml; attenuation = 5×10^{-10}. (d) Standard steroid IDMSE derivatives. 1 ng of each: 1 = androsterone, 2 = DHEA, 3 = 5α-dihydrotestosterone, 4 = pregnenolone, 5 = testosterone 6 = 20β-OH progesterone, 7 = pregn-5-ene-3β,20β-diol (di-IDMSE).

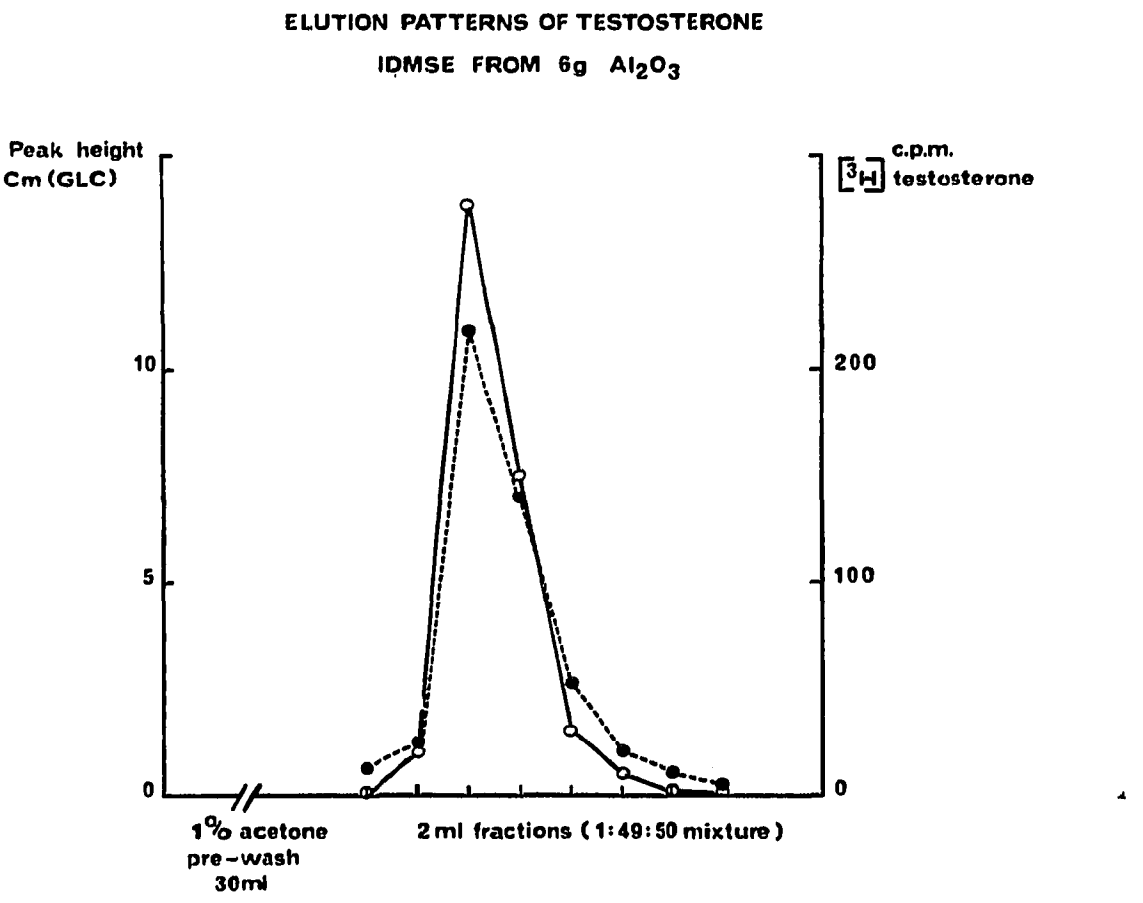

Fig. 6. Alumina column elution pattern of [^{3}H]testosterone and endogenous plasma testosterone IDMSE derivatives from plasma pool extract. ●---●, c.p.m. [^{3}H]testosterone (scintillation counter); ○—○, endogenous testosterone (peak height, GLC).

results equivalent to up to 20 ng testosterone per 100 ml. Rigorous cleaning of glassware with chromic acid generally led to a reduction of blank value to less than 6 ng/100 ml and therefore no correlation for blank was then necessary.

Ten plasma samples were obtained from 2 patients with prostatic cancer after pituitary destruction with ^{90}Y. In seven of these samples no testosterone could be detected; in two samples results of 2 ng and 5 ng per 100 ml were obtained which were below the calculated sensitivity of the method; the other samples gave a value of 7 ng per 100 ml. No testosterone was found in plasma from a male patient on oestrogen plus corticosteroid therapy (see Fig. 7).

DISCUSSION

The use of IDMSE derivatives for estimation of testosterone in plasma from normal men and women and from patients with a variety of diseases, by gas–liquid chromatography and electron capture detection is satisfactory in that reasonable accuracy, precision, sensitivity and specificity can be obtained. Because alumina columns are used instead of thin-layer chromatography, the labour involved in the preliminary purification stages is less. Furthermore, recoveries from these columns is more consistent and it is possible to assay four plasma samples in one working day.

TABLE II

RETENTION TIMES OF STEROIDAL IDMSE DERIVATIVES

Carrier gas N_2 at 85 ml/min; column temperature, 225°.

Steroid IDMSE	*Relative retention times (RRT) of steroid IDMSE derivatives*		
	XE-60 (153 cm)	*SE-30 (153 cm)*	*Mixed phase (92 cm)*
5α-Androstan-3α-ol-17-one	1.00 (11.8 min)	1.00 (10.2 min)	1.00 (16.5 min)
5β-Androstan-3α-ol-17-one	1.14	1.01	1.19
Androst-5-en-3β-ol-17-one	1.42	1.25	1.47
5β-Androstan-17β-ol-3-one	1.58	—	—
5β-Pregnan-3α-ol-20-one	1.62	—	—
5α-Androstan-17β-ol-3-one	1.69	1.38	1.74
5β-Pregnan-3β-ol-20-one	1.96	—	—
Pregn-5-en-3β-ol-20-one	2.03	1.79	2.15
Androst-4-en-17α-ol-3-one	2.11	—	—
Cholest-5-en-3β-ol	2.29	—	—
5α-Androstane-3α,17β-diol (di-IDMSE)	2.50	4.84	—
Androst-4-en-17β-ol-3-one	2.68	1.45	2.63
Androst-5-ene-3β,17β-diol (di-IDMSE)	3.50	6.24	—
Androsta-1,4-dien-17β-ol-3-one	3.62	1.49	—
5β-Pregnane-3α,20α-diol (di-IDMSE)	4.22	—	—
Pregn-4-en-20β-ol-3-one	4.53	—	4.31
Pregn-4-en-20α-ol-3-one	4.75	—	—
Pregn-5-ene-3β,20β-diol (di-IDMSE)	4.95	—	—
Pregn-5-ene-3β,20α-diol (di-IDMSE)	5.92	—	—

The isopentane/water partition is a valuable technique for removing unwanted materials from the extracts but it is at this stage of the method that the highest losses of testosterone occur (20 %). Accurate standardisation of the alumina for the second column chromatography stage is important as successful recoveries are dependent on the amount of water added for deactivation. With Woelm neutral alumina about 9 % is the optimal amount but this must be predetermined by recovery assays of [^{3}H]testosterone IDMSE which should be at least 80 %.

The conversion of BDMSE to IDMSE derivatives by halide ionic exchange as earlier described[4] was cumbersome when applied to nanogram quantities of steroid. The new incubation procedure at 37° with sodium iodide–acetone solution is complete in 30 min and the completeness of the reaction has been confirmed by mass spectrometry.

The specificity of the method is of the utmost importance but is very difficult to evaluate. The evidence presented does not preclude measurement of foreign material but suggests that this is highly unlikely. No steroids have been found that will interfere with the testosterone IDMSE peak, with the exception of 5α-androstane-3α,17β-diol (di-IDMSE). However, this latter compound is separated from the testosterone IDMSE fraction by the second alumina chromatography procedure.

The comparisons between the IDMSE method and the competitive binding assays are not always in agreement. No attempt has been made at this stage to investigate these differences except to provide an objective comparison between the two methods. However, a better correlation has been obtained than in a similar set of comparisons where the overall IDMSE results were 17.5 % lower than those from a simple competitive binding assay[14].

TABLE III

COMPARISON BETWEEN GLC AND COMPETITIVE BINDING

Sex	*Diagnosis*	*Plasma testosterone ng/100*	
		IDMSE	*Binding method*
Female	Testicular feminisation pre-op 1	779	600
	Testicular feminisation pre-op 2	894	650
	Testicular feminisation post-op 1	47	40
	Testicular feminisation post-op 2	37	40
	Testicular feminisation pre-op 1	210	412
	Testicular feminisation post-op 1	35	21
	Testicular feminisation post-op 2	22	19
	Idiopathic hirtuitism	46	59
	Idiopathic hirtuitism	44	38
	Idiopathic hirtuitism	32	18
	Idiopathic hirtuitism	39	32
	Idiopathic hirtuitism (on dexamethazone)	28	26
	Idiopathic hirtuitism (on dexamethazone)	40	43
	Idiopathic hirtuitism (on dexamethazone)	59	27
	Baldness	48	59
	Diagnosis unknown	30	29
	Normal	43	48
Male	Ca Prostate	514	552
	Ca Prostate (post orchidectomy)	38	16
	Hypoganadism	56	11
	Normal	898	998
	Normal	295	430
Female	Plasma pool 1	51	55[a]
	Plasma pool 2	36	42[a]
	Plasma pool 3	24	33[a]

[a] Carried out by Miss M. SWAIN[11], St. Thomas's Hospital, London, S.E. 1; All others done by Dr. C. ANDRE,[12,13], St. Mary's Hospital, London, W.2.

Agreement on results on both XE-60 and mixed phase columns is important evidence of specificity as incorporation of non-selective phase into selective phase columns has been shown to remove interfering material from urinary androgen metabolite TMSE derivatives[6]. Similar retention times of the plasma peak with standard testosterone IDMSE on both XE-60 and SE-30 columns also gives a measure of specificity. The latter column required considerable "priming" with up to 50 ng standard testosterone IDMSE to eliminate adsorption effects (*e.g.* peak tailing) so non-selective phases were not considered for quantitative assay because of possible contamination of plasma samples occurring (*cf.* HORNING[9]).

The calculated lower limit of sensitivity of the method (*i.e.*, 6.0 ng/100 ml plasma) is dependent on both the percentage recovery of testosterone through the method and the amount of plasma extract injected into the gas chromatograph. Although it is possible to measure as little as 0.025 ng of testosterone IDMSE (see calibration curves, Fig. 2), in practice measurements below 0.1 ng are sometimes unreliable due to amplification noise, sample background and other contributory factors such as column adsorption. For sample values above 0.8 ng a detector pulse of 150 μsec may be applied when linearity of detector response increases to at least 2 ng. However, this is accompanied by a threefold decrease in sensitivity. Androsta-1,4-dien-

17β-ol-3-one IDMSE has been found satisfactory for use as internal standard during gas chromatography because its retention time is near to that of testosterone IDMSE. With some batches of column packing some loss of peak area has been observed and consequently with these 20β-OH-progesterone IDMSE was used as internal standard instead. However, this latter steroid is not suitable if 20α-OH-progesterone is present (*e.g.* pregnancy) as these two isomers do not completely separate.

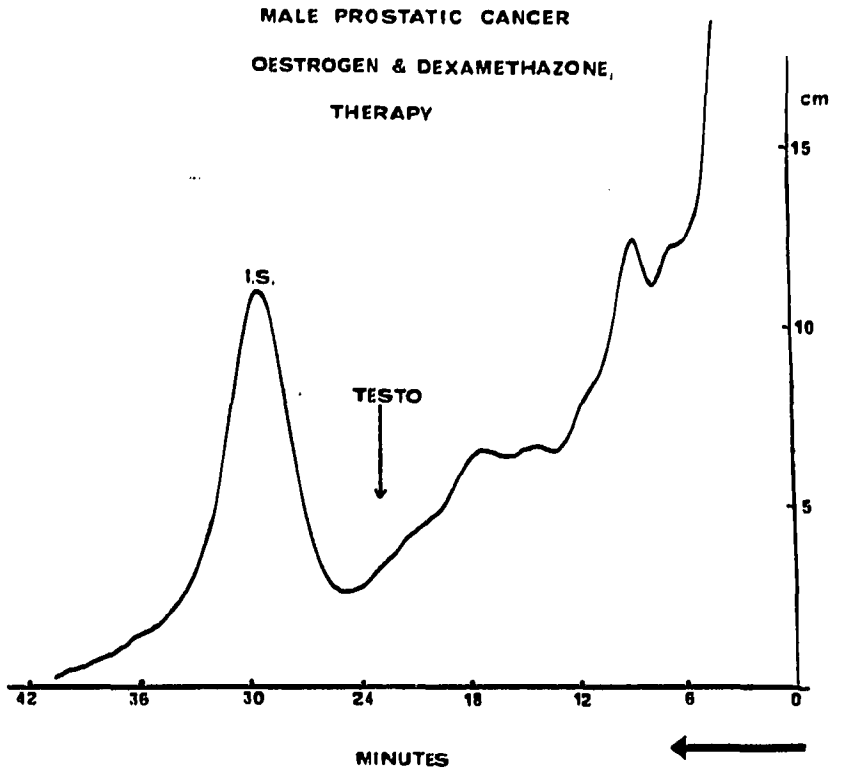

Fig. 7. Amount injected ≡ 1.6 ml plasma.

An advantage of gas–liquid chromatography is in the diversity of its application. For instance, tentative identification of other plasma steroids suggested that androsterone, aetiocholanolone, DHEA, 5α-dihydrotestosterone, 5-pregnenolone, 20α- and 20β-hydroxyprogesterone may be measured in the same extract. It is hoped to develop this aspect of the work.

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