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A PRECISE AND SENSITIVE METHOD FOR THE ANALYSIS OF STEROIDS IN SMALL URINE SAMPLES BY THIN-LAYER CHROMATOGRAPHY AND GAS–LIQUID CHROMATOGRAPHY*

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SUMMARY

A precise and sensitive method for the analysis of urinary steroids at the submicrogram level is described. After enzymatic hydrolysis and solvolysis, the steroids are extracted with chloroform and ethyl acetate, purified and separated by thin-layer chromatography and determined by gas–liquid chromatography. In particular, novel conditions of hydrolysis of the steroid conjugates are discussed, and also the localization system of steroids on Kieselgel-H thin-layer plates with the use of azo dyes. Finally, the gas chromatographic analysis of steroid standards and steroids extracted from urine is illustrated and the quantitative aspects of the proposed method are considered.

INTRODUCTION

Many methods have been described for the determination of urinary steroids by gas–liquid chromatography (GLC)[1–8]. Such methods differ from each other in the procedure for hydrolysing the steroid conjugates contained in the urine and the methods of purifying the steroid extract.

The hydrolytic procedure may be acidic, enzymatic or mixed. Acidic hydrolysis is carried out with hydrochloric[9,10], sulphuric[11,12] or perchloric acid[13]. As it has been observed that the cleavage caused by these acids is destructive and produces artefacts[7,11,12,14,15], some authors have used enzymatic splitting with β-glucuronidase and arylsulphatase[6,16]. Finally, other workers have used hydrolysis that is partly enzymatic and partly acidic[1,4,7,8], because enzymatic hydrolysis alone does not proceed to completion as enzyme inhibitors are present in the urine[17–19] and also as steroid sulphates with a 3α,5α configuration are not hydrolysed by commercial arylsulphatase[20]. The methods of analysis of urinary steroids differ further in the procedures used for purifying the extract before GLC if a high proportion of contaminating substances is present in a small amount of steroid (submicrogram level) that is to be determined. The removal of the contaminants, after washing the steroid

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extract, is carried out by various types of adsorption chromatography on alumina[7] and silicic acid[4,6,8] columns. As already shown by other workers[2,5,16,21], we also previously observed the high efficiency of thin-layer chromatography (TLC) in purifying steroid compounds[22]. We also feel that it can be used for the preparation of steroid classes prior to GLC. Having already described in a previous publication[23] the GC behaviour of a large number of steroids as their trimethylsilyl ethers, chloromethyldimethylsilyl ethers, chlorodifluoroacetates and heptafluorobutyrates, the aims of this research were to purify and separate by TLC and analyse by GLC a steroid extract by using the techniques previously described by us[22,23].

Another aim of this research, in view of the differences in the available methods for the hydrolysis of the steroid conjugates, was to establish suitable conditions for complete cleavage of the conjugated compounds.

MATERIALS AND INSTRUMENTS

The materials used were supplied by various commercial firms.

The steroid standards were obtained from Steraloid Chemical Co., Pawling, N.Y., U.S.A. Androsterone sulphate and dehydroepiandrosterone sulphate were supplied by Ikapharm, Ramat-Gan, Israel.

The dyes were ordered from KEK Laboratories, Planview, N.Y., and National Aniline Division, N.Y., U.S.A.

The Kieselgel-H was obtained from Bracco Industrie Chimiche, Milan, Italy.

The enzymes β-glucuronidase and arylsulphatase were ordered from Calbiochem, Los Angeles, Calif., U.S.A.

Regisil (bistrimethylsilyltrifluoroacetamide + 1 % trimethylchlorosilane) was obtained from Regis Chemical Co., Chicago, Ill., U.S.A.

The stationary phases for GLC, SE-30 and TMCBA (tetramethylcyclobutanediol adipate), and the Gas-Chrom P support were supplied by Applied Science Laboratories Inc., State College, Pa., U.S.A.

All the solvents used in this research were of RP-ACS type, marketed by Carlo Erba, Milan, Italy, and re-distilled. In particular, the ethyl acetate was purified according to BURSTEIN AND KIMBALL[24] and the methanol according to BRAUNSBERG AND JAMES[25].

Purified air, nitrogen and hydrogen for GLC were ordered from Siad, Bergamo, Italy.

The instrument for coating the plates for TLC was supplied by Chemetron, Milan, Italy. The tanks were ordered from the Brinkmann Chemical Co., New Jersey, N.J., U.S.A. The fibre-glass paper was supplied by Reeve Angel, Clifton, N.J., U.S.A.

A Hewlett-Packard Model 5750 gas–liquid chromatograph was used.

STANDARD PROCEDURE

Urine samples (5 ml) are placed in 50-ml centrifuge test-tubes having PTFE-lined screw caps. A 0.5-ml volume of acetate buffer of pH 5.5 is added to bring the pH of the urine to about 5.40–5.50. Then an enzymatic preparation containing β-glucuronidase and arylsulphatase is added so as to obtain 5000 Fishman Units (F.U.) and 2500 Whitehead Units (W.U.), respectively, per millilitre of incubation solution.

The urine is incubated in a water-bath at 37° for 24 h and then 10 μg of allopregnanolone (5α-pregnan-3β-ol-20-one) as internal standard are added. The urine is next extracted with 5-ml volumes of chloroform three times and 5-ml volumes of ethyl acetate three times. After shaking the extracts for 1 min and centrifuging them for 5 min at 3000 r.p.m., they are all placed in one 50-ml centrifuge test-tube, washed once with 3 ml of *N* sodium hydroxide solution and twice with 3 ml of distilled water and kept aside. The urine is then treated by BURSTEIN AND LIEBERMAN'S method[11], that is, it is brought to pH 1 with sulphuric acid, and after the addition of 1 g of sodium chloride, is extracted with 5-ml volumes of ethyl acetate three times. The organic extracts are all placed in 5-ml centrifuge test-tubes, then kept in a water-bath at 37° for 18 h. After this period, they are washed again, once with 3 ml of *N* sodium hydroxide solution and twice with distilled water. The total organic extracts obtained after enzymatic and acid hydrolysis are placed into a 100-ml flask and concentrated at 56° under nitrogen, then transferred to micro test-tubes and evaporated to dryness. The sample is re-dissolved in 50 μl of a solution of methanol–chloroform (1:1) containing the following dyes: Sudan III, *p*-aminoazobenzene, 2,4-diaminoazobenzene, 4-(2-tolylazo)resorcinol and Bismarck Brown Y. A 25-μl volume of this solution is then spotted with a semi-automatic Hamilton microsyringe on to 300-μm thick Kieselgel-H thin-layer plates, prepared by STAHL'S method[20].

The plates are developed in Brinkmann tanks, covered internally with fibreglass paper, which has previously been saturated with benzene–methanol (175:25) developing solvent. Depending on whether the 17-ketosteroids are analysed alone, in association with pregnanediol or with the steroids having three hydroxyl functions, the powder is removed, including the *p*-aminoazobenzene, and either the 2,4-diaminoazobenzene, the 4-(2-tolylazo)resorcinol or the Bismarck Brown, respectively. The powder is collected in small columns with glass microfilters. To the powder, five 2-ml volumes of an elution mixture consisting of methanol–diethyl ether (1:1) are added. The eluate is collected in micro-test-tubes and evaporated to dryness under nitrogen. The samples are then re-dissolved in 1 ml of chloroform, and 0.2 ml of Regisil and 0.1 ml of pyridine are added. The samples are left overnight at room temperature. After evaporating the samples to dryness, the trimethylsilyl ether derivatives of the steroids are dissolved in hexane and subjected to GLC on a column consisting of SE-30 and TMCBA[23] as stationary phases.

RESULTS AND DISCUSSION

Hydrolysis

The quantities of the enzymes β-glucuronidase and arylsulphatase used in doses of 5000 F.U. and 2500 W.U., respectively, per millilitre of urine are effective in splitting the conjugated compounds, glucuronides and sulphates. Thus 5 mg of dehydroepiandrosterone sulphate and 2.5 mg of phenolphthalein glucuronide submitted to enzymatic hydrolysis were completely split into free compounds (Table I). The splitting of the steroid conjugates depends on the quantity of enzymes used, and good results were obtained in the present research with a high enzyme concentration. Lower hydrolysis values were produced with a decrease in the quantity of enzyme used.

TABLE I

HYDROLYSIS OF GLUCURONIDATE AND SULPHATE COMPOUNDS BY β-GLUCURONIDASE AND ARYLSULPHATASE ACCORDING TO THE METHOD DESCRIBED

Compound	*Amount incubated (mg)*	*Free amount expected (mg)*	*Amount found after hydrolysis (mg)*	*Recovery (%)*
Dehydroepiandrosterone sodium sulphate	5.0	3.38	3.31 ± 0.02[a]	98
Phenolphthalein sodium glucuronide	2.582	1.716	1.700 ± 0.011[a]	99

[a] Each value represents the average of three separate determinations ± S.D.

TABLE II

HYDROLYSIS OF URINARY STEROIDS BY β-GLUCURONIDASE AND ARYLSULPHATASE IN VARIOUS CONCENTRATIONS

The samples were urine taken from the same subject on different days. Figures in the table are steroid values expressed as mg/24 h.

Sample	*Androsterone*		*Etiocholanolone*		*Dehydroepiandrosterone*	
	I[a]	*II*[a]	*I*[a]	*II*[a]	*I*[a]	*II*[a]
I	2.264	1.360	2.160	1.360	0.812	0.324
II	2.758	1.497	2.048	1.891	1.260	0.615
III	3.293	3.034	4.129	3.256	1.613	0.510

[a] Concentrations of β-glucuronidase and arylsulphatase per millilitre correspond to 5000 F.U. and 2500 W.U. (I) and 1000 F.U. and 500 W.U. (II).

TABLE III

HYDROLYSIS OF DIFFERENT AMOUNTS OF ANDROSTERONE SODIUM SULPHATE BY ARYLSULPHATASE IN VARIOUS CONCENTRATIONS ACCORDING TO THE METHOD DESCRIBED

The arylsulphatase used was from the commercial preparation supplied by Calbiochem: β-glucuronidase associated with arylsulphatase extracted from *Helix pomatia* juice.

Sample	*Amount of enzyme (W.U. per ml of incubation mixture)*	*Amount of androsterone sulphate added (μg)*	*Expected amount of androsterone freed on complete hydrolysis (μg)*	*Actual amount of androsterone found (μg)*	*Degree of hydrolysis (%)*
1	2500	1000	678	4.3	0.63
2	2500	1000	678	4.7	0.69
3	2500	1000	678	3.8	0.56
4	5000	1000	678	6.2	0.91
5	5000	1000	678	5.3	0.78
6	5000	50	33.9	0.7	2.07
7	5000	50	33.9	0.75	2.22
8	5000	50	33.9	1.1	3.25

Using enzymatic concentrations of 5000 F.U. and 2500 W.U. per millilitre of urine, as proposed in the present method, compared with those used by other workers, *i.e.*, 1000 F.U. and 500 W.U.[2,4,6–8], we showed that a decrease in the amount of free steroids occurred at the lower doses of enzymes (Table II). Consequently, the other

workers did not obtain complete enzymatic hydrolysis as they did not use adequate enzyme concentrations. Nevertheless, even under the best conditions of enzymatic cleavage, the splitting of some steroid sulphates is not completed. Thus we submitted 1-mg and 50-μg quantities of androsterone sulphate to enzymatic hydrolysis with different concentrations of sulphatase and observed very low values of free androsterone, varying between 0.56 % and 3.25 % of the total amount expected (Table III). Our results are not in accordance with those obtained by other workers, who claimed to have obtained complete hydrolysis of the steroid conjugates, including the 3α-sulphates, by using enzyme concentrations the same as those used in the present research[16]. The fact that androsterone sulphate is only weakly hydrolysed by the sulphatases contained in *Helix pomatia* juice is in accordance with the knowledge

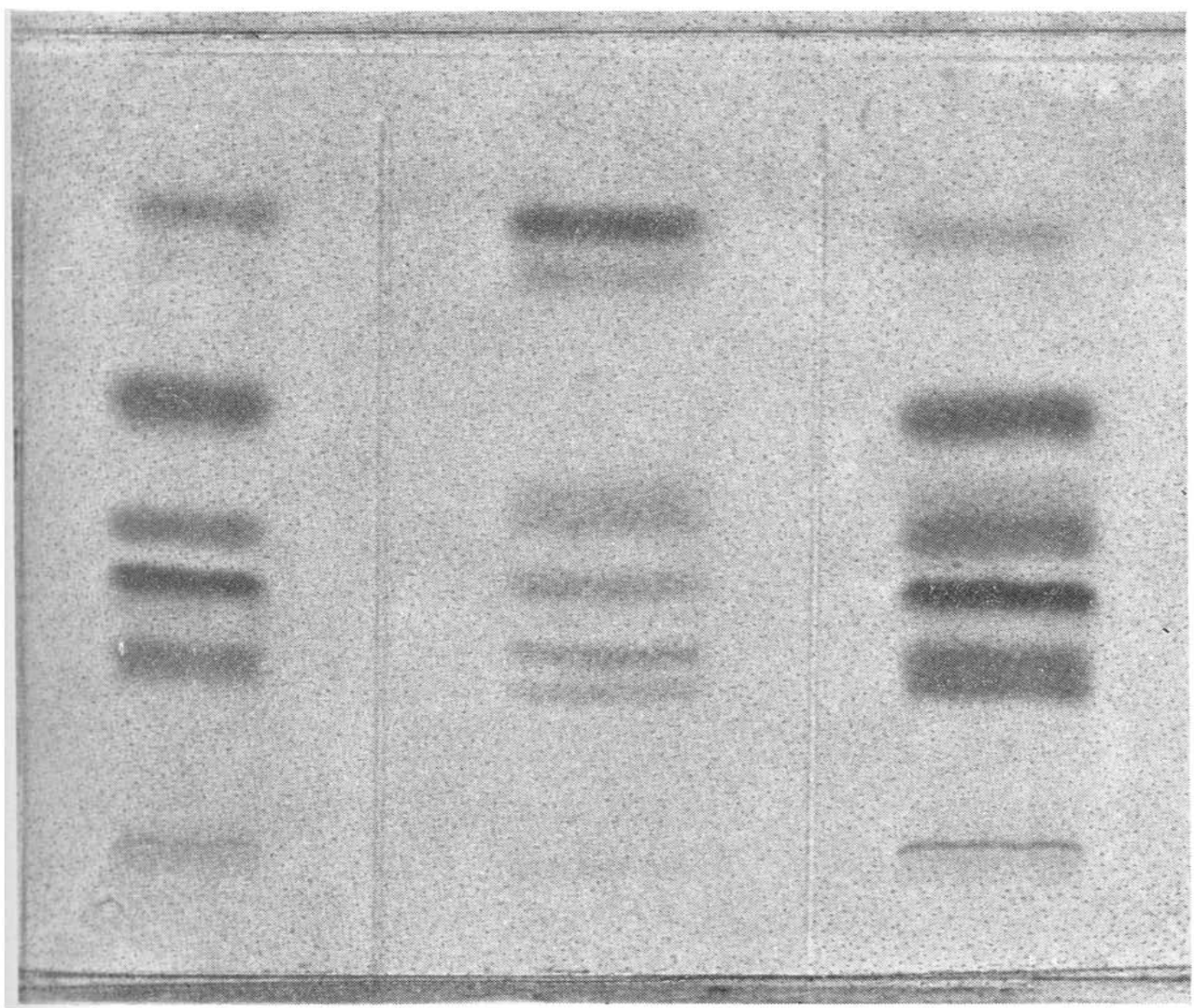

Fig. 1. Separation of steroids and azo dyes on Kieselgel-H thin-layer plates using benzene–methanol (175:25) as the mobile phase. On the left of the plate are spotted, from top to bottom, Sudan III, *p*-aminoazobenzene, 2,4-diaminoazobenzene, 4-(2-tolylazo)resorcinol and Bismarck Brown Y. In the centre are spotted Sudan III, androsterone, dehydroepiandrosterone, etiocholanolone, pregnanediol, estradiol, pregnanetriol and estriol. On the right of the plate, the steroids and azo dyes are spotted together. Phosphomolybdic acid was used to reveal the steroids. It is possible to observe that steroids having different functional groups can be easily localized by using the above dyes.

that steroid sulphates with a 3α,5α configuration are practically not hydrolysed by the above enzymes[20]. Therefore, in order to ensure complete hydrolysis of the steroid sulphates, we used solvolysis as described by BURSTEIN AND LIEBERMAN[11] in addition to enzymatic hydrolysis.

Thin-layer chromatography

After extracting the steroids with chloroform and ethyl acetate[27], the steroids contained in the extract are purified and separated on Kieselgel-H plates, using benzene–methanol (175:25) as the mobile phase. The steroids migrate in differentiable classes, according to their hydroxyl and ketone functions, and are easily localized with azo dyes. These are added to the extract before TLC and do not interfere with the migration of the steroids themselves. As shown in Fig. 1, the 17-ketosteroids are located between *p*-aminoazobenzene and 2,4-diaminoazobenzene, and the 17-ketosteroids and pregnanediol occur in the zone between *p*-aminoazobenzene and 4-(2-tolylazo)resorcinol. With Bismarck Brown, it is possible to localize the position of steroids having three hydroxyl functions.

Elution of steroids from TLC plates

Using labelled steroids, it was found that extensive destruction of the steroids occurred during evaporation and concentration of the eluate obtained from the Kieselgel plates[28]. To avoid such a loss, the steroids were eluted from the powder by using small columns with glass microfilters and the solvent was collected and evaporated under nitrogen in micro test-tubes[29]. We used diethyl ether–methanol (1:1) as the elution solvent. To test the efficiency of the solvents proposed by us, 5 μg of a steroid mixture were spotted on Kieselgel-H plates and then eluted with diethyl ether–methanol (1:1) and determined by GLC. Recoveries of the various steroids were of the order of 99–100 % (Table IV). Such recoveries are comparable with those obtained by MATTHEWS *et al.*[30] and GÄNSHIRT[31], who reported high recoveries of steroids spotted on thin-layer plates but in greater quantities (about 50–100 μg).

Gas–liquid chromatography

The steroids are subjected to GLC analysis as their trimethylsilyl ethers on a two-phase column previously described by us[23]. A gas chromatogram in which many

TABLE IV

RECOVERIES OF VARIOUS STEROIDS SPOTTED ON KIESELGEL-H THIN-LAYER PLATES, ELUTED WITH DIETHYL ETHER–METHANOL (1:1) AND DETERMINED AS THEIR TRIMETHYLSILYL ETHERS BY GAS–LIQUID CHROMATOGRAPHY

Steroid	*Amount of steroid spotted (μg)*	*Amount of steroid recovered (μg)*[a]	*Standard deviation*	*Recovery (%)*
Androsterone	5	5.05	±0.62	100
Etiocholanolone	5	4.74	±0.19	94.8
Dehydroepiandrosterone	5	4.85	±0.17	97
Pregnanediol	5	4.95	±0.45	99
Estriol	5	4.74	±0.24	94.8

[a] Each value represents the average of four different samples.

steroid standards are separated is shown in Fig. 2. The azo dyes, if present in the steroid mixture and subjected to the silylation process, do not alter the retention time of the steroids.

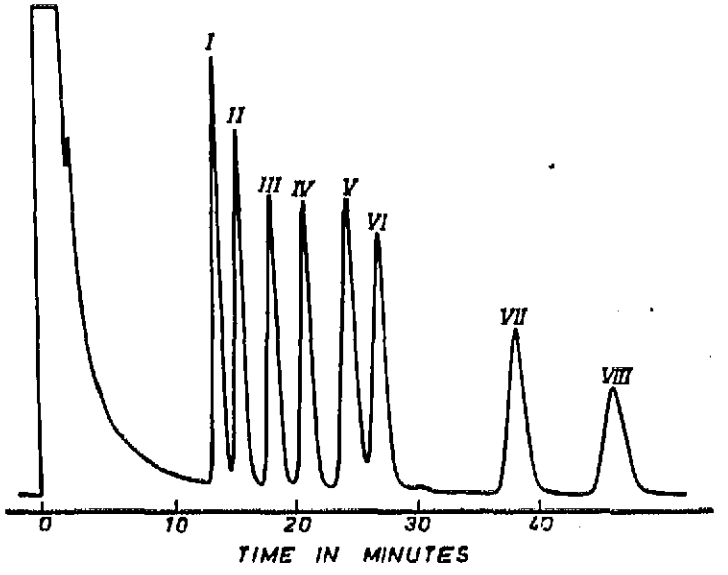

Fig. 2. Separation of steroid hormones as their trimethylsilyl ethers on a two-phase column composed of SE-30 and TMCBA. The compounds are located in the following order: androsterone (I), etiocholanolone (II), dehydroepiandrosterone (III), estradiol (IV), estrone (V), pregnanediol (VI), estriol (VII) and pregnanetriol (VIII). A Hewlett-Packard Model 5750 gas chromatograph was used, equipped with a flame ionization detector. Separation was carried out under the following conditions: temperature of the inlet heater, 270°; column temperature, 230°; detector temperature, 270°; outlet flow-rate, 24 ml/min. Coiled borosilicate glass columns, 6 ft. × 5 mm I.D., were used.

TABLE V

RECOVERIES OF VARIOUS STEROIDS DISSOLVED IN 5 ml OF AN AQUEOUS SOLUTION AND ANALYSED BY THE METHOD DESCRIBED

Steroid	*Amount of steroid added (μg)*	*Amount of steroid recovered (μg)*[a]	*Standard deviation*	*Recovery (%)*
Androsterone	5	4.87	±0.34	96.9
Etiocholanolone	5	4.60	±0.40	91.1
Dehydroepiandrosterone	5	4.81	±0.32	96.0
Pregnanediol	5	4.86	±0.35	96.9

[a] Each value represents the average of five individual determinations.

Recovery tests and use of the method

To evaluate the efficiency of the above method, aqueous solutions containing 5 μg of various steroids were submitted to the analytical procedure described. Recoveries of 91–96.9 % were obtained (Table V). Furthermore, 5 μg of dehydroepiandrosterone and pregnanediol were added to 5 ml of urine and subjected to hydrolysis and extracted by the method described. Recoveries of these steroids varied between 75 and 96 % (Table VI). To correct for eventual losses of steroids contained in the urine, allopregnanolone was added to the urine as an internal standard before beginning the extraction procedure. The method described is therefore quantitative, even for small amounts of urine. Samples of 5 ml of urine from normal subjects were

TABLE VI

RECOVERIES OF 5 μg OF DEHYDROEPIANDROSTERONE AND PREGNANEDIOL DISSOLVED IN TWO SAMPLES OF 5 ml OF URINE, HYDROLYSED AND EXTRACTED ACCORDING TO THE METHOD DESCRIBED

Sample	*Steroid*	*Amount of steroid present in urine (μg)*[a]	*Amount of steroid expected (μg)*	*Amount of steroid found (μg)*	*Recovery (%)*
I	Dehydroepiandrosterone	1.02	6.02	5.54	92.0
	Pregnanediol	1.46	6.46	6.20	96.0
II	Dehydroepiandrosterone	0.80	5.80	4.40	75.5
	Pregnanediol	1.00	6.00	4.80	80.0

[a] The amount of steroid present in the urine was determined in the same samples of urine without adding the steroids.

TABLE VII

URINARY EXCRETION OF STEROID HORMONES IN NORMAL HUMAN SUBJECTS

Figures given in the table are steroid values expressed in mg/24 h.

Subjects	*Age*	*Androsterone*	*Etiocholanolone*	*Dehydroepiandrosterone*	*Pregnanediol*	*Method*
B.G.	52	0.820 ± 0.05[a]	0.980 ± 0.300	0.230 ± 0.110	0.031 ± 0.062	This work
P.L.	58	2.340 ± 1.540	1.730 ± 0.630	1.260 ± 0.860	0.408 ± 0.337	This work
S.A.	58	0.796 ± 0.298	1.290 ± 1.117	0.331 ± 0.287	0	This work
10[b]	>50	0.38 (0.0–0.73)[c]	1.53 (0.14–2.7)[c]	0.6 (0.0–1.1)[c]	—	SOLOMON *et al.*[8]
13[b]	>50	—	—	—	0.15 ± 0.02	ROMANOFF *et al.*

[a] Each value represents the average of four determinations of urine samples ± S.D., collected on different days.

[b] Number of subjects studied.

[c] Mean ranges.

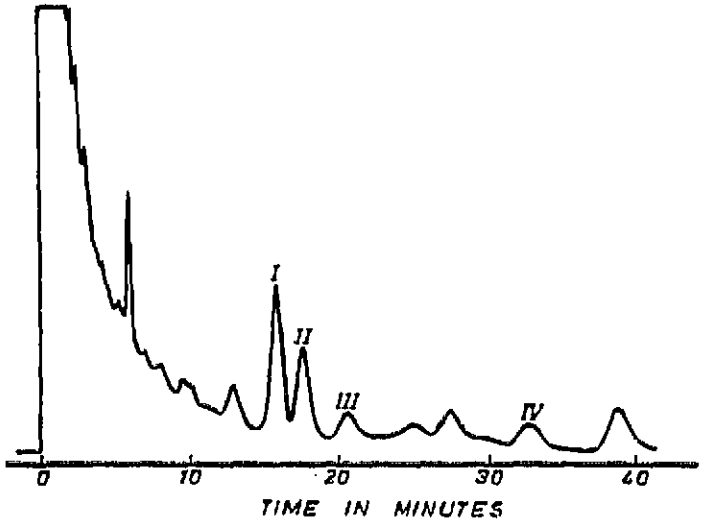

Fig. 3. Separation of steroids extracted from human urine and analysed as their trimethylsilyl ethers according to the described method. The compounds identified were androsterone (I), etiocholanolone (II), dehydroepiandrosterone (III) and pregnanediol (IV). Conditions for the steroid separation are the same as in Fig. 2, except for the column temperature, which was 225°.

subjected to the analysis, and the results are given in Table VII. Fig. 3 shows a gas chromatogram of the urinary steroids.

CONCLUSION

The use of the hydrolytic procedure and the quantitative extraction of steroids, the removal of contaminants by TLC and the extreme sensitivity of the GLC system allowed us by the method described to analyse sub-microgram amounts of steroids in small volumes of urine. The present procedure is advantageous also for its lower cost compared with other methods that deal with 50 or 100 ml of urine[4-11]. The method is therefore recommended for the analysis of various steroids, in particular 17-ketosteroids and pregnanediol, in 5 ml of urine obtained from human subjects or from small laboratory animals.

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REFERENCES

1 M. SPARAGANA, E. H. KEUTMAN AND W. B. MASON, *Anal. Chem.*, 35 (1963) 1231.
2 M. A. KIRSCHNER AND M. B. LIPSETT, *Steroids*, 3 (1964) 277.
3 L. P. CAWLEY, B. O. MUSSER, W. FAWCETTE, S. BECKLOFF AND H. LEARNED, *Clin. Chem.*, 11 (1965) 1009.
4 J. T. FRANCE, R. RIVERA, N. L. MCNIVEN AND R. I. DORFMAN, *Steroids*, 5 (1965) 687.
5 C. R. BERRET AND C. MCNEIL, *Clin. Chem.*, 12 (1966) 399.
6 W. L. GARDINER AND E. C. HORNING, *Biochim. Biophys. Acta*, 115 (1966) 524.
7 H. C. CURTIUS AND M. MÜLLER, *J. Chromatogr.*, 30 (1967) 410.
8 D. SOLOMON, D. STRUMMER AND P. P. NAIR, *Amer. J. Clin. Pathol.*, 48 (1967) 295.
9 J. B. BROWN AND H. A. BLAIR, *J. Endocrinol.*, 17 (1958) 411.
10 I. J. DREKTER, A. HEISLER, G. R. SCISM, S. STERN AND S. PEARSON, *J. Clin. Endocrinol. Metab.*, 12 (1952) 55.
11 S. BURSTEIN AND S. LIEBERMAN, *J. Biol. Chem.*, 233 (1958) 331.
12 G. JACOBSOHN AND S. LIEBERMAN, *J. Biol. Chem.*, 237 (1962) 1468.
13 J. C. DE PAOLI, E. NISHIZAWA AND K. B. EIK-NES, *J. Clin. Endocrinol. Metab.*, 23 (1963) 81.
14 M. F. JAYLE, E. E. BAULIEU, R. HENRY AND R. SCHOLLER, in M. F. JAYLE (Editor), *Analyse des Stéroides Hormonaux*, Vol. II, Masson, Paris, 1962, p. 17.
15 J. A. MOORE AND E. HEFTMANN, in O. EICHLER AND A. FARAH (Editors), *The Adrenocortical Hormones, Handbuch der Experimentellen Pharmakologie*, Vol. XIV/I, Springer-Verlag, Berlin, 1962, p. 294.
16 F. A. VANDENHEUVEL, *Can. J. Biochem.*, 45 (1967) 191.
17 O. CREPY, M. F. JAYLE AND F. R. MESLIN, *Acta Endocrinol.*, 24 (1957) 233.
18 W. R. SLAUNWHITE, JR. AND A. A. SANDBERG, *Endocrinology*, 62 (1958) 283.
19 I. E. BUSH AND V. B. MAHESH, *Biochem. J.*, 71 (1959) 705.
20 A. B. ROY, *Biochem. J.*, 62 (1956) 41.
21 R. NOIRET, *J. Chromatogr.*, 34 (1968) 415.
22 P. P. NAIR AND A. PINELLI, *J. Chromatogr.*, 47 (1970) 513.
23 A. PINELLI AND P. P. NAIR, *J. Chromatogr.*, 43 (1969) 223.
24 S. BURSTEIN AND H. L. KIMBALL, *Steroids*, 2 (1963) 209.
25 H. BRAUNSBERG AND V. H. T. JAMES, *Anal. Biochem.*, 1 (1960) 452.
26 E. STAHL (Editor), *Thin-Layer Chromatography*, Academic Press, London, 1965, p. 5.
27 S. BURSTEIN, *Science*, 124 (1956) 1030.
28 D. R. IDLER, N. R. KIMBALL AND B. TRUSCOTT, *Steroids*, 8 (1966) 865.
29 F. A. VANDENHEUVEL, *J. Lab. Clin. Med.*, 69 (1967) 343.
30 J. S. MATTHEWS, A. L. PAREDA AND A. P. AGUIBERA, *J. Chromatogr.*, 9 (1962) 331.
31 H. GÄNSHIRT, in E. STAHL (Editor), *Thin-Layer Chromatography*, Academic Press, London, 1965, p. 53.
32 L. P. ROMANOFF, M. P. CRACE, M. N. BAXTER AND G. PINCUS, *J. Clin. Endocrinol. Metab.*, 26 (1966) 1023.