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EVALUATION OF A GAS–LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF URINARY STEROIDS USING HIGH-RESOLUTION OPEN-TUBULAR GLASS CAPILLARY COLUMNS

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SUMMARY

A gas chromatographic method is described using high-resolution open-tubular glass capillary columns for the simultaneous estimation of the following neutral steroids in urine: androsterone, etiocholanolone, dehydroepiandrosterone, 11-oxoandrosterone, 11-oxoetiocholanolone, pregnanolone, 11β-hydroxyandrosterone, 11β-hydroxyetiocholanolone, *allo*-pregnanediol, pregnanediol, Δ^5-pregnenediol, pregnanetriol and cholesterol. The method involves an ether extraction of the urine after enzymatic hydrolysis and the gas chromatographic separation of the steroids as their trimethylsilyl ether derivatives on a 50 m × 0.25 mm I.D. OV-101 capillary column. Quantitation is effected by reference to the peaks derived from three mass internal standards added to the hydrolysed urine. Data are presented that show that the precision and sensitivity of the method are adequate for clinical purposes. Experiments using reference steroids demonstrated essentially quantitative recoveries and the specificity as determined by combined gas chromatography–mass spectrometry was found to be satisfactory for eleven of these neutral steroids. Preliminary studies using an auto-solid sampler and an automated gas chromatograph indicate that the method is readily amenable to routine application.

INTRODUCTION

Gas–liquid chromatography (GLC) is one of the most widely used and effective techniques for the analysis of hormonal steroids. A recent and significant development in this field of biochemical analysis has resulted from advances made in the preparation of open-tubular glass capillary columns suitable for high-temperature analysis. Initially, attempts were made to use stainless-steel capillary columns for

steroid analysis[1] but these were not entirely successful. Subsequent studies showed that capillaries made of glass were necessary if decomposition and adsorption of steroids at the nanogram and subnanogram level were to be avoided. The difficult problem of preparing open-tubular glass columns coated with thermostable stationary phases was first overcome by Grob[2]. Several reliable procedures for preparing these thermally stable columns have since been described[3–7]. When used with suitable injection systems, glass capillary columns can perform multicomponent steroid separations at the submicrogram level with very much greater resolving power than can be obtained with conventional packed columns[8–10]. A number of investigators have demonstrated that the major neutral steroid metabolites extracted from human urine can be effectively separated as their trimethylsilyl (TMS) ether or O-methyl-oxime-trimethylsilyl ether (MO-TMS) derivatives on open-tubular glass columns coated with non-polar stationary phases[10–15].

In a previous paper Ros and Sommerville[15] described a method for the simultaneous estimation of 27 neutral steroid metabolites in human urine as their MO-TMS derivatives on open-tubular glass columns coated with the stationary phase OV-101. The method was evaluated for precision and specificity for the determination of 18 of these compounds but no data were given relating to the accuracy of the assay procedure. It is often stated that accurate quantitative results are difficult to obtain with open-tubular columns. The purpose of this study was to develop a reliable quantitative method for the simultaneous determination of the major androgen and progesterone metabolites in urine that was suitable for routine application.

MATERIALS AND METHODS

Reference steroid compounds were purchased from Steraloids (Croydon, Great Britain) and their purity checked by GLC and gas chromatography–mass spectrometry (GC–MS). All solvents were obtained from BDH (Poole, Great Britain) and were of AnalaR grade. Hexane was washed with concentrated sulphuric acid and water, dried over anhydrous sodium sulphate, and redistilled. Diethyl ether was redistilled twice before use. The purity of these solvents was checked by GLC.

Bis(trimethylsilyl)acetamide (BSA), trimethylchlorosilane (TMCIS), and hexamethyldisilazane were obtained from Pierce, Rockford, Ill., U.S.A. 2-Chloro-1,1,2-trifluoroethyl methyl ether was obtained from Fluorochem, Glossop, Great Britain. The juice from *Helix pomatia* (Industrie Biologique Française, Gennevilliers, France) contained 100,000 units of β-glucuronidase (Fisherman) and 800,000 units of sulphatase (Roy) per millilitre.

Procedure

24-h urine samples were collected and stored at −20° until analysed. A 4-ml aliquot of urine was adjusted to pH 4.8 with dilute acetic acid, 0.8 ml of 1 *M* sodium acetate buffer (pH 4.8) and 0.04 ml of *Helix pomatia* extract were added and the solution incubated at 37° for 48 h. 4 μg of each of the three internal standards, *viz.* epietiocholanolone, 5α-androstan-3β,17β-diol and epicoprostanol, were then added, and the urine extracted twice with 20 ml of diethyl ether. The combined ether extracts were washed twice with 2 ml of 2 *N* NaOH and twice with 2 ml of distilled water, dried over anhydrous sodium sulphate and evaporated to dryness on a rotary vacuum

evaporator. The dried extract was transferred with methanol to a screw-capped tube with a PTFE-lined cap, and then taken to dryness under nitrogen.

The TMS ether derivatives were prepared by dissolving the extract in 0.1 ml of chloroform and reacting for 1 h at 60° with 0.1 ml of BSA and 20 μl of TMCIS. The reaction mixture was taken to dryness at 60° under dry nitrogen and finally taken up in 0.4 ml of hexane. 4 μl of this solution was taken for analysis by GLC.

Gas–liquid chromatography

GLC was carried out on a Pye Unicam Series 104, Model 64, gas chromatograph (Pye Unicam, Cambridge, Great Britain) equipped with dual flame ionization detectors. In order to prevent possible thermal decomposition and component loss during analysis, an all-glass system was used. The stainless-steel detector inlet pipe was replaced by a 3-cm length of (1/16 in. O.D. × 0.5 mm I.D.) glass-lined stainless-steel tubing (Scientific Glass Engineering, Melbourne, Australia) and this was terminated in the GLC oven by a Swagelok T-union. An ancillary flow of argon was added at the outlet end of the column via the T-union in order to eliminate "dead volume" effects in the detector pipe and also to obtain optimum conditions for the flame ionization detector. Samples were injected without splitting into the capillary column, with an all-glass solid injection system. This injector, which is based on the device used in the analysis of steroids on packed columns[16], is illustrated in schematic form in Fig. 1. The inlet end of the column was connected to the injection system by means of a Gaslok connector (Scientific Glass Engineering). A similar connecting system using a PTFE seal was used to join the outlet of the column to the Swagelok

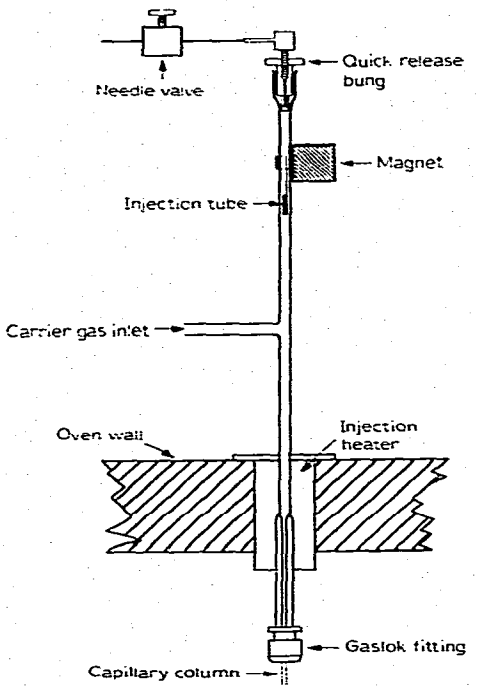

Fig. 1. Schematic diagram of the injection system used for introducing solid samples into the capillary column.

T-union. The injection tube was a 1-cm-long thin-walled glass tube (2.5 mm O.D. × 1.5 mm I.D.) open at both ends. Attached to this was a 3-cm-long sealed capillary tube containing a metal pin. The injection tube was held on a magnet while the sample in solution was injected into the tube from a 10-μl syringe and the solvent evaporated under a stream of dried nitrogen. The injection tube was then transferred into the top of the injection system by the magnet and held there until the quick release bung (Pye Unicam) had been replaced and the column flow conditions allowed to stabilize. The magnet was then removed to allow the injection tube to fall into the flash heater zone of the gas chromatograph. After completing the chromatographic analysis, the injection tube was removed from the injection system with the magnet. The purpose of the needle valve connected to the quick release bung at the top of the injector was to purge the system with a constant stream of approximately 4 ml/min of carrier gas.

Column preparation

The glass capillary columns (50 m × 0.25 mm I.D.) were drawn from 2-m lengths of pyrex tubing (7 mm O.D. × 2.3 mm I.D.) using an apparatus similar to that designed by Desty and co-workers[17]. Prior to drawing and coiling the columns, the tubes were washed with 5% aqueous sodium hydroxide, distilled water, methanol, acetone and dried under vacuum. The inner surface of each capillary was etched with gaseous hydrogen fluoride formed from the thermal decomposition of 2-chloro-1,1,2-trifluoroethyl methyl ether, and then silanized in the gaseous phase using a mixture of hexamethyldisilazane and TMClS according to the procedures of Novotný and Tesařik[3]. The column was then heated in a GLC oven for 18 h at 200° with a helium flow-rate of 2 ml/min. The coating of the column with stationary phase was carried out in two steps using the dynamic method. 1 ml of a 1% (w/v) solution of OV-101 in chloroform contained in a coating reservoir was forced through the column with nitrogen at a speed of 2 cm/sec. After the coating solution had left the column, the nitrogen flow was increased and maintained for several hours to dry the solvent off the column. The column was conditioned for 18 h at a temperature that was initially programmed at 0.5°/min from 50° to 200° and with a helium flow-rate of 4 ml/min. The above coating procedure was then repeated using 1 ml of a 7% (w/v) solution of OV-101 in chloroform. The column was finally conditioned for 48 h at a temperature which was initially programmed at 0.25°/min from 50° to 290° and with a helium flow-rate of 1 ml/min.

The efficiency of the columns for *n*-octacosane determined at 240° ranged from 1800 to 2200 theoretical plates per meter. The GLC tracings illustrated in the text were obtained on a 50 m × 0.25 mm I.D. column which had an efficiency of 98,000 theoretical plates.

Operating conditions

The injection tube containing the extract was introduced into the flash heater zone of the injection system, which was maintained at a temperature of 280°. After 2 min the column was programmed at 32°/min from 150° to 190° and then at 1°/min to a final temperature of 280°. The carrier gas flow-rate at 200° was 2 ml/min and the auxillary argon flow-rate to the detector was 40 ml/min. The detector temperature was 300° and the hydrogen and air flow-rates to the detector were 30 and 220 ml/min.

respectively. The amplifier attenuation was 10^{-11} A. The GLC tracings were obtained on a two pen Telsec 700T recorder (Telsec Instruments, Oxford, Great Britain) set at 20 and 50 mV and with a chart speed of 20 cm/h.

Gas chromatography–mass spectrometry

This was carried out using an MS-1073 double beam, double focusing, mass spectrometer (AEI Scientific Apparatus, Manchester, Great Britain) interfaced to a Pye Unicam Series 104 gas chromatograph with a single-stage membrane separator[18]. The column and operating conditions were the same as used in the GLC assay. An additional helium gas stream of 15 ml/min was introduced at the outlet of the column via a Swagelok T-union to optimize flow conditions in the separator. The mass spectrometer operating conditions were as follows: nominal electron energy, 70 eV; ion source temperature, 200°; membrane separator temperature, 230°.

Quantitation

The steroids eluted up to and including 11-oxoetiocholanolone were quantitated using the mean peak height measurements of the two internal standards epietiocholanolone and 5α-androstan-3β,17β-diol. The remaining steroids were quantitated using the mean peak height measurements of the two internal standards 5α-androstan-3β,17β-diol and epicoprostanol. The amount of steroid excreted per 24 h was calculated from the following equation:

$$\text{Steroid excreted (mg/24 h)} = \frac{S}{IS} \cdot \frac{X}{V_1} \cdot \frac{V}{m} \cdot \frac{1}{1000}$$

where S = peak height of steroid in the extract; IS = mean peak height of two internal standards in the extract; X = μg of internal standard added; V_1 = urine volume in millilitres taken for analysis; V = 24-h urine volume in millilitres; and m = calibration constant. The calibration constant m in the above equation was determined for each steroid by analysing a series of standard reference steroid mixtures containing from 0.5 to 8 μg of each steroid. The reference steroids were added to 4-ml aliquots of distilled water and then taken through the assay procedure. Calibration curves were drawn for each steroid by plotting the ratio peak height steroid TMS: average peak height of two internal standards against weight ratio μg steroid:μg internal standard. The calibration constants were calculated from the slopes of the curves.

RESULTS

Table I lists the trivial, systematic and abbreviated names of the neutral steroids determined in the assay, together with the three internal standards used for quantitation. The methylene unit (MU) values of the steroid TMS ethers measured under temperature programmed conditions[19] are also given in the table, as are the calibration constants for these compounds. The gas chromatographic separation of an authentic steroid mixture after TMS ether formation is shown in Fig. 2. Each steroid peak corresponds to 40 ng injected into the column. The calibration curve for each steroid was linear and passed through the origin. Three typical curves are illustrated

ABLE I

U VALUES AND CALIBRATION CONSTANTS OF STEROID TMS ETHER DERIVATIVES

eroid		*Abbreviation*	*MU value*	*Calibration constants*
ivial name	*Systematic name*			
pietiocholanolone	3β-Hydroxy-5β-androstan-17-one	e-Et	24.45	--
ıdrosterone	3α-Hydroxy-5α-androstan-17-one	An	24.57	1.04
iocholanolone	3α-Hydroxy-5β-androstan-17-one	Et	24.74	0.96
ehydroepiandrosterone	3β-Hydroxy-5-androsten-17-one	DHA	25.26	0.89
-Oxoandrosterone	3α-Hydroxy-5α-androstan-11,17-dione	O-An	25.37	0.82
-Oxoetiocholanolone	3α-Hydroxy-5β-androstan-11,17-dione	O-Et	25.45	0.80
α-Androstan-3β,17β-diol		An-D	26.23	--
egnanelone	3α-Hydroxy-5β-Pregnan-20-one	Pn	26.43	0.86
β-Hydroxyandrosterone	3α,11β-Dihydroxy-5α-androstan-17-one	H-An	26.71	1.09
β-Hydroxyetiocholanolone	3α,11β-Dihydroxy-5β-androstan-17-one	H-Et	26.92	0.85
ω-Pregnanediol	5α-Pregnan-3α,20α-diol	a-Pd	27.67	1.09
egnanediol	5β-Pregnan-3α,20α-diol	Pd	27.78	1.00
-Pregnenediol	5-Pregnen-3β,20α-diol	Δ^5-Pd	28.35	0.81
egnanetriol	5β-Pregnan-3α,17α,20α-triol	Pt	29.16	0.78
picoprostanol	5β-Cholestan-3α-ol	e-Co	30.25	--
ıolesterol	5-Cholesten-3β-ol	Ch	30.90	0.86

* Denotes internal standards.

in Fig. 3. The calibration constants determined from the slope of the calibration curve ranged from 0.78 to 1.09 (see Table I). These values obtained on the same capillary column were very reproducible and showed little change over a period of several months. Similar calibration constants were obtained from authentic standards which had not been put through the assay procedure. This demonstrated the absence both of selective losses in the extraction procedure and of interferenee from the method blank.

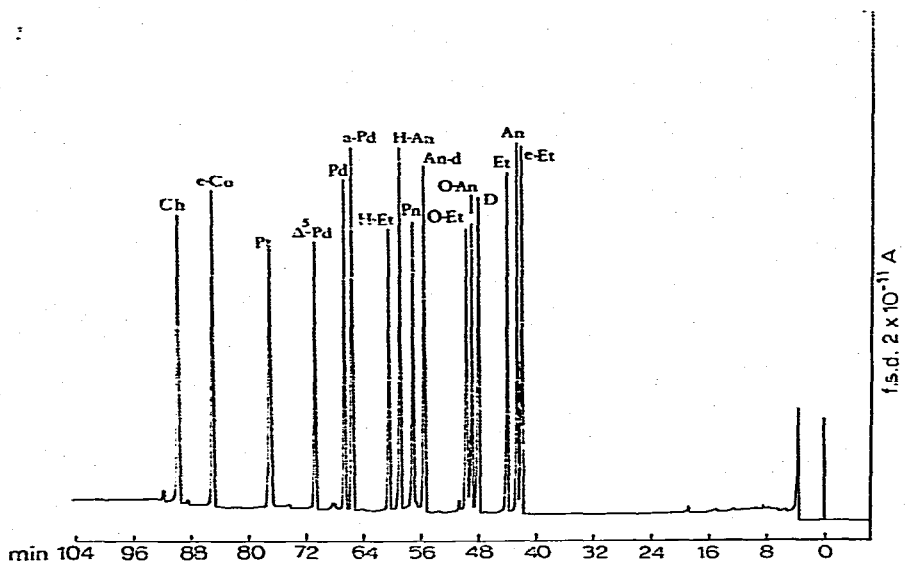

Fig. 2. GC tracing of an authentic steroid mixture as TMS ethers.

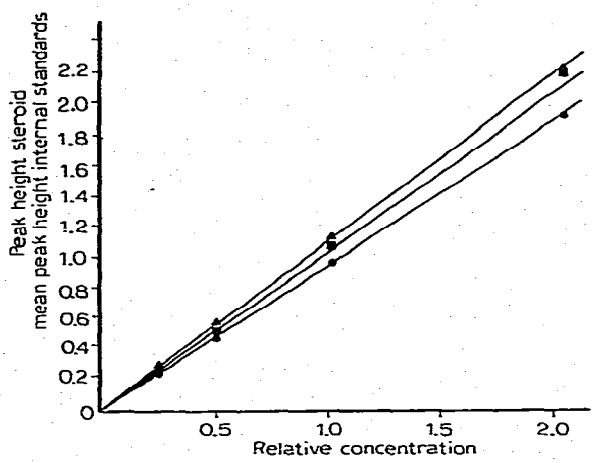

Fig. 3. Calibration curves for androsterone (▲—▲), 11β-hydroxyetiocholanolone (●—●) and pregnanediol (■—■). Each point on the curve represents the mean of duplicate determinations. The values at the relative concentration of 0.125 are not shown.

Fig. 4 shows the recovery from water and added enzyme of an authentic standard mixture containing 4 μg of each steroid. A comparison of this tracing with that obtained from the direct analysis of a mixture of authentic steroid TMS ethers (Fig. 2) shows a number of additional peaks, which are mainly derived from the enzyme preparation.

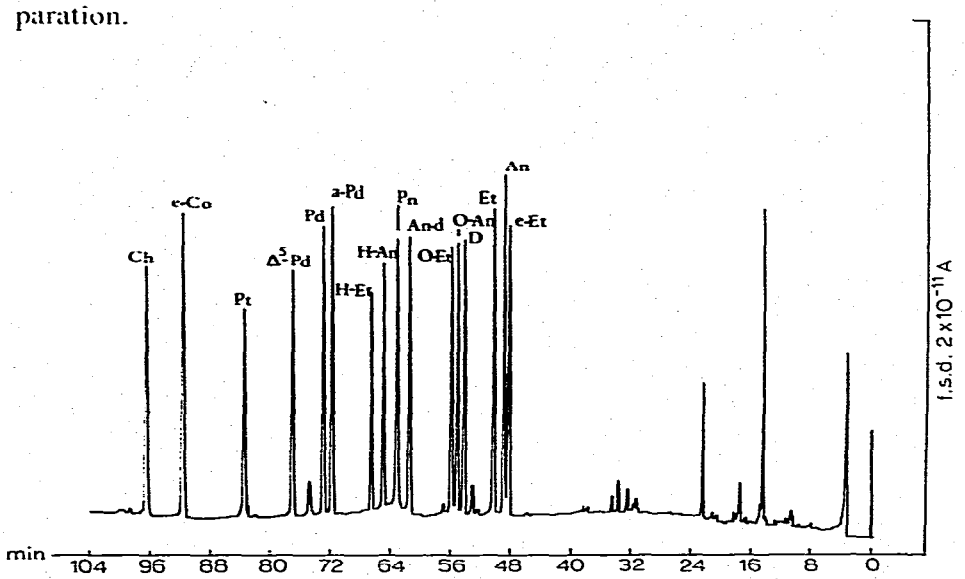

Fig. 4. GC tracing of an authentic steroid mixture after recovery from water and added enzyme.

Accuracy

The accuracy of the method was determined by carrying out replicate analyses on authentic steroid mixtures added to water and to a single 24-h urine specimen. The amounts added are equivalent to 0.25 mg and 1 mg of each steroid contained in

a 24-h specimen with a urine volume of 2 l. The corrected mean recoveries and standard deviation for each steroid taken through the assay procedure are given in Table II. The mean recoveries for all steroids from water ranged from 91.8 to 109.4% and from the urine specimen ranged from 90.3 to 108.5%. The absolute recoveries of added steroid from water and urine ranged from 84.6 to 97.3%.

TABLE II

CORRECTED RECOVERIES OF REFERENCE STEROIDS ADDED TO 4-ml ALIQUOTS OF WATER AND 4-ml ALIQUOTS OF A SINGLE 24-h URINE SPECIMEN

Recoveries are expressed as a mean of six replicate determinations.

Steroid	*Amount added (μg)*	*Recoveries from water (%)*		*Recoveries from urine (%)*	
		Mean	*S.D.*	*Mean*	*S.D.*
Androsterone	2.0	97.6	2.4	97.2	3.6
	0.5	102.7	7.5	105.6	2.4
Etiocholanolone	2.0	99.6	3.7	96.7	1.2
	0.5	98.7	6.6	104.3	1.49
Dehydroepiandrosterone	2.0	95.3	4.8	96.6	4.4
	0.5	97.8	5.2	101.1	7.7
11-Oxoandrosterone	2.0	92.6	8.7	93.2	6.3
	0.5	101.2	4.3	94.6	7.4
11-Oxoetiocholanolone	2.0	97.9	5.1	96.7	3.9
	0.5	99.4	4.2	95.5	5.5
Pregnanolone	2.0	103.3	3.6	90.3	2.8
	0.5	98.9	3.4	99.4	4.7
11β-Hydroxyandrosterone	2.0	94.8	4.6	93.8	5.9
	0.5	102.8	5.8	103.7	6.8
11β-Hydroxyetiocholanolone	2.0	104.2	4.4	96.8	3.7
	0.5	101.3	7.6	105.5	4.8
allo-Pregnanediol	2.0	100.6	2.2	100.7	5.3
	0.5	99.1	3.7	106.2	5.4
Pregnanediol	2.0	100.3	1.6	99.9	3.0
	0.5	99.2	3.3	104.6	3.7
Δ^5-Pregnenediol	2.0	93.7	5.5	98.8	5.3
	0.5	98.6	6.0	99.7	6.8
Pregnanetriol	2.0	91.8	8.6	89.7	3.6
	0.5	100.2	5.9	88.5	7.9
Cholesterol	2.0	99.7	5.0	101.6	8.4
	0.5	109.4	9.1	108.5	10.6

Precision

The precision of the method was evaluated according to the method of Snedecor[20]. A series of urines as analysed in duplicate and the standard deviations from the means were calculated from the formula, S.D. $= \sqrt{\Sigma d^2/2N}$ where d = difference between duplicates and N is the number of duplicate determinations. The results are presented in Table III.

Sensitivity

Specific studies were not conducted to determine the sensitivity of the method.

However, the assay has been used for the quantitation of steroids whose mean urinary excretion levels are as low as 0.10 mg/24 h (*e.g.* 11-oxoandrosterone) without any obvious loss in precision (see Table III). Thus, it is apparent that the sensitivity is not a limiting factor at least down to this level. (A urinary excretion of 0.10 mg/24 h corresponds to approximately 2–3 ng of the derivatized steroid injected into the column.)

TABLE III

PRECISION OF THE METHOD FROM ESTIMATES OF 20 DUPLICATE DETERMINATIONS

Steroid	*Range (mg/24 h)*	*S.D. (mg/24 h)*
Androsterone	0.81–2.03	0.081
Etiocholanolone	1.10–2.34	0.092
Dehydroepiandrosterone	0.13–0.59	0.029
11-Oxoandrosterone	0.02–0.19	0.067
11-Oxoetiocholanolone	0.20–0.79	0.044
Pregnanolone	0.07–0.34	0.022
11β-Hydroxyandrosterone	0.65–1.49	0.012
11β-Hydroxyetiocholanolone	0.05–0.39	0.034
allo-Pregnanediol	0.03–0.69	0.043
Pregnanediol	0.27–2.65	0.013
Δ^5-Pregnenediol	0.08–0.43	0.060
Pregnanetriol	0.35–1.35	0.089
Cholesterol	0.31–1.46	0.081

Specificity

An assessment of the specificity of the method has been made by combined GC–MS of a number of extracts. Scans taken over the peaks have not so far indicated any impurities unresolved from any of the internal standards and all but two of the urinary steroids. These steroids, *viz.* 11-oxoandrosterone and Δ^5-pregnenediol, have in general not been present at a high enough level to allow a satisfactory assessment of their purity in the extracts by scanning.

Application

Fig. 5 shows a typical GC tracing from a normal urine collected on day 20 of the menstrual cycle, and Fig. 6 shows a similar tracing from a normal adult male urine. The mean steroid excretion values determined in twelve normal male and twenty female subjects are presented in Table IV. The variation in excretion of three of the urinary metabolites of progesterone, *viz.* pregnanediol, *allo*-pregnanediol and pregnanolone, in a patient with pre-menstrual tension syndrome is illustrated in Fig. 7. Urines were collected and analysed from day 13 of the menstrual cycle and throughout the whole of the following cycle. From day 15 to day 25 of the second cycle, the patient received a daily dose of 200 mg of progesterone administered rectally as suppositories. A number of blood samples were taken during both menstrual cycles for the determination of plasma progesterone levels. The increase in plasma progesterone and urinary excretion of the three urinary progesterone metabolites during the luteal phase of the first cycle suggest a normal ovulatory cycle. The administration of pro-

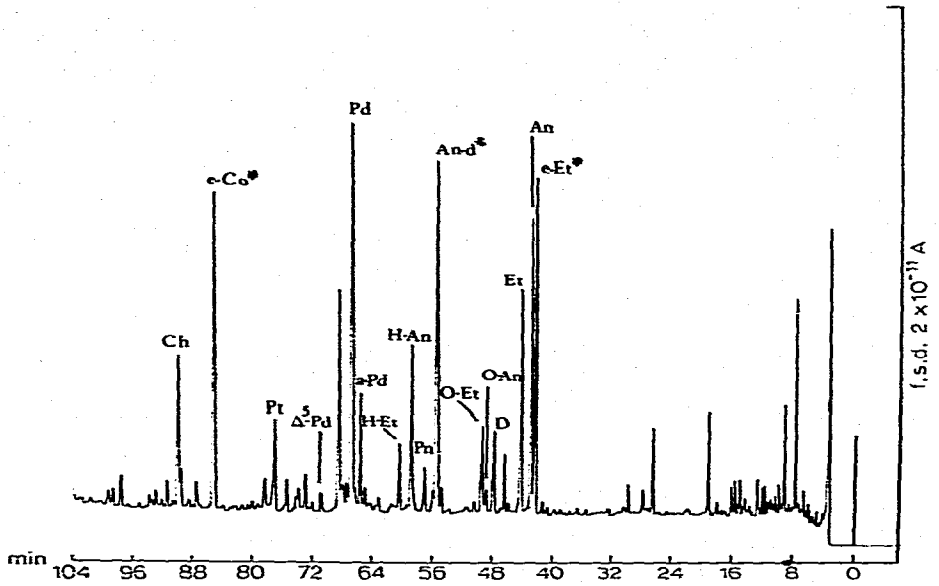

Fig. 5. A typical GC tracing from a normal female urine collected on day 20 of the menstrual cycle.

gesterone for the treatment of the pre-menstrual tension symptoms resulted in a rapid increase in the plasma progesterone levels with an expected increase in each of the urinary progesterone metabolites. Fig. 8 shows a GC tracing from this patient's urine collected on the fourth day of progesterone therapy.

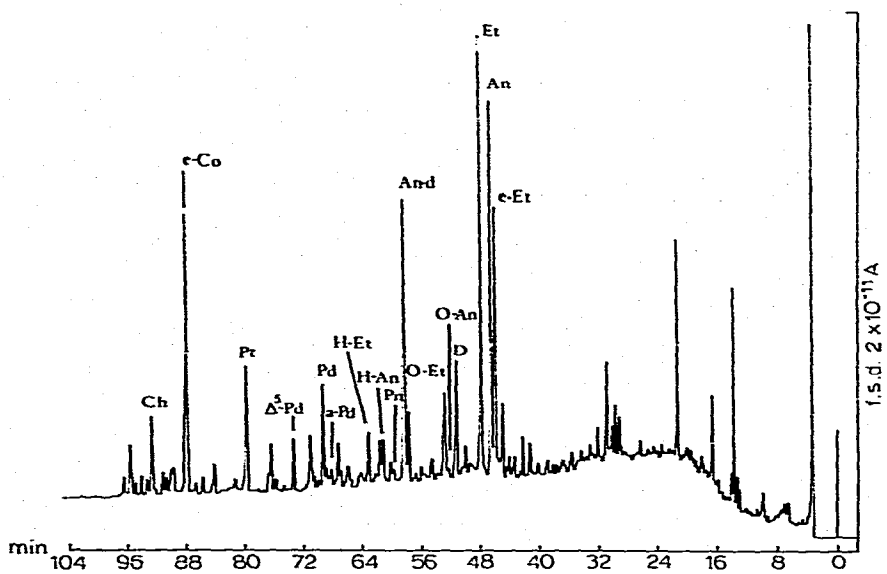

Fig. 6. A typical GC tracing from a normal adult male urine.

TABLE IV

URINARY STEROID EXCRETION (mg/24 h) IN NORMAL WOMEN DURING THE FOLLICULAR AND LUTEAL PHASE OF THE MENSTRUAL CYCLE AND IN NORMAL MEN

Steroid	*Female (aged 19–38)*				*Male (aged 20–35)*	
	Follicular phase (day 6)		*Luteal phase (day 20)*		*Mean*	*Range*
	Mean	*Range*	*Mean*	*Range*		
Androsterone	1.80	0.98–2.67	2.05	1.31–3.10	2.03	1.06–3.22
Etiocholanolone	2.35	1.19–4.13	2.40	1.23–5.80	2.52	1.17–4.83
Dehydroepiandrosterone	0.29	0.08–0.68	0.54	0.19–1.21	0.35	0.10–0.68
11-Oxoandrosterone	0.07	0.04–0.13	0.11	0.04–0.24	0.10	0.05–0.17
11-Oxoetiocholanolone	0.42	0.22–0.60	0.51	0.28–0.96	0.42	0.20–0.70
Pregnanolone	0.27	0.07–0.53	0.52	0.13–0.72	0.14	0.06–0.22
11*β*-Hydroxyandrosterone	0.70	0.26–1.21	0.59	0.41–0.96	0.50	0.27–0.83
11*β*-Hydroxyetiocholanolone	0.41	0.14–0.75	0.26	0.13–0.48	0.31	0.05–3.72
allo-Pregnanediol	0.10	0.07–0.15	0.63	0.29–1.09	0.11	0.08–0.22
Pregnanediol	0.65	0.33–1.14	2.52	1.41–4.13	0.43	0.28–0.63
Δ^5-Pregnenediol	0.46	0.21–1.11	0.43	0.19–1.23	0.38	0.12–0.89
Pregnanetriol	0.49	0.25–0.70	0.92	0.77–1.46	0.67	0.32–0.99
Cholesterol	0.44	0.26–1.47	0.51	0.38–1.76	0.67	0.33–1.86

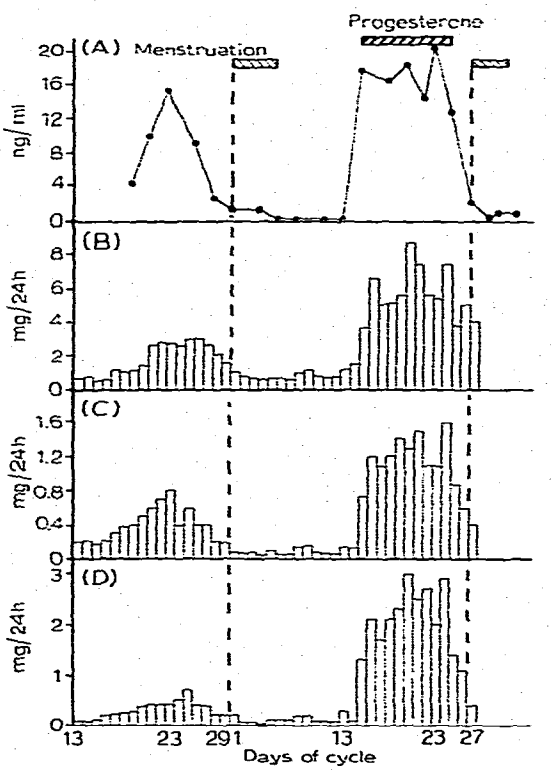

Fig. 7. Plasma progesterone levels (A) and urinary excretion of pregnanediol (B), *allo*-pregnanediol (C) and pregnanolone (D) in a patient with pre-menstrual tension syndrome before, during and after progesterone therapy.

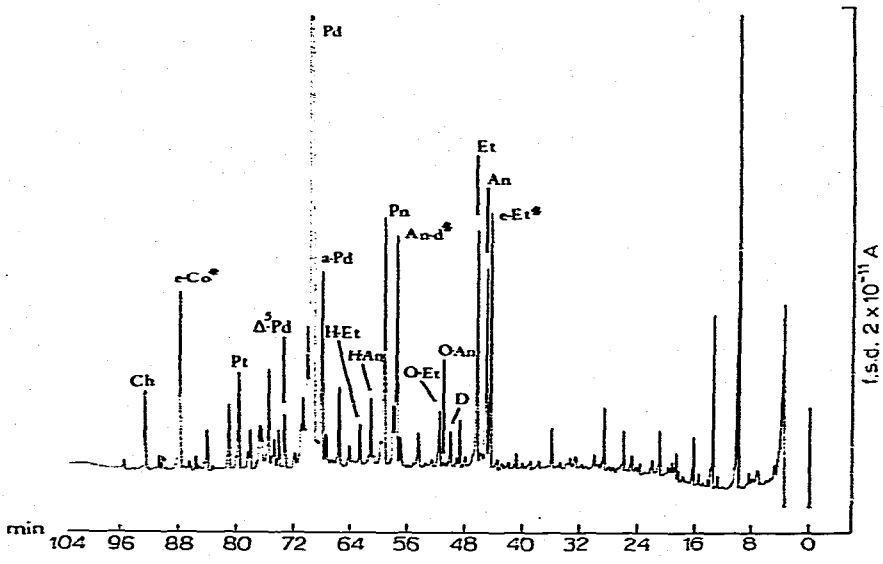

Fig. 8. GC tracing from a patient with pre-menstrual tension syndrome on the 4th day of progesterone therapy.

DISCUSSION

A number of alternative methods have been described in the literature for preparing wall-coated open-tubular glass capillary columns suitable for steroid analysis[2–7]. These methods differ in the procedure used for modifying the inner walls of the column and the technique for coating with the stationary phase. An initial treatment of the capillary is necessary both to deactivate the glass surface and also to promote the deposition of a homogeneous film of phase on the capillary wall. The etching and chemical surface modification method recommended by Novotný and Tesařík[3] for preparing non-polar columns was found to give reliable and reproducible columns coated with the stationary phase OV-101. The plate efficiency of these columns was increased when the dynamic coating procedure was carried out in two steps. Columns prepared in this way have been in continuous use for periods of over eight months with no measurable deterioration in performance.

The introduction into capillary columns of samples containing only submicrogram amounts of high-boiling-point compounds, such as derivatized steroids dissolved in volatile solvents, cannot be satisfactorily accomplished with conventional splitting systems. In order to overcome this problem, a number of improved injection devices have been developed[8,15,21,22]. The all-glass solid injection system described in this paper is both simple and effective. Steroid samples can be injected quantitatively from dilute solutions without loss of column efficiency. Dead volume effects in the injector were eliminated by using the principle of direct sampling without splitting developed by Grob and Grob[8]. During injection of the dried sample the column was maintained at a temperature of 150°, which is low enough to trap and concentrate the high-boiling-point components at the beginning of the column. High-resolution separations were then obtained under temperature-programmed conditions

after the column had been initially heated rapidly to 190°. Non-volatile components present in the urine extracts are retained in the injection tube. This protects the injection system and column from contamination, thus preventing possible absorption and decomposition of steroidal components and loss of column resolution. The injection system can easily be removed for cleaning but this was only found to be necessary at 3–4-week intervals.

The accuracy of the method is dependent on the use of mass internal standards that compensate for losses during work-up of the urine, and in sampling, column and detector variables of GC. The choice of internal standards is limited to steroids that do not occur naturally in urine. Ideally they should be eluted near to and have physicochemical properties similar to the steroids being quantitated. It is also imperative that the standards are resolved from both steroidal and non-steroidal components contained in the urine extract. In a multicomponent analysis involving the simultaneous estimation of relatively large numbers of steroids, all of these requirements cannot adequately be satisfied. Nevertheless, the use of the three internal standards in our method, which are eluted at the beginning, middle and end of the programmed analysis, is a compromise that has proved to give acceptable results.

The GLC tracings illustrated in this paper were obtained with a single-pen potentiometric recorder. For routine analysis a two-pen instrument was used. This had the advantage of recording on two voltage channels set at different sensitivities enabling those urinary steroid components present in lower concentration to be determined with greater accuracy.

The sensitivity of the MS1073 mass spectrometer used in this study enabled spectra to be obtained from a peak containing as little as 10 ng of the steroid derivative. However, a satisfactory assessment of peak homogeneity could not be obtained from scans on such small amounts. A better method would involve the quantitative mass spectrometric determination of the low-level steroids in a number of urines by a specific single-peak monitoring technique and comparison of these results with those obtained using the flame ionization detector.

The use of a data system together with the mass spectrometer considerably reduces the labour of data acquisition and comparison of spectra of this procedure is now being used to examine further urine extracts. In addition to providing data on a peak homogeneity, the mass spectrometer is also providing information that will help to establish the identity of a number of unidentified steroid components present in the chromatograms.

With regard to the practicability of the method, this is limited by the number of urine extracts that can be gas chromatographed in a working day. Since no time-consuming purification or fractionation steps are involved in the assay, it is possible for one technician to extract and derivatize 50 urines a day, but only five extracts can be analysed in this time. However, preliminary studies using a modified Pye Unicam auto-solid injector with a Pye Unicam Series 106 automated gas chromatograph indicate that it should be possible to assay routinely 100 urine specimens per week.

Data presented shows that the precision, accuracy, specificity and sensitivity of the method is satisfactory and that the assay is suitable for routine clinical application. Individual urinary steroid values obtained for normal men and normal women during the follicular and luteal phases of the menstrual cycle are comparable with published values obtained by other methods.

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