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DETERMINATION OF TESTOSTERONE IN HUMAN URINE BY MEANS OF HORIZONTAL THIN-LAYER AND GAS–LIQUID CHROMATOGRAPHY

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SUMMARY

A method has been developed for the determination of testosterone in human urine by using enzymic hydrolysis, ether extraction, solvent partition and horizontal thin-layer chromatography to purify the urinary extracts, followed by gas–liquid chromatography or SACHS' colorimetric method. Comparison of the two methods of estimation of testosterone in the final extract shows that determination by gas–liquid chromatography is more reproducible than the colorimetric method when applied to the same eluates from the horizontal thin-layer chromatography. The reliability criteria studied show that this method is specific for routine analysis of testosterone in urine of normal men and normally menstruating women. Results of determinations on human urine are presented.

INTRODUCTION

It has always been accepted that testosterone (17β-hydroxyandrost-4-en-3-one) is the most potent of the naturally occurring androgens which it is possible to determine in tissue and biological fluids and thus can give useful information about the androgenic state of the subject. Although it is metabolised to 17-ketosteroids, the urinary estimation of these compounds is rarely useful in the assessment of human androgen production because the main precursors of the 17-ketosteroids are only weakly androgenic. Very recently it has been demonstrated, in rats and humans, that certain essential organs metabolise testosterone preferentially into 5α-dihydrotestosterone[1–3]. In some biological assay systems dihydrotestosterone is even more powerful as an androgen than testosterone itself. But, at present, methods for its determination have not been described. Therefore, the quantitation of testosterone still has a prominent role in the study of androgenic function. Many methods have been described to determine the urinary levels of this compound[4–13], but a lot of them show evident technical disadvantages, particularly for the separation of testosterone (Ts) from epitestosterone (Epi-Ts) which is not derived directly from the hormone[14]. In addition, the results obtained are not all in agreement[15,16].

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The present paper concerns a rather simple procedure for the determination of urinary testosterone. After enzymic hydrolysis and extraction with ether, the fraction containing Ts and Epi-Ts is separated and purified by horizontal thin-layer chromatography (HTLC). Further simple chromatographic partition provides a reasonable separation of the two epimers. Finally, the Ts was determined by gas–liquid chromatography (GLC) with a flame ionization detector (FID) and electronic integration or by a spectrophotometric colour reaction.

MATERIALS AND METHODS

Solvents and reagents

The solvents and reagents used were of analytical grade. The solvents were redistilled before use and tested by GLC. Silica Gel HF_{254} (Merck) was sieved and then washed before use with absolute ethanol. [4-^{14}C]Testosterone (S.A. 58.2 mCi per mmole) obtained from the Radiochemical Centre of Amersham, was used in tracer quantities as an internal standard and for estimating recoveries. Ketodase (β-glucuronidase, 500 U/ml) was obtained from Warner Chilcott.

Horizontal thin-layer chromatography

The Desaga outfit and the Desaga B.N.-Kammer for horizontal migration were employed. Glass plates, 20 × 20 cm, were prepared as previously described[17,18]. The urinary extracts were quantitatively transferred with acetone (0.2 ml, 3 times) to a thin-layer plate; spotting was approximately 1.5 cm from the lower edge and 2–2.5 cm from the side. The following solvent systems have been used: (a) benzene–ethyl acetate (60:40); (b) dichloromethane–ethyl acetate (90:10) and (c) benzene–ethyl acetate (80:20). The plates were developed, at room temperature, using horizontal migration, with a system of cooling by running water. After UV absorption, the area of silica gel containing the labelled Ts was loosened with a microspatula, aspirated into a sintered glass disc (1 cm diameter, No. 2 grade), eluted under positive pressure with 5 × 0.3 ml absolute ethanol and, finally, the eluate evaporated to dryness.

Gas–liquid chromatography

A Fractovap model D (Carlo Erba), glass column, gas chromatograph equipped with double flame ionization detector was used. Chromosorb W (80–100 mesh) was acid washed, silanised, and coated with 3 % SE-30; the packing was blown into the glass coiled columns 80 cm long and 2 mm I.D. Glass columns, of the same dimensions, but packed with silicone-treated Gas-Chrom P (100–120 mesh) coated with 1 % XE-60 were also employed. The samples were introduced into the glass vaporisation chamber by means of a Hamilton liquid microsyringe. The carrier gas was nitrogen (filtered through molecular sieve and with a flow-rate of 60 ml/min) and the flow rates of the flame gases were adjusted to achieve satisfactory sensitivity and base-line stability. The temperatures were 300° for the flash heater, 228° for the columns and 240° for the detectors. The peak areas were measured by triangulation and by an electronic decade counting system (max. count rate: 1500 c.p.s.) with a Keinzle (model D-11 E) digital printer, and by using a corresponding external standard run before and after each urinary sample.

Identification and determination of radioactivity

Radioactivity due to the ^{14}C was identified on thin-layer plates by autoradiography (with overnight exposure of the plates to Agfa-Gevaert no-screen X-ray films) or by using a Thin-layer Radio-Scanning apparatus (Desaga), with a ^{14}C counting efficiency of 15–18 %. Liquid scintillation counting (LSC) was done in 15 ml of scintillation solution (42 ml of Liquifluor in 1000 ml of redistilled toluene) by means of a Nuclear-Chicago Mark I Liquid Scintillation Spectrometer. The counting efficiency (mean: 89 % for carbon) for each sample was determined by reference to a calibration curve, which was plotted from a set of quenched standards by using an external ^{133}Ba source and a channels-ratio method.

Colour reaction for testosterone and its spectrophotometric determination

The SACHS' colour reaction[19] at 620 mμ was used. Absorption spectra were recorded in a Beckman D.U. model spectrophotometer.

Standard analytical procedure

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

Hydrolysis and extraction. Samples of 100 ml of male and 200 ml of female urine from a 24 h collection were used for each determination. After enzymic hydrolysis

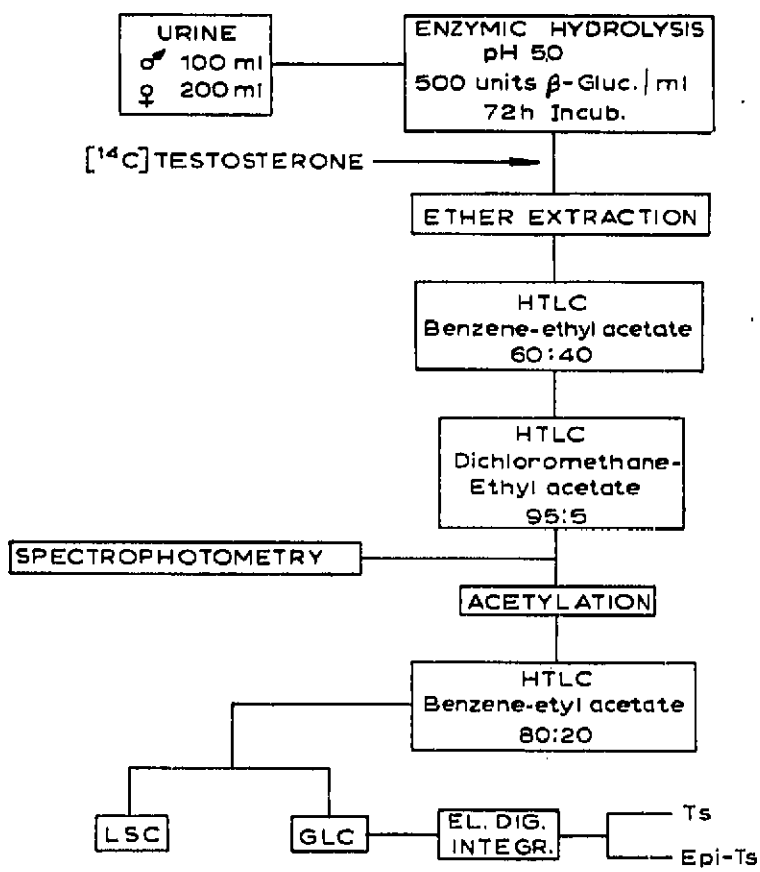

Fig. 1. Flow diagram of the method used for routine determination of urinary testosterone.

(urine adjusted to pH 5.0, addition of 500 U of ketodase per ml urine and, finally, incubation for 72 h at 37°), the urine was cooled and extracted three times with 1 vol of ether. The combined ether extracts were washed three times with 0.2 vol of 1 *N* NaOH and three times with 0.2 vol of distilled water. The ether extracts were dried with anhydrous Na_2SO_4 and evaporated to dryness on a water bath under nitrogen.

HTLC. The extracts were applied to the silica gel plates alongside a standard of Ts as a reference. Development was with solvent system (a). After detection by UV-light absorption of the reference Ts, the areas of the chromatogram corresponding to urinary Ts were then eluted with absolute ethanol. The eluates were evaporated, redissolved in acetone and rechromatographed in solvent system (b). Identification and elution of the steroid compound was repeated and the dry extract was acetylated by the addition of 0.2 ml of pyridine and 0.1 ml of acetic anhydride, the mixture being left at room temperature for 12 h. The reagents were removed under nitrogen on a water bath at 55° and the extract was rechromatographed using solvent system (c). The area corresponding to urinary Ts-acetate was eluted, then evaporated, redissolved in a known quantity of absolute ethanol and small portions were removed for LSC. The residual liquid was evaporated again and then subjected to GLC.

RESULTS AND DISCUSSION

Hydrolysis

Previously described methods of determining urinary Ts have used enzymic hydrolysis with a different concentration of β-glucuronidase at 37° for 48 h. In the present study incubation at 37° for 72 h with a concentration of 500 U/ml of ketodase

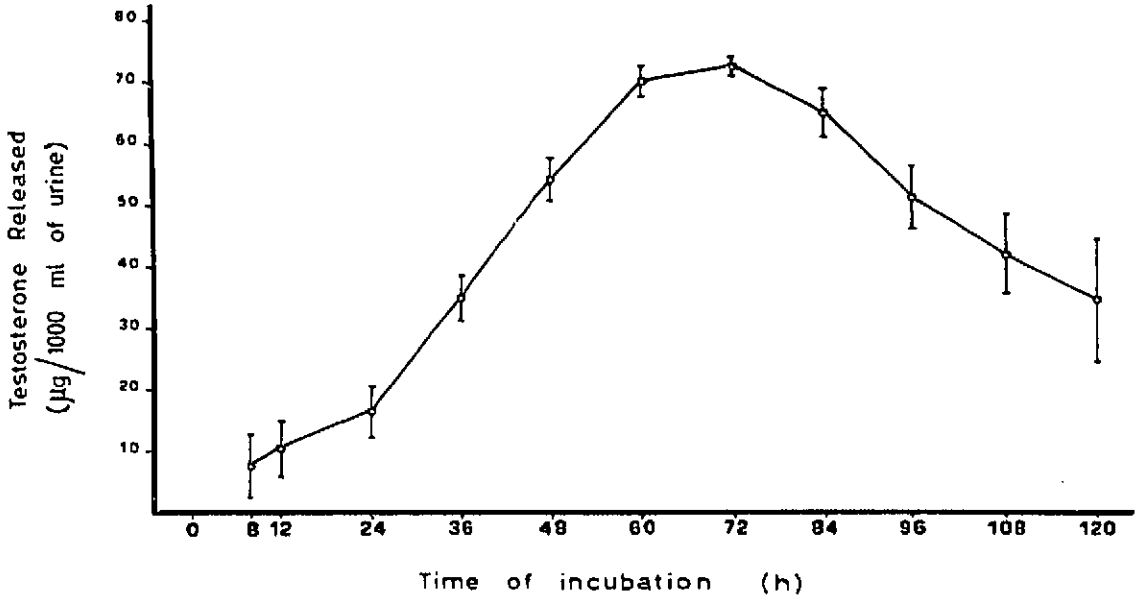

Fig. 2. Amount of testosterone released in relation to the time of incubation; constant enzyme activity. Urine samples were obtained from a pool of normal men. Each point of the curve represents the mean and the standard deviation of 5 determinations.

was found to give reproducible results. Using the same concentration of enzymic activity but with smaller or longer incubation periods, we obtained a considerable reduction in the amount of Ts released and a progressive increase in the standard deviation. This is evident from Fig. 2. The stability of Ts under our conditions of hydrolysis was confirmed by duplicate analyses. A 5 μg sample of authentic Ts was added to 100 ml of urine (100 ml of the same urine is the blank). In 7 experiments the mean amount of Ts recovered, as estimated by GLC, was 60.3 $\pm$ 6.5 % (S.D.).

Thin-layer chromatography

Fig. 3 shows the preliminary purification of Ts and Epi-Ts obtained with solvent system (a). Visualisation of the plates with ethanol–sulphuric acid (50:50), makes it possible to pick out different spots, some of these are visible even under UV light. The second HTLC in system (b) clearly separated Ts from Epi-Ts (Fig. 4). Finally,

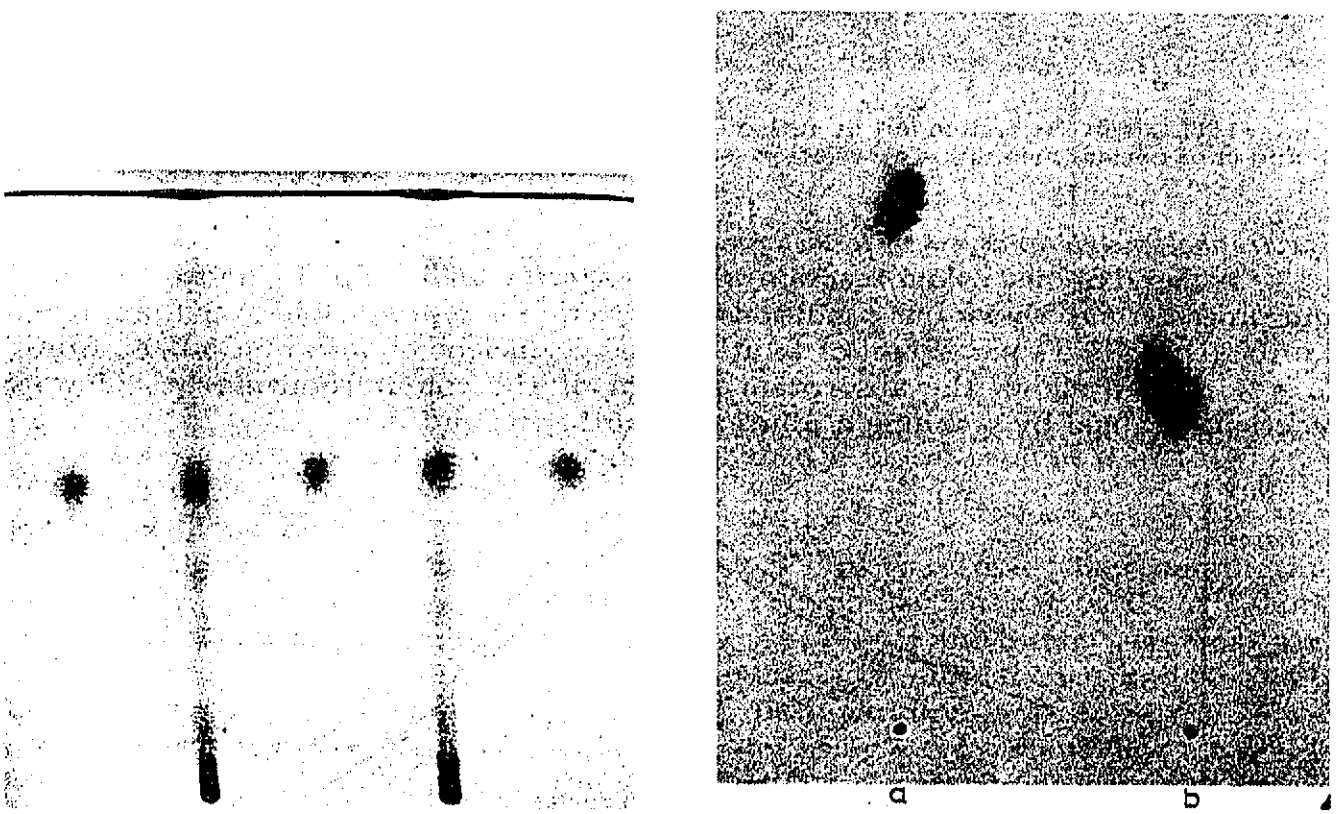

Fig. 3. Horizontal thin-layer chromatography (under UV light), in solvent system (a), of urinary extracts obtained from two normal men (scale: 3/1).

Fig. 4. Separation of testosterone (a) from epitestosterone (b). HTLC with continuous flow of solvent system (b) scale: 3/2).

Fig. 5 shows the separation of Ts-acetate in system (c); this was satisfactory for GC quantitation.

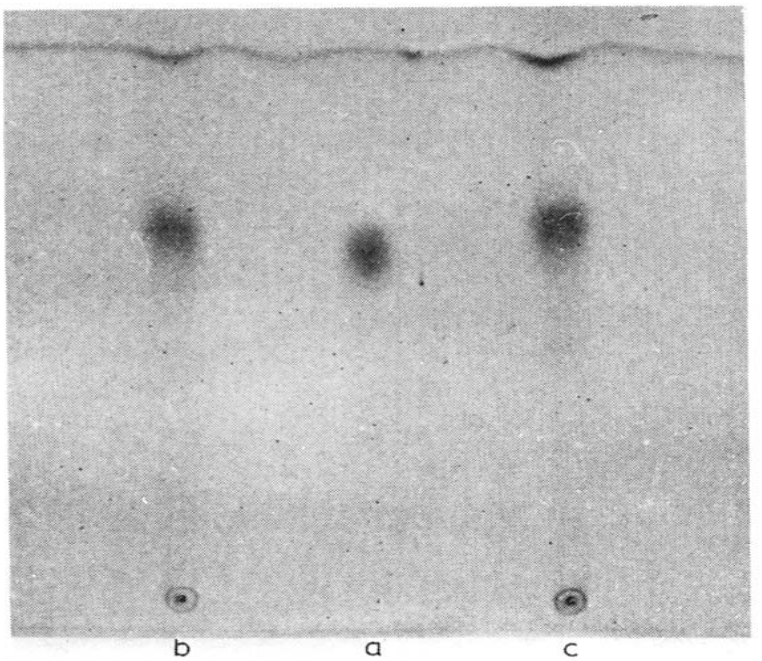

Fig. 5. HTLC (solvent system (c)) of authentic testosterone acetate (a) and two urinary extracts (b and c) of normal men, after acetylation (scale: 3/1).

Spectrophotometric analysis of testosterone

We used SACHS' colour reaction which is specific for Ts, Epi-Ts, androstenedione and progesterone. Under our conditions the reaction is reproducible over the range of 1.0 to 20.0 μg of Ts per 1.0 ml of solution; the coefficient of variation ranged from 3 to 5%. Fig. 6 shows the absorption spectra of the colour resulting from SACHS' reaction obtained by scanning a solution of authentic and urinary Ts.

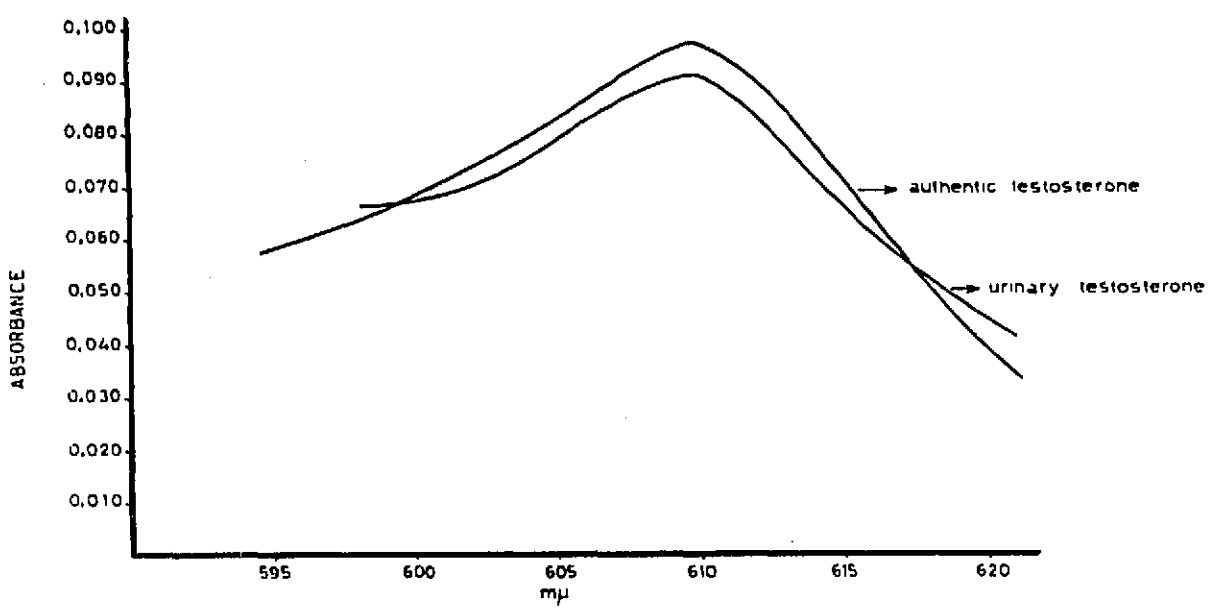

Fig. 6. Absorption spectra of the colour (SACHS' reaction) of authentic testosterone and of that obtained from a pool of normal male urine.

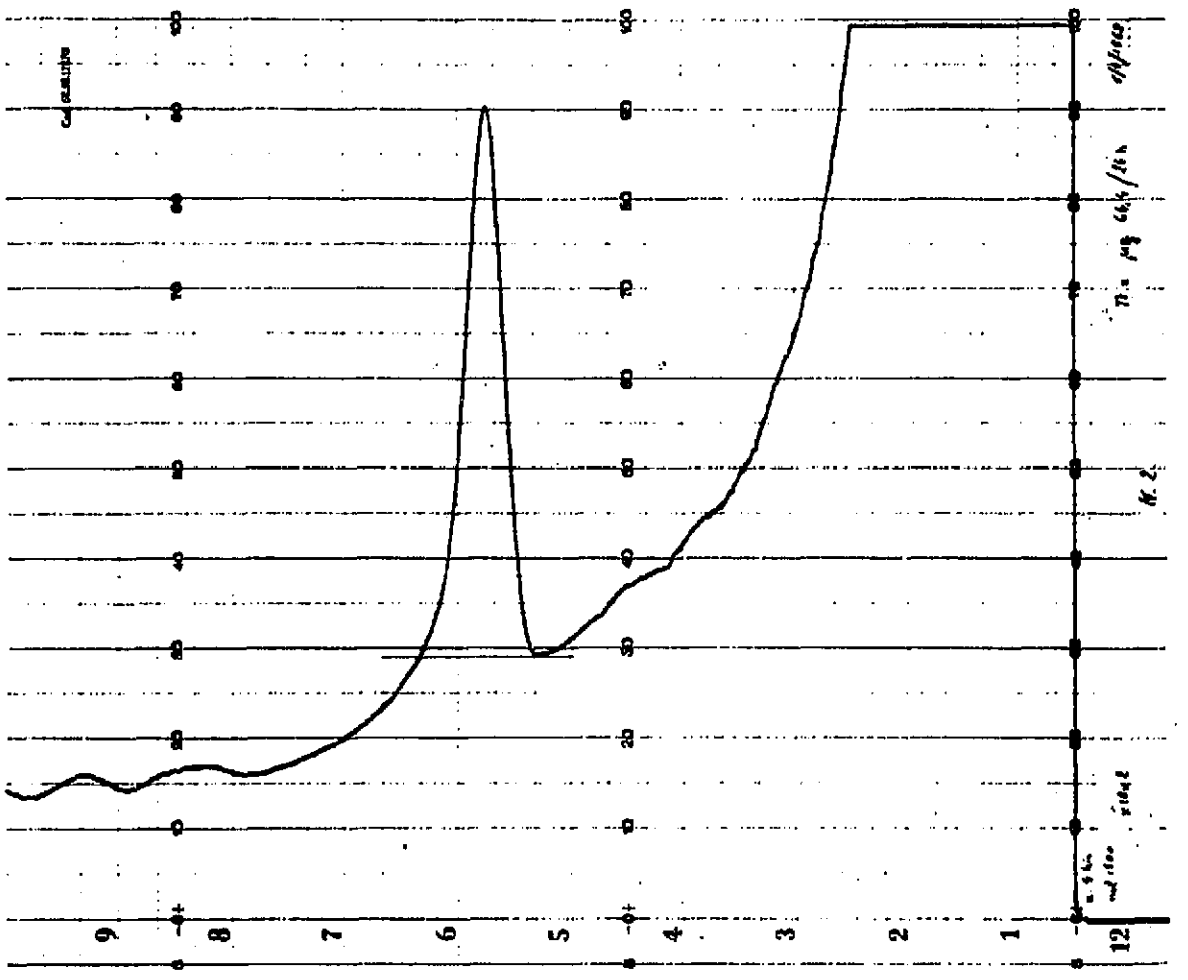

Fig. 7. Gas chromatographic analysis of a 3/10 testosterone urinary extract from normal men. Retention time, 10.2 min (att., × 10 ×2). Other conditions as described in the text.

Gas–liquid chromatographic analysis

Initially micro-columns coated with 1 % SE-30 were used; however the impurities were found to have a retention time (t_R) similar to that of Ts. A column coated with 3 % SE-30 provided better separations. Figs. 7 and 8 show representative gas chromatograms obtained from urinary extracts of normal men and normally menstruating women. In previous methods[4,9] the separation of Ts from Epi-Ts was not achieved. However, we have obtained this by using a continuous flow method in the HTLC (Fig. 4) and also with the GLC analysis. Figs. 9 and 10 show the separation of Ts from Epi-Ts both of a standard solution and of urinary extracts from normal men. The reproducibility of the injection technique and of the GLC has been tested by repeated analysis of standard Ts-acetate. The results are shown in Table I. It will be seen that the coefficient of variation is lower when the peak area is determined by electronic integration: 1.8–3.5 %. Fig. 11 shows the calibration curve based upon numerous replicate chromatograms which have been obtained by the analysis of known quantities of authentic Ts-acetate. There is satisfactory linearity between the mass of injected Ts expressed in integrator units and the mass calculated by the triangulation method, respectively.

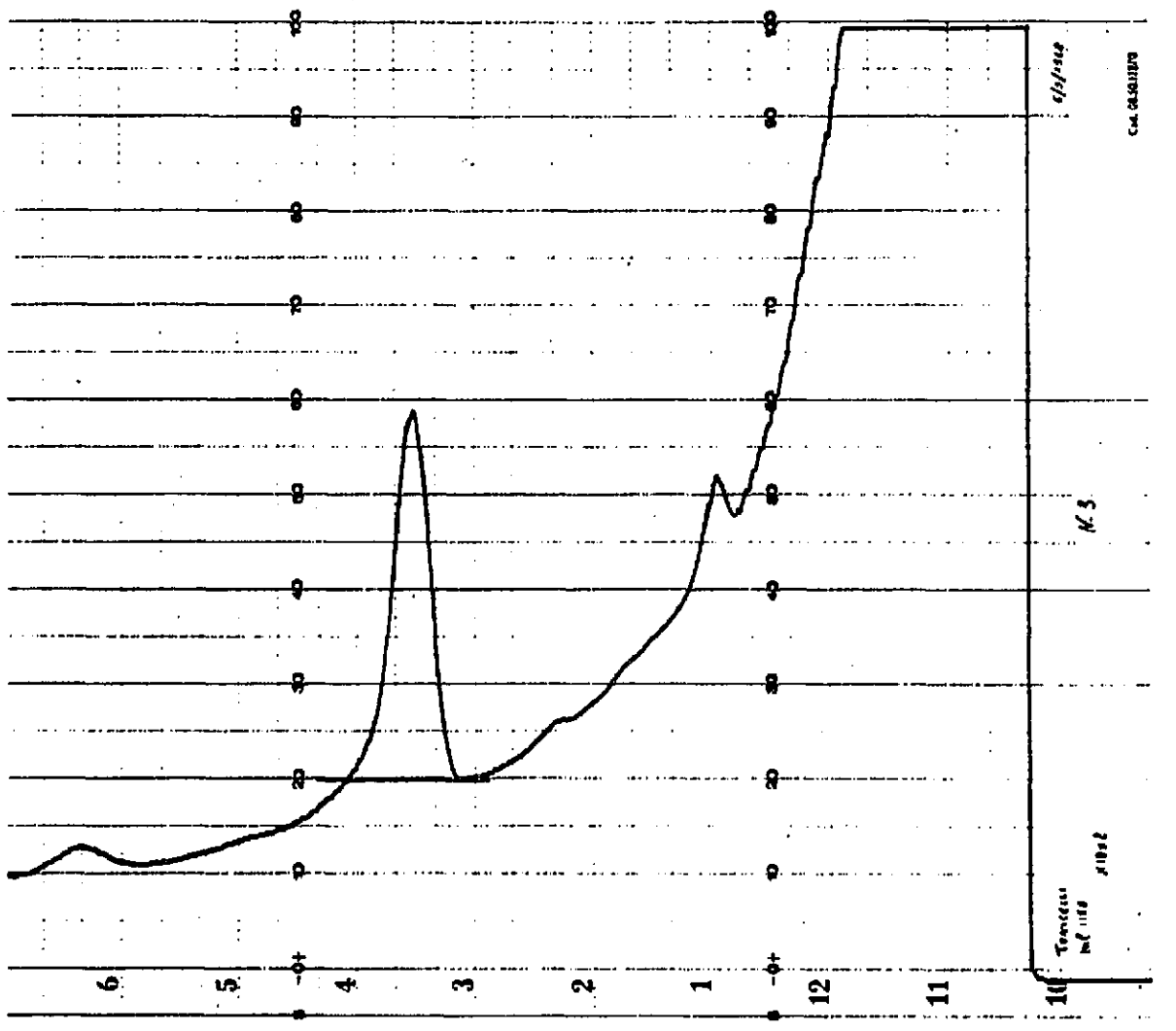

Fig. 8. Gas chromatographic analysis of 9/10 testosterone urinary extract from normally menstruating women in luteal phase. Retention time, 10.15 min (att., × 10 × 2). Other conditions as described in the text.

TABLE I

REPRODUCIBILITY OF GAS–LIQUID CHROMATOGRAPHY (INCLUDING LIQUID INJECTION OF PURE DERIVATIVE) USING A FLAME IONISATION DETECTOR AND ELECTRONIC DIGITAL INTEGRATOR

Chart speed: 1/2 cm/min.

	Authentic testosterone-acetate (μg)	
	0.010	*0.020*
Number of samples	15	20
Integration units		
Mean ± S.D.	32.328 ± 1.142	62.858 ± 1.136
Coeff. of variation	3.5%	1.8%
Peak areas (mm²)		
Mean ± S.D.	542 ± 43	984 ± 47
Coeff. of variation	7.9%	4.7%

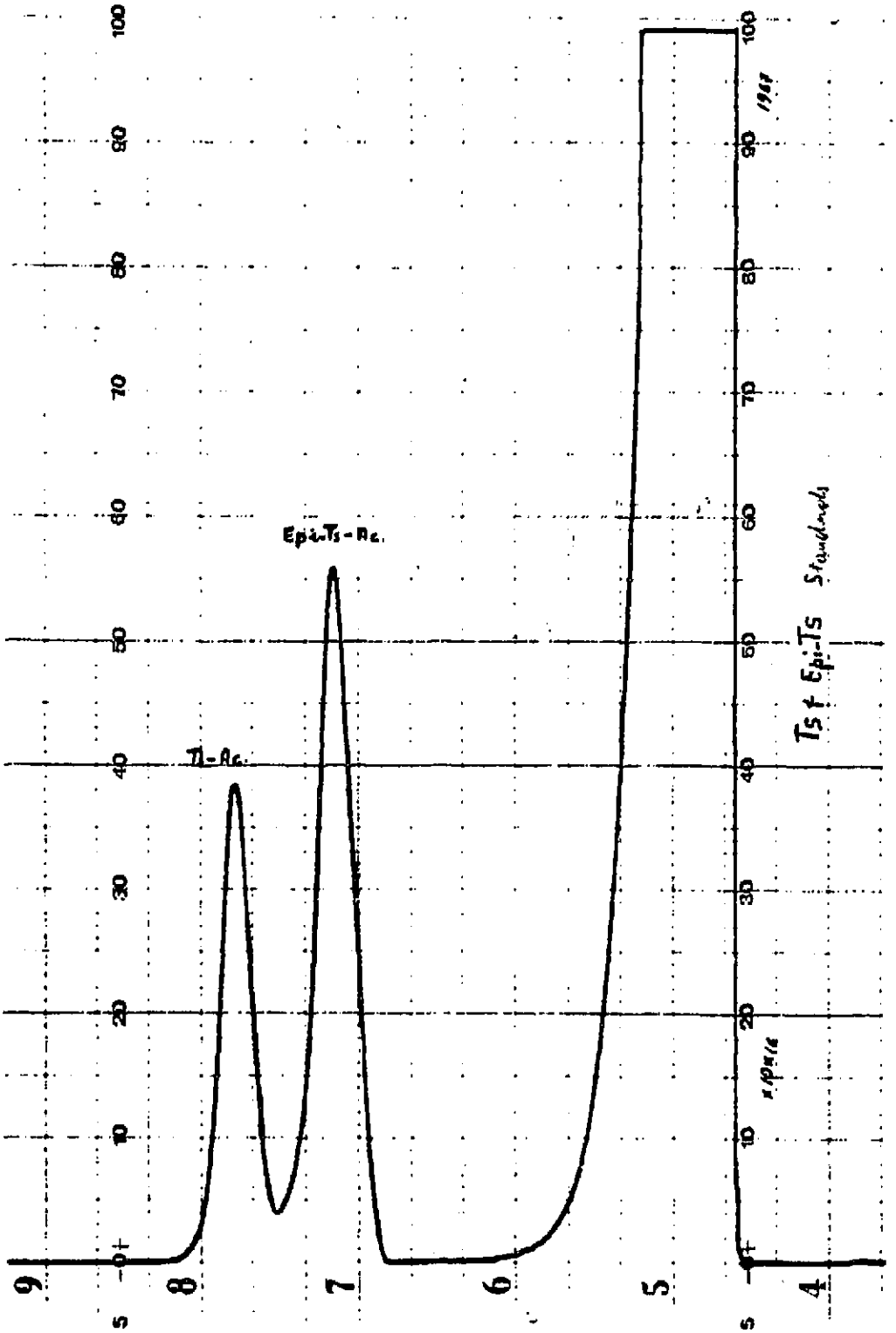

Fig. 9. Gas–liquid chromatographic analysis of authentic testosterone acetate (about 30 ng) and epitestosterone acetate (about 35 ng). Conditions as described in the text.

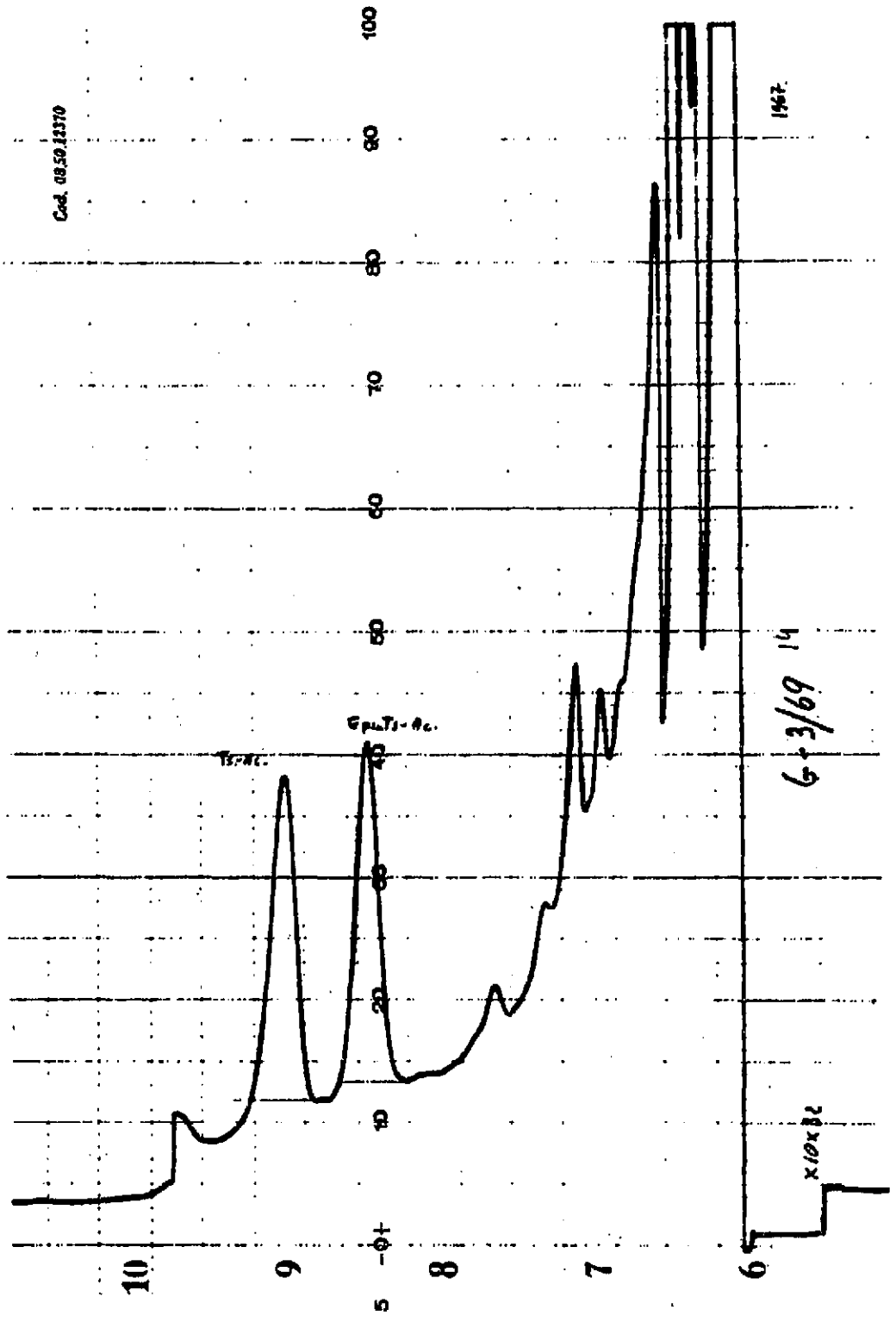

Fig. 10. Gas–liquid chromatographic analysis of urinary testosterone acetate and epitestosterone acetate in normal men after chromatographic partition in solvent system (a). Conditions as described in the text.

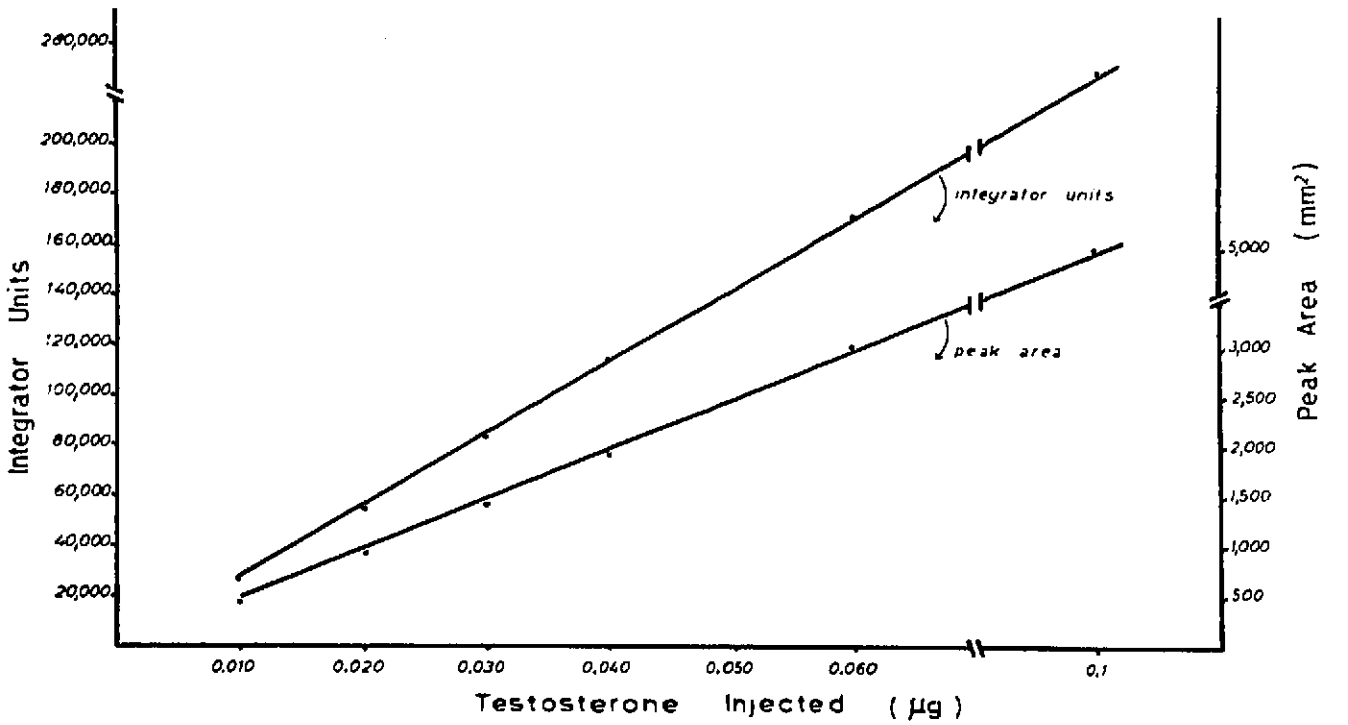

Fig. 11. Calibration curve: linearity and range of detector response to μg quantities of authentic testosterone using integrator units and peak areas, respectively.

Reliability criteria of the method

Accuracy. This was estimated by recovery experiments. A 5.0 μg sample of unlabelled authentic Ts was added to 100 ml of water, and 0.01 μg of labelled Ts were added to 100 ml of female urine. The recoveries obtained by different methods of detection are shown in Table II. Recoveries of 75.1 % and 88.4 % were obtained by using GLC and by LSC. However, the recoveries obtained using SACHS' reaction were only 66.8 %.

Precision. This may be estimated from the difference between duplicate analyses, according to SNEDECOR[20]. The standard deviation from 21 pairs of estimates was $\pm$ 3.2 μg for a mean of 58.7 μg/24 h (male urine). In 18 pairs of estimates from female

TABLE II

ACCURACY OF THE METHOD USED FOR DETERMINING URINARY TESTOSTERONE

Experimental details are given in the text and the number of observations is given in parentheses.

Method of detection	*Amount of testosterone added after hydrolysis (μg)*	*Mean percentage recovery $\pm$ S.D.*
Liquid scintillation counting (radioactivity due to [^{14}C]testosterone)	0.01 (31)	88.4 $\pm$ 3.7
Gas–liquid chromatography (as testosterone acetate)	5.0 (18)	75.1 $\pm$ 6.8
Spectrophotometric method (SACHS' reaction)	5.0 (23)	66.8 $\pm$ 8.2

urine, the standard deviation was ± 1.2 μg for a mean of 8.4 μg/24 h. The coefficients of variation were 4.8 % and 7.3 % for male and female urine, respectively.

Specificity. The identity of the compound determined by the present method was examined at the different stages of analysis. HTLC of the extract containing Ts-acetate showed only one component when the plates were sprayed with the detection reagent. In 75 chromatograms from different samples of male and female urine, only one spot could be detected corresponding in colour and position to Ts. Oxidation of 15 eluates obtained from the second migration produced a compound identical with androstenedione both on HTLC and in GLC on columns coated with SE-30 and XE-60. In all experiments, formation of the acetate of Ts in the final eluate from the second HTL-partition gave a compound of the same R_F and t_R as authentic Ts-acetate. Finally, testosterone could not be detected in 9 urine samples from three women subjected to bilateral ovariectomy and adrenalectomy and accordingly it was concluded that the non-steroidal materials present in urines of these patients did not interfere with the method.

Sensitivity. About 0.5 μg of testosterone could be measured accurately in the final residue by using SACHS' spectrophotometric method, and 0.04 μg as steroid acetate by GLC. The sensitivity, calculated according to the method of BROWN *et al.* [21], was 0.7 μg in a 24 h collection of urine.

Urinary excretion of testosterone in normal subjects

In 100 determinations from 25 normal males aged from 21 to 46 years, the mean excretion of Ts was 61.4 ± 15.7 μg (S.D.) in a 24 h urine (range: 32.4–87.1 μg/24 h). In 24 determinations from 5 young men aged 17–22 years, the mean urinary excretion was 91.7 ± 88 μg (S.D.) per 24 h. When the urinary Ts level was determined in normal women during the follicular phase of the menstrual cycle (111 determinations in 37 women aged 20–41 years), the mean value was 6.0 ± 3.1 μg (S.D.) per 24 h (range: 1.0–92 μg/24 h), while during the luteal phase (69 determinations in 23 women aged 18–40 years) it averaged 12.2 ± 4.3 μg (S.D.) per 24 h (range: 5.1–16.8 μg/24 h). Thus there was a significant difference between the amounts eliminated in male and female urine and between the follicular and luteal phase of the menstrual cycle. Fig. 12 shows the daily Ts excretion pattern during the menstrual cycle of a 21-year-old normally menstruating girl.

CONCLUSIONS

The evidence obtained by GLC shows that testosterone is the major steroid compound present in the final urinary extract. Our experimental results obtained after the more efficient separation and purification of Ts from contaminants and closely related steroids by selective HTLC and GLC, support this finding. However it is necessary to confirm the concept that by using HTLC or GLC it is possible to obtain a satisfactory separation of Ts from its epimer, the latter being the main steroid which interferes with the determination of Ts and is the cause of higher results being obtained with some other methods[4,6,7]. There is considerable evidence to show that the present method is specific for Ts.

The determination of Ts by the colorimetric method is possible only with male urines because of the low sensitivity of the SACHS' reaction. The fact that the re-

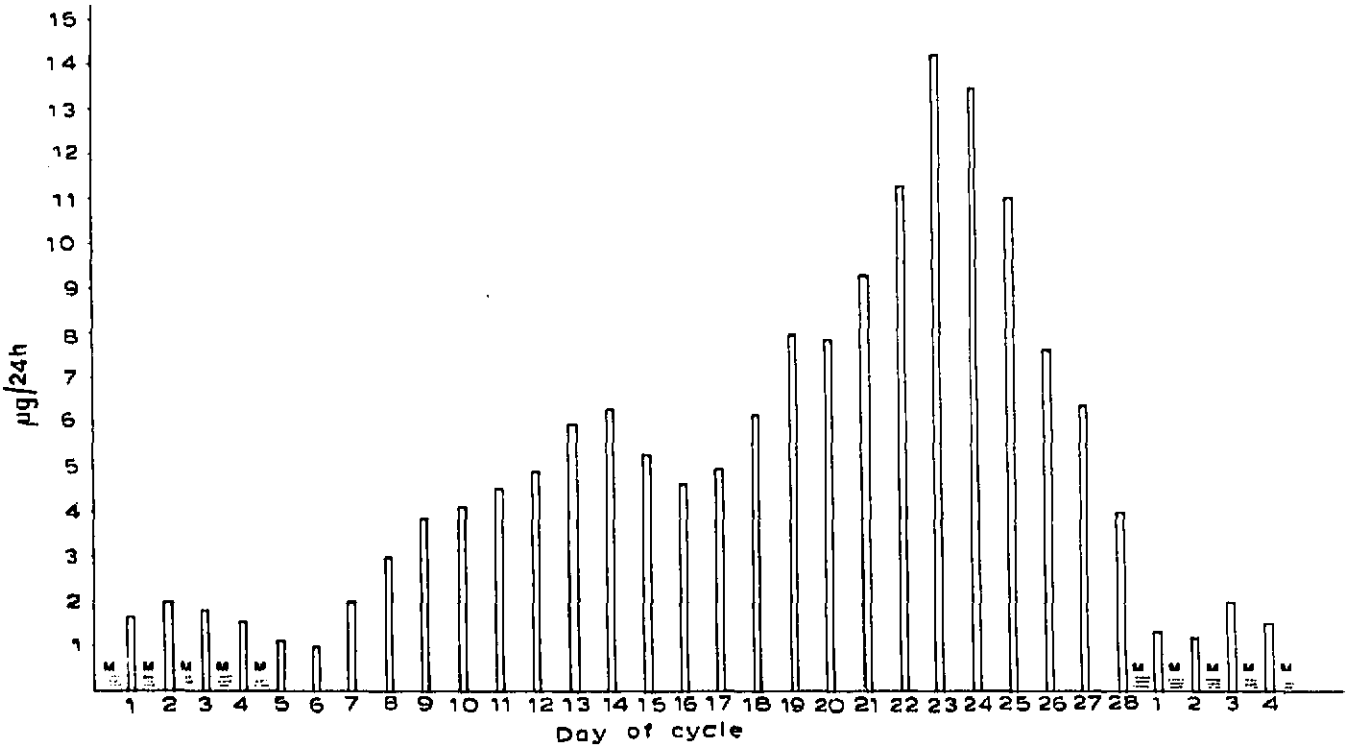

Fig. 12. Daily testosterone excretion pattern during the normal menstrual cycle of a 21-year-old girl.

covery values obtained by GLC on replicates of materials used for determinations by the SACHS' reaction were always higher suggests that impurities from the thin layer may have interfered in the colorimetric determination. GLC represents therefore the procedure of choice.

The values obtained by the method described in this paper are in agreement with the results of some previously reported techniques. However, the upper limits of our results were generally lower than those which have been obtained by some of the previous methods. Finally, the reliability criteria of the method described here permits its use for routine analyses to determine urinary testosterone in normal men, in the follicular and luteal phase of the normal menstrual cycle and in different cases of endocrine diseases.

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