A new method for the gas-chromatographic analysis of the urinary 11-deoxy-17-ketosteroids

The past few years have seen marked progress in the gas-chromatographic analysis of urinary 17-ketosteroids[1-8]. This is probably due to the greater selectivity and speed of this method in comparison with other systems of analysis. Owing to the extreme sensitivity of the method, however, rigorous purification is essential during the extraction from urines, with the result that the time required for the examination is increased.

The object of the present work was to devise a method for the determination of urinary 17-ketosteroids, which would exhibit the advantages of speed and simplicity normally associated with gas chromatography, while at the same time avoiding the need for laborious preliminary purification.

The urinary catabolites examined were confined to androsterone, etiocholanolone and dehydroepiandrosterone; the reasons for this were partly technical, *i.e.* the possibility of obtaining optimum separation of a few steroids in the central part of the chromatogram, and partly clinical, *i.e.* the fact that information about the rates of elimination of these three steroids is sufficient in most cases to act as a guide to diagnosis.

Extraction

50 ml of urine acidified to pH 4.5 with acetic acid and buffered with acetate buffer are subjected to enzymatic hydrolysis by *Helix pomatia* (1,500 U of β-glycuronidases and 12,000 U of sulfatases per ml of urine) for 24 h at 38°. The sample is then extracted with 50 ml and 2 × 25 ml of diethyl ether, and the extract is washed with 2 × 25 ml of *N* NaOH and with 2 × 25 ml of distilled water. The ether extract is dried with anhydrous sodium sulfate, filtered, and evaporated to dryness under vacuum. The dry extract is then taken up in 2 × 3 ml of acetone in Teflon containers, and the solvent is evaporated off in a current of nitrogen.

Preparation of trimethylsilyl (TMS) derivatives

1 ml of hexamethyldisilazane and 0.1 ml of trimethylchlorosilane are added to the extract, and the container is closed with a Teflon stopper and kept at 60° for 1 h. The solvents are evaporated off in a current of nitrogen, and the extract is redissolved and centrifuged at 3,000 r.p.m. for 5 min. The supernatant liquid is decanted into another container and evaporated to dryness.

Gas-chromatographic analysis

The gas-chromatographic analysis is carried out by means of a Fractovap Model C* chromatograph with an AID/f analytical unit having a flame-ionization detector.

The chromatographic column is made of glass, and is in the form of a spiral: I.D. 2 mm, length 2 m; packing: 1 % QF-1 on silanized Gas-Chrom P, 100–120 mesh. The conditions for analysis are: column temperature 190°, evaporator temperature 260°, flow rate of N_2 25 ml/min, H_2 0.3 kg/cm^2 and air 1.2 kg/cm^2.

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Injection of the extract

1 μl of a 0.5–2 μg/μl solution of the TMS derivatives in hexane is introduced with a Hamilton microsyringe, the attenuation values being stabilized at 10 × 8.

Quantitative estimation

The areas of the peaks are compared with those of the corresponding standards, which are introduced at a concentration of 1 μg/μl for each steroid. The values obtained in this way are converted into mg/24 h with the aid of the values for the dilution of the extract and for the diuresis.

Results

The resolution of the three standards introduced is shown in Fig. 1, while Figs. 2 and 3 show an example of the gas-chromatographic analysis of an urinary extract. The retention times listed in Table I are taken from the first line of the solvent peak to the maximum of the peak in question.

TABLE I

RETENTION TIMES (IN MINUTES) OF URINARY 17-KETOSTEROIDS

Steroid	*Retention time (min)*
Andro TMS	7.63
Etio TMS	8.82
DHEA TMS	10.39

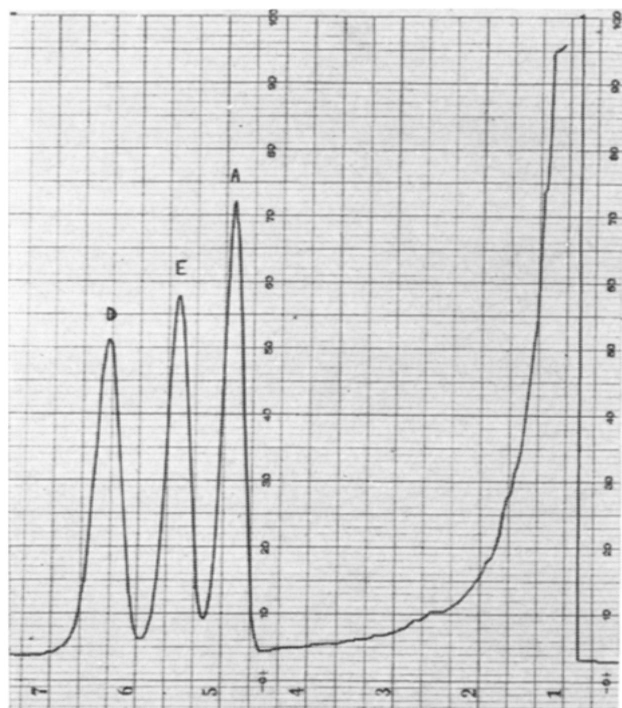
The results of recovery tests carried out after the addition of known quantities of the three steroids to urine were subjected to a statistical analysis, the results of which are shown in Table II.

The time required for the quantitative determination even of several samples from the hydrolysate is about 3 h.

Discussion

It should first be pointed out that for various reasons, considerable difficulty has been encountered in the use of gas-chromatographic methods for the analysis of the urinary 17-ketosteroids. In the first place, it was necessary to eliminate the non-steroid lipids before the extract was introduced into the apparatus; in the best-known of these methods[6], this was achieved by a preliminary separation by thin-layer chromatography, with the result that the time required for the examination was considerably increased. If, on the other hand, this preliminary purification is omitted, a satisfactory separation of the three principal 17-ketosteroids is impossible owing to the presence of interfering peaks[1]. Moreover, it is only recently that the difficulty of obtaining good discrimination between the three 17-ketosteroids by gas chromatography has been partially overcome by the conversion of the steroids into their trimethylsilyl ethers and by the use of highly polar columns.

In our method, the enzymatic hydrolysis gives a less pigmented urinary extract in comparison with acid hydrolysis, while the transfer of the urinary extract into



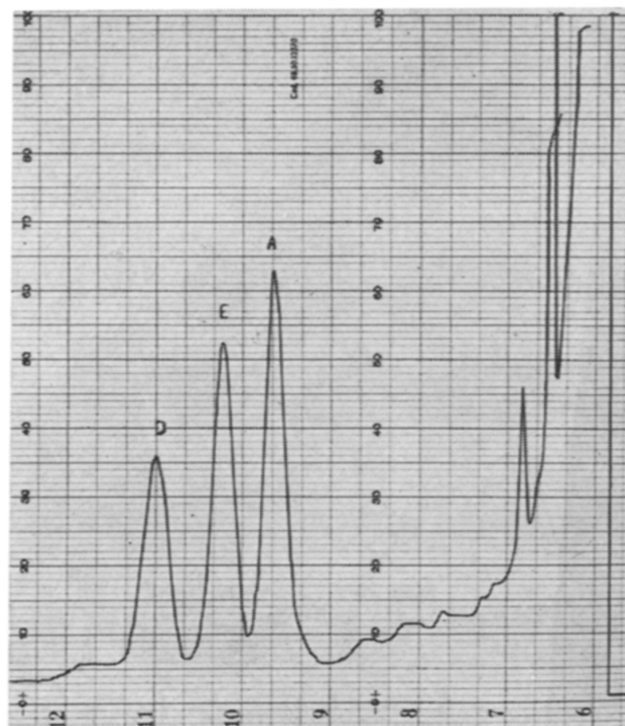


Fig. 1. Analysis of a mixture of androsterone, etiocholanolone, and dehydroepiandrosterone as their TMS derivatives at a concentration of 1 μg/μl. A = Androsterone; E = etiocholanolone; D = dehydroepiandrosterone.

Fig. 2. Analysis of a sample of male urine. A = Androsterone; E = etiocholanolone; D = dehydroepiandrosterone.

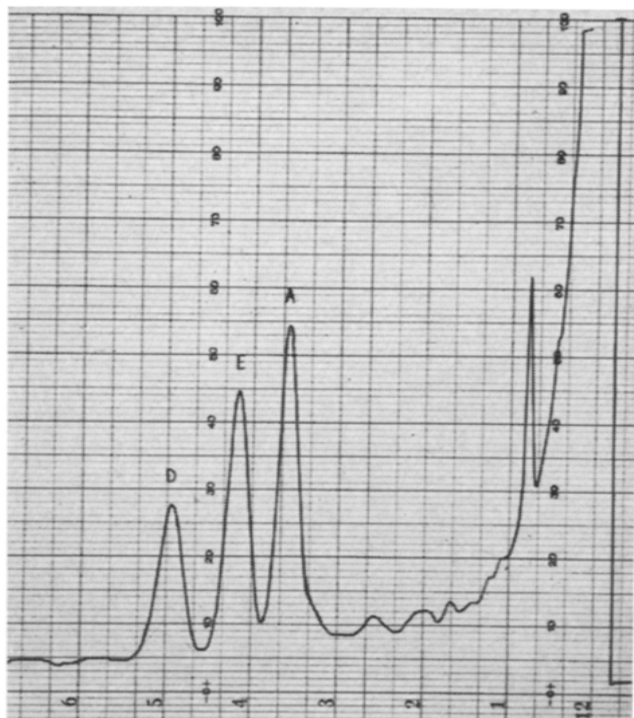


Fig. 3. Analysis of a sample of male urine. A = Androsterone; E = etiocholanolone; D = dehydroepiandrosterone.

TABLE II

RECOVERY OF THE URINARY 17-KETOSTEROIDS

A = androsterone; E = etiocholanolone; D = dehydroepiandrosterone.

Steroids µg/50 ml of urine							*Mean*	σ	*Amount recovered*	%
Basic	A	127.15	125.98	129.67	130.29	135.47	129.71	3.60	—	—
	E	96.69	104.08	94.19	126.73	120.09	108.35	14.38	—	—
	D	41.20	48.03	45.21	54.00	61.53	49.99	7.93	—	—
+ 50 µg of each	A	169.18	169.85	176.89	175.19	176.73	173.56	3.76	43.85	87.70 ± 14
	E	161.64	159.89	157.06	174.72	171.31	164.92	7.66	56.57	113.14 ± 30
	D	94.19	94.02	84.77	97.95	105.49	95.28	7.48	45.29	90.58 ± 29
+ 100 µg of each	A	228.57	218.52	224.48	244.89	230.61	229.41	9.79	99.70	99.70 ± 19
	E	200.00	210.98	189.01	215.38	191.20	201.31	11.66	92.96	92.96 ± 23
	D	149.76	150.70	142.46	149.45	149.45	148.36	3.31	98.37	98.37 ± 7
+ 200 µg of each	A	365.38	366.95	343.79	329.67	334.37	348.03	17.32	218.31	109.15 ± 17
	E	304.94	291.99	326.92	298.90	307.69	306.08	13.11	197.73	98.86 ± 13
	D	222.91	241.83	251.17	238.61	236.81	238.26	10.19	188.27	94.13 ± 10

the Teflon containers by means of acetone before the formation of the TMS derivative subsequently eliminates many of the pigments without interfering with the recovery of the 17-ketosteroids.

Still in connection with technical expedients, it should be noted that the fact that the detector system is kept at high sensitivity permits the use of very small quantities of solvent, which would otherwise interfere with the detection of the steroid peaks.

The results of the recovery tests reported in Table II provide sufficient evidence of the accuracy of the method, even when compared with the results obtained by much more time-consuming methods involving preliminary purification on a thin layer[6] (Table III), and they appear better than the results obtained by other methods that omit this preliminary treatment of the extract.

TABLE III

COMPARISON OF THE CONCENTRATIONS FOUND BY US IN A POOL OF MALE URINE (A) AND THOSE REPORTED BY KIRSCHNER AND LIPSETT[6] (B)

	A	*B*
Andro	2.59 mg/l	2.40 mg/24 h
Etio	2.16 mg/l	2.18 mg/24 h
DHEA	0.99 mg/l	0.50 mg/24 h

In conclusion, owing to the decrease in the time required, the satisfactory recovery, and the clarity of the gas chromatogram reading, our method confirms the possibility of developing and using gas chromatography for the analysis of steroids, and at the same time exhibits the characteristics of simplicity, accuracy, and specificity that are demanded for clinical examinations.

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