CHROM. 5843

DETERMINATION OF URINARY ANDROSTANEDIOL AND TESTOSTERONE IN NORMAL MEN BY GAS–LIQUID CHROMATOGRAPHY

G. CHARRANSOL, F. BOBAS-MASSON, S. GUILLEMANT AND P. MAUVAIS-JARVIS
Laboratoire de Biochimie Médicale, Faculté de Médecine Pitié-Salpétrière, 91, Boulevard de l'Hôpital, Paris 13ème (France)*

(First received September 23rd, 1971; revised manuscript received November 15th, 1971)

SUMMARY

A method using gas–liquid chromatography for the determination of urinary androstanediol and testosterone in normal men is reported. After hydrolysis and extraction, testosterone was separated from androstanediol by Girard's T reagent. After further purification, the trimethylsilyl ethers were formed and measured by gas–liquid chromatography. The average androstanediol and testosterone excretions in twenty-nine normal males (aged 20–40 years) were 112 μg/24 h $\pm$ 42 (S.D.) (range 46–205) and 52 μg/24 h $\pm$ 26.2 (S.D.) (range 5–95), respectively. The method appeared to be a clinically useful one.

INTRODUCTION

In human beings, testosterone glucuronide seems to be essentially formed in the splanchnic compartment[1], whereas 5α-androstane-3α,17β-diol (androstanediol) glucuronide, which is the main metabolite of 17β-hydroxy-5α-androstan-3-one (dihydrotestosterone), may originate from extra-hepatic sources such as the accessory sex organs and skin[2–4]. *In vivo* studies in this laboratory[5] have demonstrated that 50 to 70% of urinary androstanediol recovered in the urine of normal males arises from extra-hepatic tissues, the other part being formed in the liver. Also, the comparison of urinary values of testosterone and androstanediol glucuronides may have great importance in evaluating the metabolism of testosterone outside the liver. This metabolism, according to several authors, is possibly related to the activity of androgen in target tissues and thus the determination of the extra-hepatic production of urinary androstanediol might be a good index of androgenicity in humans.

EXPERIMENTAL

Material and apparatus

All solvents were further purified and redistilled before use according to JAYLE's methods[6].

* Director: Prof. P. DESGREZ.

Testosterone and androstanediol, used as reference steroids, were obtained from Roussel UCLAF. An extract of *Helix pomatia* (1 ml containing 210,000 units of β-glucuronidase and 50,000 units of sulphatase) was supplied by Industrie Biologique Française, France. [4-^{14}C]Testosterone (specific activity 29.2 mCi/mole) was obtained from the Radiochemical Centre, Amersham, and was used in tracer quantities for estimating recoveries; the purity of the [4-^{14}C]testosterone was checked in the paper chromatographic system hexane–benzene (1:1), developed for 6 h at 25°; the testosterone peak was detected by scanning on a Packard Model 385. 5α[1,2-$^{3}H_2$]androstane-3α,17β-diol (specific activity 42 Ci/mmole) was synthesized[7] from 5α[1,2$^{3}H_2$]dihydrotestosterone; androstanediol was purified by gradient elution chromatography on an alumina column, and crystallized to constant specific activity; after purification, androstanediol was treated in a similar manner to testosterone for estimating recoveries.

Thin-layer chromatography was carried out on Silica Gel F_{254} (Merck) precoated layers, thickness 0.25 mm. A Camag ultraviolet lamp was used at 254 nm. An F & M Model 402 (Hewlett-Packard) gas chromatograph fitted with dual flame ionization detectors was used for gas–liquid chromatography (GLC). Different columns were used for testosterone and androstanediol determinations, *viz.* for testosterone two glass columnns, 4 ft. × 0.34 cm I.D., were packed with Gas-Chrom Q (80–100 mesh) coated with 3 % SE-30 and 3 % OV-1 and for androstanediol the same columns were packed with Gas-Chrom Q coated with 3 % XE-60 and 1 % NGS.

The carrier gas was nitrogen, with a flow-rate of 40 ml/min. For testosterone the temperatures were 275° for the flash heater, 238° for the columns and 255° for the detectors. For androstanediol the temperatures were 260° for the flash heater, 200° for the columns and 240° for the detectors.

All samples were diluted with 0.1 ml of carbon disulphide and 4 μl were injected into the column with a Hamilton syringe.

Derivatives for GLC were prepared with the following reagent: 10 ml of hexamethyldisilazane (HMDS) (Girdel), 1 ml of trimethylchlorosilane (TMCS) (Girdel) and 89 ml of tetrahydrofuran (purified over sodium). This reagent was stable for several weeks when kept in a glass-stoppered flask.

Extraction of steroids from urine

Urine was collected for a period of 24 h on Merseptyl "Houde", and stored at —20° until processed. A 1/10 aliquot of urine was analysed; the pH was adjusted to 5 with 2 *N* acetic acid which was buffered with acetoacetate buffer in order to maintain the pH of the urine at 5. In addition, approximately 5000 c.p.m. of [^{14}C]-testosterone, approximately 10,000 c.p.m. of [^{3}H]androstanediol, and 1000 U/ml of *Helix pomatia* were added to each urine sample. This mixture was incubated at 37° for 24 h. After this incubation period, 500 U/ml of *Helix pomatia* were added and incubated for a further period of 24 h. The hydrolyzed urine sample was extracted twice for 1 h with equal volumes of methylene chloride. The combined methylene chloride extract was washed once with sodium carbonate (10 %) and twice with distilled water and then evaporated to dryness in an evaporator.

Then 25 ml of ethyl alcohol, 2.5 ml of acetic acid, and 300 mg of Girard's T reagent were added to the dried extract. This was incubated overnight at 37°. After this incubation period the reaction was stopped with 50 ml of ice-cold distilled water

the pH was adjusted to 6.5 with 10 *N* sodium hydroxide. The hydroxysteroids were extracted twice with equal volumes of ether and the combined ether extract was washed with sodium carbonate (10 %) and with distilled water and then evaporated to dryness. The aqueous fraction after extraction of the hydroxysteroids contains the ketosteroids. The ketosteroids were hydrolyzed by adjusting the pH to 1 with hydrochloric acid and storing for 2 h at room temperature. After hydrolysis, the ketosteroids were extracted by the same process that was used for hydroxysteroids.

Purification of androstanediol by thin-layer chromatography

In normal human urine, the rate of excretion of androstanediol is greater than that of testosterone, and good purification of the dry hydroxysteroid extract can be carried out by quantitatively transferring it with ethyl acetate to a thin-layer plate. Benzene–ethyl acetate (1:1) was used as the solvent system. The plate was developed at room temperature using ascending migration. Standard androstanediol was applied on both sides of the extract. The area containing androstanediol was identified by spraying the standard with alcohol–sulphuric acid (1:1) and heating for 5 min at 100°. The area on the thin-layer plate corresponding to the androstanediol standard was scraped off quantitatively and the silica gel eluted twice with ethyl acetate. The ethyl acetate extracts were pooled and dried. Ethyl acetate (1 ml) was added and two aliquots (0.05 ml) were taken for assay in a scintillation spectrometer for estimation of androstanediol losses during the procedure. The remainder was transferred to a stoppered and silanized tube. A standard curve was run at the same time with pure androstanediol and prepared with concentrations approximating as closely as possible to those of the samples to be analysed.

TMS ether formation. To each tube 1.5 ml of trimethylsilylating reagent was added. The tubes were tightly stopped, briefly agitated in a vortex mixer and stored overnight at 37°. Excess reagent was evaporated under reduced pressure, 0.1 ml of carbon disulphide was added, and 4 μl of the mixture were injected into the gas–liquid chromatograph.

Purification of testosterone

The ketosteroid extract contains microgram quantities of testosterone in the presence of 17-ketosteroids. For good separation, it was necessary to introduce two steps of purification, *viz.* paper and thin-layer chromatography (TLC).

Paper chromatography. The Zaffaroni system hexane–benzene (1:1) was used. The extract was spotted on a 5-cm wide Schleicher & Schüll filter paper. Reference testosterone was spotted as a guide on the strip. The chromatogram was developed for 6 h and dried. The guide strip containing reference testosterone was examined under a UV lamp. The relevant parts of the chromatogram were cut out and eluted with ethyl acetate and agitated for 30 min three times. The combined ethyl acetate extracts were evaporated to dryness, dissolved in 10 ml of water and partitioned twice against 20 ml of ether. The combined ether extract was evaporated to dryness.

Thin-layer chromatography. The solvent system used was hexane–ethyl acetate (1:1). The extract was transferred to a thin-layer plate. Standard testosterone was applied on both sides of the extract. The chromatogram was developed and the area containing the testosterone was examined under a UV lamp. The area corresponding

to the testosterone standard was scraped off, and eluted twice with 2.5 ml of ethyl acetate. The ethyl acetate extracts were pooled and dried, 1 ml of ethyl acetate was added and two aliquots (0.05 ml) were taken for assay in a scintillation spectrometer for estimation of the testosterone losses during the procedure. The remainder was transferred to a silanized tube and prepared for GLC in the same way as the androstanediol extract.

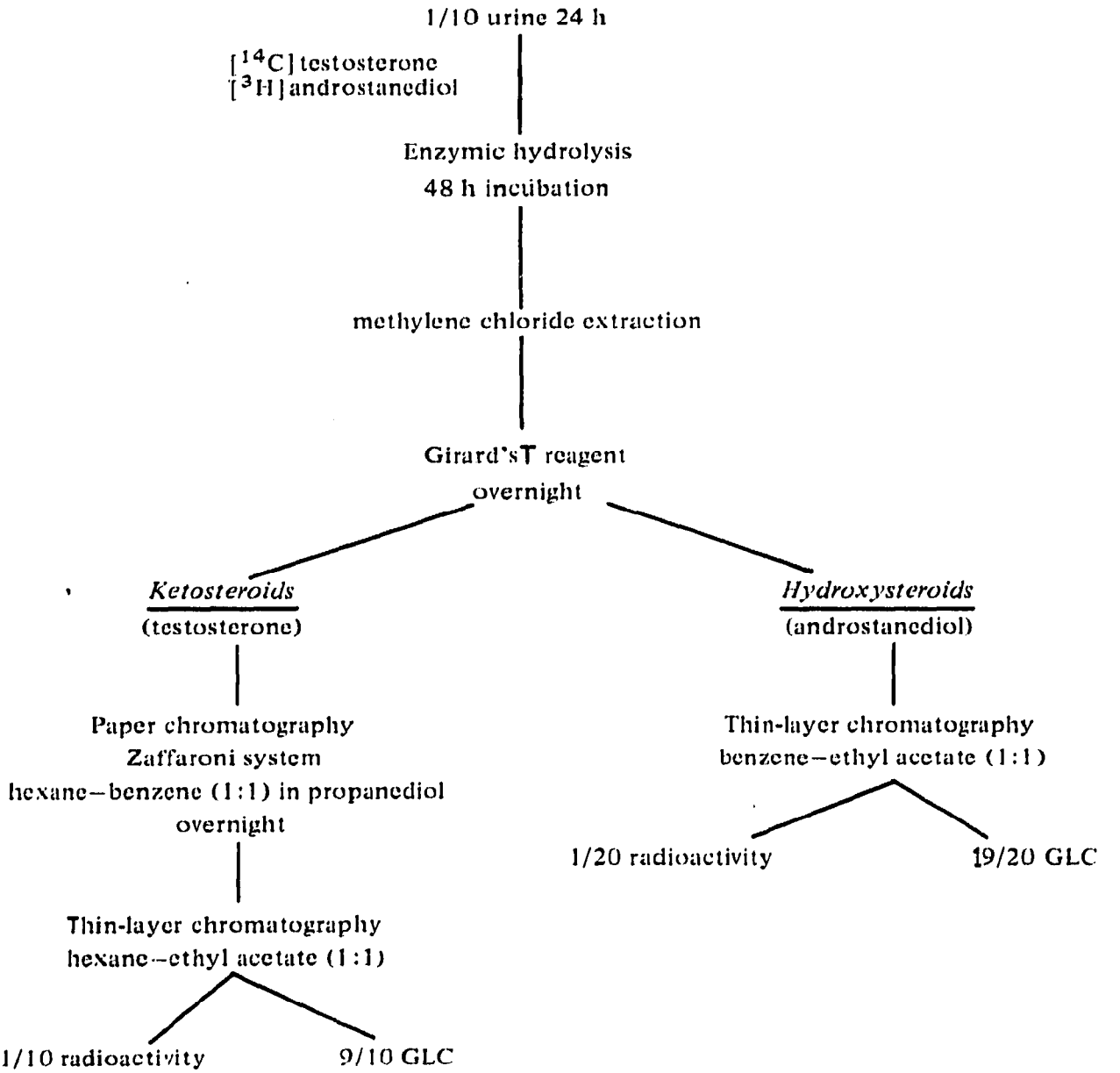

Fig. 1. Diagram of the method used for routine determination of urinary testosterone and androstanediol.

RELIABILITY CRITERIA OF THE METHOD

Sensitivity

About 5 μg of androstanediol and testosterone may be measured accurately in a 24-h collection of urine and 0.02 μg of both steroids may be detected in a flame ionization detector.

Recovery

The results of recovery in 29 samples analysed averaged 73 ± 7% (S.D.) of androstanediol and 56 ± 6% (S.D.) of testosterone. Lower testosterone recoveries were the result of the paper chromatography purification step. In addition, the urine of a prepubertal girl was investigated before and after addition to a 60-ml urine sample containing different concentrations of unlabelled androstanediol and testosterone. After calculation of steroid loss resulting from the experimental procedure, the respective recoveries of androstanediol and testosterone were as follows: Androstanediol added: 0.5, 1.0, 2.0 μg; recovered: 0.4, 1.0, 2.0 μg. Testosterone added: 0.5, 1.2, 2.5 μg; recovered: 0.4, 1.2, 2.7 μg.

Reproducibility

The volume of a 24-h normal male urine collection was divided into ten equal parts, and only six parts were analysed. The concentrations of androstanediol and testosterone were determined in each sample. The mean value was 93 μg/24 h ± 3.35 (S.D.) for androstanediol and 37 μg/24 h ± 2.4 (S.D.) for testosterone.

Specificity

The specificity of the method depends on the elimination of non-steroidal impurities by TLC and the adequate separation of the urinary steroids by paper chromatography and GLC. In GLC, the addition of authentic standards to the samples increased only the corresponding peak without the appearance of any additional peaks. The mobilities of urinary steroids in the systems employed are shown in Table I relative to testosterone and in Table II relative to androstanediol.

TABLE I

CHROMATOGRAPHIC MOBILITY AND RETENTION TIME OF URINARY KETOSTEROIDS

RT = Mobility relative to testosterone obtained by paper chromatography using the solvent system hexane–benzene (1:1).
RTT = Relative retention time with respect to testosterone trimethylsilyl ether obtained by GLC on 3% SE-30 at 240°.

Steroid	*RT*	*RTT*
Testosterone	1	1
Epitestosterone	1.29	0.85
Dehydroepiandrosterone	2	0.74
Androsterone	2.5	0.61
Etiocholanolone	2.29	0.64
11β-Hydroxyetiocholanolone	0.11	0.92
11-Ketoetiocholanolone	0.64	0.70
11β-Hydroxyandrosterone	0.23	0.90
11-Ketoandrosterone	0.72	0.72

RESULTS

Fig. 2 shows the chromatogram of an androstanediol urinary extract from a normal man, and Fig. 3 that of a testosterone urinary extract from a normal man after formation of the trimethylsilyl ethers. In 29 determinations from normal males

aged from 20 to 40 years, the mean excretion of androstanediol was 112 μg/24 h $\pm$ 42 (S.D.), and that of testosterone was 52 μg/24 h $\pm$ 26.2 (S.D.).

TABLE II

CHROMATOGRAPHIC MOBILITY OF URINARY HYDROXYSTEROIDS

RTA = Relative retention time with respect to 5α-androstanediol trimethylsilyl ether obtained by GLC.

Steroid	*RTA*	
	3% XE-60, 200°	*1% NGS, 185°*
5α-Androstane-3α,17β-diol	1.0	1.0
5β-Androstane-3α,17β-diol	1.18	1.33
Androst-5-ene-3β,17β-diol	1.46	1.70
5α-Androstane-3β,17β-diol	1.50	1.59
5α-Pregnane-3α,20α-diol	2.18	2.28
5β-Pregnane-3α,20α-diol	2.66	3.01

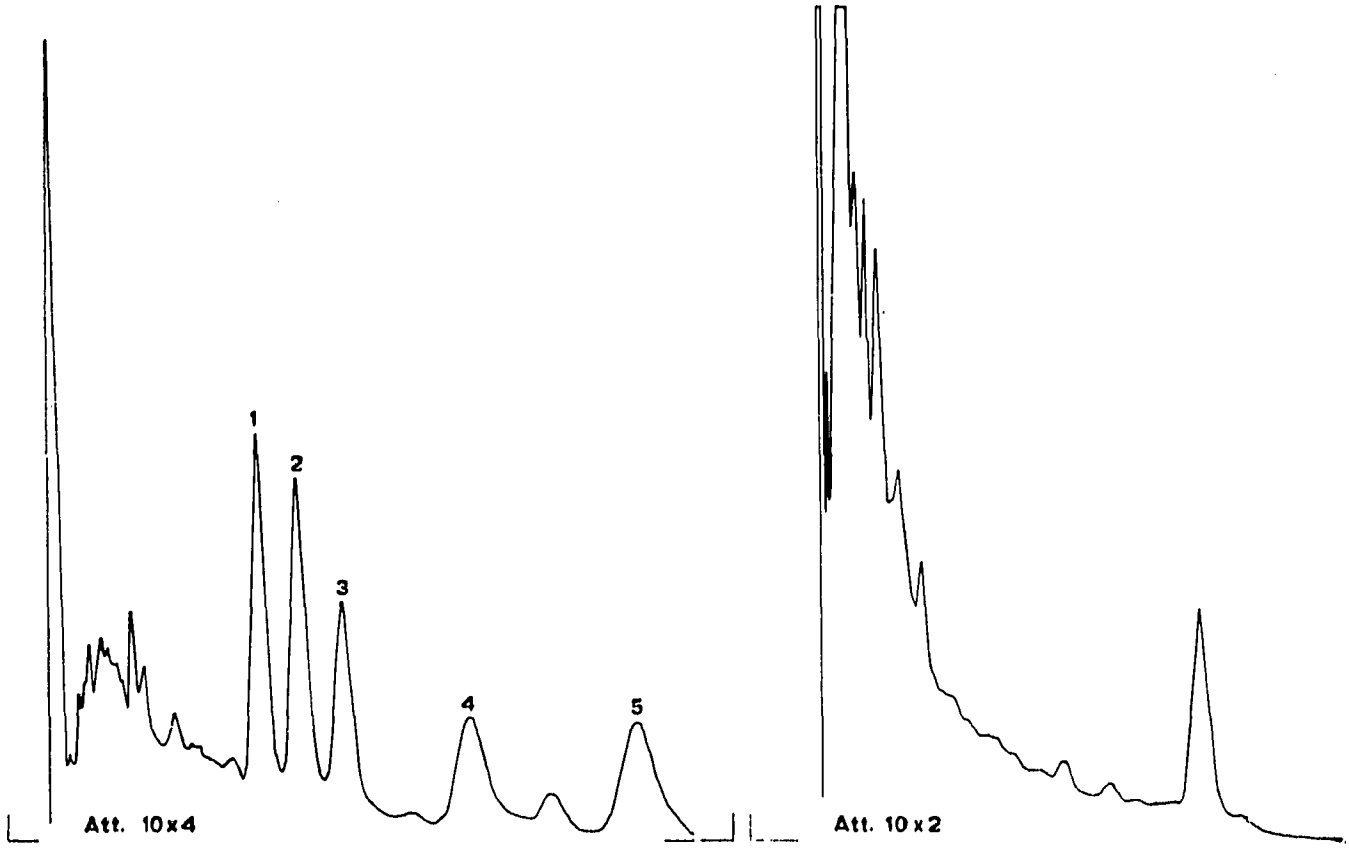

Fig. 2. Chromatogram of an androstanediol urinary extract from a normal man. 1 = 5α-Androstanediol; 2 = 5β-androstanediol; 3 = androstenediol; 4 = 5α-pregnanediol; 5 = β-pregnanediol. Conditions were as described in the text.

Fig. 3. Chromatogram of a testosterone urinary extract from a normal man after formation of the trimethylsilyl ethers.

DISCUSSION

The method described in this paper for the determination of urinary conjugated testosterone and androstanediol seems to be suitable for use in human subjects. From a technical point of view, testosterone is essentially excreted as a glucuronide and only a small amount of this androgen is excreted as sulphate and free steroid[8]. In the present experiment, indeed, no significant decrease in the 24-h excretion of testosterone was observed when extraction of free steroid was performed before the enzymatic hydrolysis of the urine. The procedure was also carried out for androstanediol.

In previous papers, the values of urinary testosterone reported cover a wide range. Those obtained in this experiment are in good agreement with the values obtained by other authors[9–11]. As regards androstanediol, the results obtained in normal men[7] are very close to ours. This could be foreseen since the technique described in this paper is very similar to that used by these authors.

From a clinical point of view, the mean values of urinary androstanediol obtained in normal men are more than twice as high as those of urinary testosterone. These results are in perfect accordance with the calculations made in this laboratory from experiments using the simultaneous administration of radioactive testosterone by oral, percutaneous and intravenous routes[5].

From these *in vivo* experiments, as from the present results, more than 50 % of the androstanediol may arise from extra-hepatic sources. On the other hand, urinary determinations done in pathological situations such as the testicular feminization syndrome (in preparation) seem to confirm the hypothesis already presented[5].

Thus, the simultaneous determination of urinary testosterone and androstanediol deserves attention and may be used for the calculation of an index of extra-hepatic testosterone metabolism, particularly of the extra-hepatic 5α-reduction of testosterone in target cells. This index could provide a simple means of determining the biological significance of active androgen at the level of target tissues.

REFERENCES

1 R. HORTON AND J. F. TAIT, *J. Clin. Invest.*, 45 (1966) 301.
2 K. M. ANDERSON AND S. LIAO, *Nature*, 219 (1968) 277.
3 N. BRUCHOVSKY AND J. D. WILSON, *J. Biol. Chem.*, 243 (1968) 5933.
4 E. E. GOMEZ AND S. L. HSIA, *Biochemistry*, 7 (1968) 24.
5 P. MAUVAIS-JARVIS, J. P. BERCOVICI, O. CREPY AND F. GAUTHIER, *J. Clin. Invest.*, 49 (1970) 31.
6 M. F. JAYLE, *Analyse des Stéroïdes Hormonaux*, Vol. 2, Masson, Paris, 1962, p. 458.
7 L. BERTHOU, L. G. BARDOU AND H. H. FLOCH, *J. Steroid Biochem.*, 2 (1971) 141.
8 A. M. CAMACHO AND C. J. MIGEON, *J. Clin. Endocrinol.*, 23 (1963) 301.
9 P. VESTERGAARD, E. RAADO AND S. VEDS, *Clin. Chim. Acta*, 14 (1966) 540.
10 M. LUISI, C. FASSORA, C. LEVANTE AND F. FRANCHI, *J. Chromatogr.*, 58 (1971) 213.
11 S. B. PAL, *Clin. Chim. Acta*, 33 (1971) 215.