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QUANTITATIVE ANALYSIS OF STEROID PROFILES FROM URINE BY CAPILLARY GAS CHROMATOGRAPHY

I. ACCURACY AND REPRODUCIBILITY OF THE SAMPLE PREPARATION

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SUMMARY

A method is described for the determination of steroid profiles from urine by means of gas chromatography using high-efficiency glass capillary columns. The accuracy and reproducibility of essential steps in the sample preparation (extraction of steroids and steroid conjugates by means of XAD-2, enzymatic hydrolysis with *Helix pomatia* juice, solvolysis in acidified ethyl acetate and alkali wash) are established using different endogenously labelled urine samples, obtained from normal subjects to whom labelled steroids had been administered. Preliminary results are given on the reproducibility of the derivatization procedure (formation of methoxime-trimethylsilyl (MO-TMS) ethers), the gas chromatographic analysis and the whole method. Two procedures for the purification of MO-TMS steroid derivatives are compared. Application of the method to urine samples of patients with various endocrine disorders is included.

INTRODUCTION

Many papers have been published in recent years on different aspects of the determination of steroid profiles from urine by gas chromatography (GC).

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NOMENCLATURE AND ABBREVIATIONS OF STEROIDS AND KEY TO FIGURES

Identification number of peaks in the figures	Trivial name	Abbreviation	Systematic name
1	Androsterone	A	3α-Hydroxy-5α-androstane-17-one
2	Etiocholanolone	E	3α-Hydroxy-5β-androstane-17-one
3	Dehydroepiandrosterone	DHEA	3β-Hydroxy-5-androstene-17-one
4	11-Keto-androsterone	11-O-A	3α-Hydroxy-5α-androstane-11,17-dione
	11-Keto-etiocholanolone	11-O-E	3α-Hydroxy-5β-androstane-11,17-dione
5	Estradiol	E-II	3,17β-Dihydroxy-1,3,5(10)-estratriene
6	11β-Hydroxy-androsterone	11-OH-A	3α,11β-Dihydroxy-5α-androstane-17-one
7	11β-Hydroxy-etiocholanolone	11-OH-E	3α,11β-Dihydroxy-5β-androstane-17-one
8	Pregnanediol	PD	3α,20α-Dihydroxy-5β-pregnane
9	Allopregnanediol	aPD	3α,20α-Dihydroxy-5α-pregnane
10	Pregnanetriol	PT	3α,17α,20α-Trihydroxy-5β-pregnane
11	THS		3α,17α-21-Trihydroxy-5β-pregnane-20-one
12	THDOC		3α,21-Dihydroxy-5β-pregnane-20-one
13	Estriol	E-III	3,16α,17β-Trihydroxy-1,3,5(10)-estratriene
14	aTHS		3α,17α,21-Trihydroxy-5α-pregnane-20-one
	aTHDOC		3α,21-Dihydroxy-5α-pregnane-20-one
15	THE		3α,17α,21-Trihydroxy-5β-pregnane-11,20-dione
16	THA		3α,21-Dihydroxy-5β-pregnane-11,20-dione
17	THB		3α,11β,21-Trihydroxy-5β-pregnane-20-one
18	aTHB		3α,11β,21-Trihydroxy-5α-pregnane-20-one
19	THF		3α,11β,17α,21-Tetrahydroxy-5β-pregnane-20-one
	aTHE		3α,17α,21-Trihydroxy-5α-pregnane-11,20-dione
20	aTHF		3α,11β,17α,21-Tetrahydroxy-5α-pregnane-20-one
21	α-Cortolone		3α,17α,20α,21-Tetrahydroxy-5β-pregnane-11-one
22	β-Cortolone		3α,17α,20β,21-Tetrahydroxy-5β-pregnane-11-one
	β-Cortol		3α,11β,17α,20β,21-Pentahydroxy-5β-pregnane
23	α-Cortol		3α,11β,17α,20α-21-Pentahydroxy-5β-pregnane
	Estrone	E-I	3-Hydroxy-1,3,5(10)-estratriene-17-one
	Cortisone		17α,21-Dihydroxy-4-pregnene-3,11,20-trione
	Androstenedione		4-Androstene-3,17-dione
	Testosterone	T	17β-Hydroxy-4-androstene-3-one

Most of them deal with the analytical procedure, for example, the development of techniques for sample preparation [1—8], the formation of suitable derivatives [9—14], or the preparation of glass capillary columns [15—20]. Other papers [21—35] describe applications of this method to problems of clinical interest, showing the great possibilities of the techniques employed. It is likely that in the future steroid profiling by means of GC using high-efficiency glass capillary columns will be used more often, since no other technique gives qualitative and quantitative information on each steroid present in the sample from only one determination.

In our opinion, until now not enough attention has been paid to quantitative aspects of the analysis, especially sample preparation i.e. extraction of steroids and steroid conjugates from urine, hydrolysis, solvolysis and alkali wash. It is realized that when large deviations from the normal pattern have to be established, no quantitative method is required. However, small deviations can only be detected with reliable quantitative methods. Some clinical problems underline the need for such a method.

In some papers methods for sample preparation were tested with only a few selected steroids [3, 23—26], or steroid conjugates [6, 23—28], and the results generalized to all classes of steroids and all types of steroid conjugates. In a series of papers a method for the quantitative GC analysis of steroid profiles from urine will be described. This method will be evaluated for all classes of steroids and all types of steroid conjugates normally present in urine samples. In this first paper we describe the quantitative evaluation of essential steps in the preparation of the sample. Preliminary results on derivatisation and GC analysis are given.

Sample preparation procedures can only be evaluated correctly if urine samples are used in which "endogenously" labelled conjugates are present, the samples being obtained from normal subjects to whom labelled steroids had been administered orally or by continuous infusion. As these labelled steroids take part in normal metabolism, all metabolites of these compounds will appear in the urine as labelled conjugates. To our knowledge such investigations have been described only by Bradlow [1] and Setchell et al. [2] for a small number of steroids. In this study normal subjects were given cortisol*, androstenediol and estrone, androstenedione and DHEA, or DHEA—S and DHEA. The main metabolites of cortisol [36] are THE, aTHE, THF, aTHF, α- and β-cortolones and cortols, 11-O-A and 11-O-E, 11-OH-A and 11-OH-E. Androsterone, etiocholanolone and testosterone are the most important metabolites of androstenedione. Estrone is partly converted into estriol, estradiol and other estrogens. Infusion with DHEA—S results in the excretion of DHEA, androsterone and etiocholanolone. Using urine samples obtained from normal subjects to whom the above-mentioned steroids had been administered, the whole polarity range of steroids and all types of steroid conjugates, normally present in urine samples, are included.

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*Trivial names and abbreviations used in this paper are listed in Table I. A capital G or S added to the steroid name indicates the glucuronide or sulphate conjugate, respectively.

EXPERIMENTAL

Materials

Non-radioactive steroids were purchased from Steraloids (Pawling, N.Y., U.S.A.) and Ikapharm (Ramat Gan, Israel). Radioactive steroids, [4-^{14}C]-androstenedione (60 mCi/mmole), [4-^{14}C]dehydroepiandrosterone sulphate, ammonium salt (54 mCi/mmole), [1,2-^{3}H]cortisol (0.36 Ci/mmole), [6,7-^{3}H]-estrone (44 Ci/mmole) and [7-^{3}H]dehydroepiandrosterone (16.6 Ci/mmole), were all purchased from the Radiochemical Centre (Amersham, Great Britain) and used only after a chromatographic purity check. Servachrom XAD-2 (Serva, Heidelberg, G.F.R., particle size 300—1000 μm) was purified according to the method described by Setchell et al. [12]. In some experiments Amberlite XAD-2 (Rohm & Haas, Pa., U.S.A., particle size 300—1000 μm) was used after purification according to the method of Shackleton et al. [6]. *Helix pomatia* juice was obtained from Industrie Biologique Française (Gennevilliers, France). Lipidex-5000, obtained from Packard-Becker (Groningen, The Netherlands), was purified according to the method of Axelson et al. [37]. Solvents (ethanol, ethyl acetate, hexane) were p.a.-grade (Merck, Darmstadt, G.F.R.) and used without further purification. Pyridine (Merck) was redistilled before use over P_2O_5 and stored over potassium hydroxide. Methoxyamine hydrochloride was purchased from Applied Science Labs., (State College, Pa., U.S.A.). Hexamethyldimethylsilazane (HMDS) was obtained from Pierce (Rockford, Ill., U.S.A.). Dimethoxypropane was purchased from Aldrich (Beerse, Belgium). The silylation mixture, BSA (N,O-bistrimethylsilylacetamide), TSIM (N-trimethylsilylimidazole) and TMCS (trimethylchlorosilane) in the ratio 3:3:2 (v/v) was purchased from Supelco (Bellefonte, Pa., U.S.A.). Scintillation cocktail NE 262 was supplied by Nuclear Enterprises (Edinburgh, Great Britain). Urine samples were obtained from normal subjects to whom had been administered ^{3}H-labelled cortisol (1 μCi, oral), ^{3}H-labelled estrone/^{14}C-labelled androstenedione, ^{3}H-labelled DHEA/^{14}C-labelled DHEA—S or ^{3}H-labelled DHEA/^{14}C-labelled androstenedione (30 μCi ^{3}H and 15 μCi ^{14}C, all given by continuous infusion for, respectively, 2 and 12 h).

Sample preparation

Extraction of steroids and steroid conjugates from urine. Urine samples (24 h) were collected in polyethylene bottles. After the volume had been recorded, they were stored at —20° before processing. Urine (10 ml) was passed through a column (10 × 1 cm I.D.) containing 8 g of XAD-2 resin. The column was fitted with a 25-ml reservoir and a PTFE stopcock. After being washed with 20 ml of distilled water, the column was allowed to drain completely. Steroid conjugates were eluted with 40 ml of methanol. The flow-rate was kept constant (0.5—1.0 ml/min) throughout the entire procedure. Columns can be re-used after washing three times with 40 ml of distilled water and removing air bubbles.

Hydrolysis and solvolysis. Methanol was evaporated under nitrogen at 60°. The residue was redissolved in 10 ml of sodium acetate buffer (0.5 *M*, pH 5.0), to which 0.1 ml of *Helix pomatia* juice was added containing 10,000

Fishman units of β-glucuronidase and 80,000 Roy units of sulphatase. Hydrolysis of the steroid glucuronides was performed for 18 h at 37°. After hydrolysis the sample was brought to pH 1 with concentrated hydrochloric acid, and NaCl (1.5 g) was added [38]. Liberated steroids and steroid sulphates were extracted by shaking with 25 ml of ethyl acetate for 60 min. The aqueous phase was removed by suction and solvolysis took place for 18 h at 45°. Only when the accuracy of the hydrolysis and solvolysis had to be determined was ethyl acetate evaporated under nitrogen at 60°. The residue was redissolved in 10 ml of distilled water and liberated steroids were extracted three times each with 10 ml of ethyl acetate.

Alkali wash. The ethyl acetate fraction was washed twice with 5 ml of 8% aqueous sodium bicarbonate (to remove acids but not phenols) and two times with 5 ml of distilled water. It was shown that some steroids were partly lost in the aqueous phase. These steroids could be recovered by XAD-2 extraction as already described. Finally, the combined organic layers were evaporated under a stream of nitrogen at 60°.

Formation of derivatives

Preparation of methoxime-trimethylsilyl (MO-TMS) derivatives. The dry residue was redissolved in 1 ml of ethyl acetate, transferred to a silylation vial and 1 ml of a solution of *n*-alkanes C_{24} and C_{32} in hexane (3 mg/100 ml) was added. Solvents were evaporated under a stream of nitrogen at 60°. To the dry residue 100 μl of a solution of methoxyamine hydrochloride (100 mg/ml) in dry pyridine were added. The vial was closed with a PTFE-lined cap and heated for 1 h at 60°. Excess pyridine was removed under nitrogen at 60°. After the addition of 100 μl of the mixture BSA—TSIM—TMCS (3:3:2, v/v), persilylation was achieved in 4 h at 80° for all compounds, except for cortols and cortolones; full silylation of these latter compounds took 24 h at 80°.

Purification of derivatives. Two methods were employed for purification of the sample after derivatisation.

Method A. Excess silylation reagents and compounds with polarities similar to those of steroids, were removed on a Lipidex-5000 column (70 × 4 mm) containing 0.25 g of dry gel, prepared in the solvent system hexane—HMDS—pyridine—dimethoxypropane (97:1:2:10, v/v) [2, 37, 39]. The sample was transferred onto the top of the column by adding 400 μl of the solvent to the reaction mixture. The vial was washed with 500 μl of the solvent, which was also passed through the column. For rapid filtration a nitrogen pressure of 0.5 kg/cm^2 was applied, resulting in a flow of 3 ml/min. MO-TMS derivatives were recovered in the first 3.5 ml of effluent. Solvents were evaporated under nitrogen at 60°. The sample was redissolved in 1 ml of hexane, of which 1 μl was injected into the gas chromatograph.

Method B [40]. To the reaction mixture 1 ml of methylene chloride was added. Excess reagents were removed by washing the methylene chloride with 1 ml of 0.1 *N* sulphuric acid and twice with 1 ml of distilled water. The sample was dried over anhydrous sodium sulphate and 1 μl of the supernatant was injected into the gas chromatograph. A flow diagram of the complete method is shown in Fig. 1.

Liquid scintillation counting. The accuracy and reproducibility of the

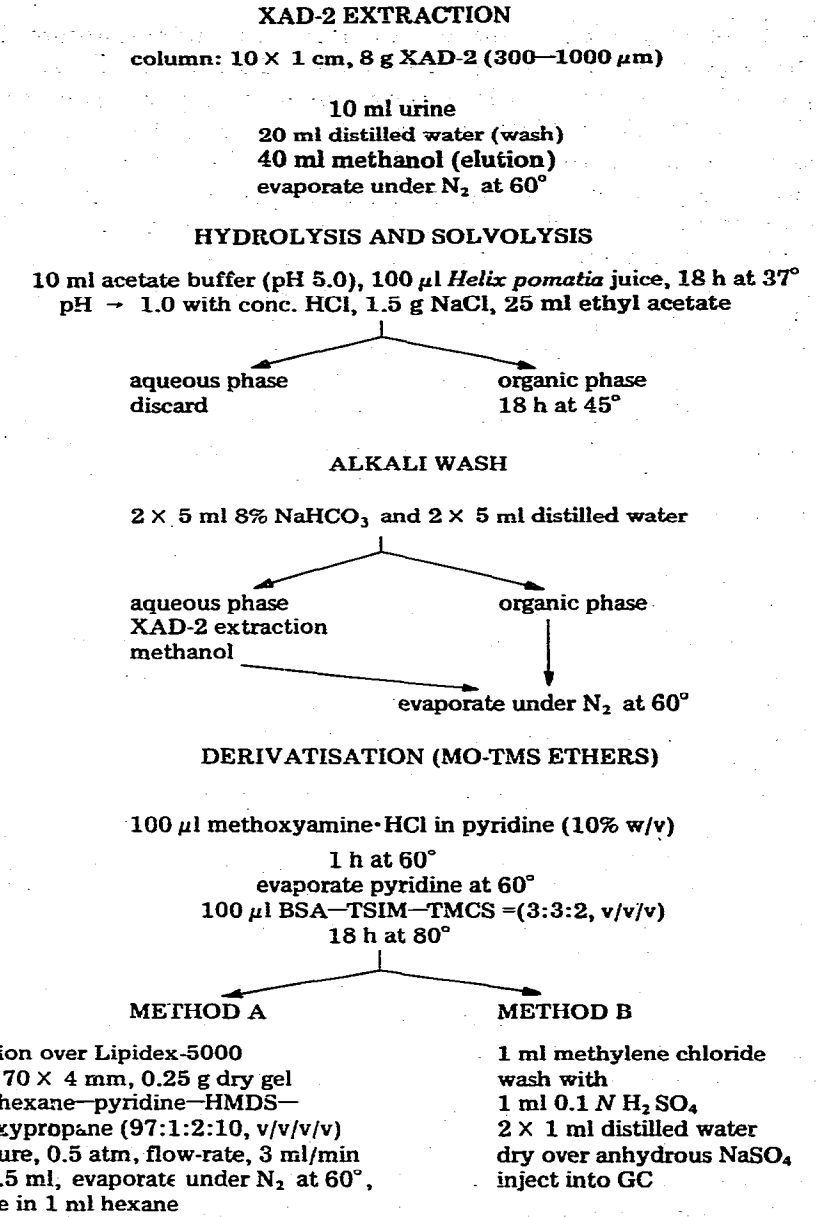

Fig. 1. Flow diagram of the sample preparation and derivatization of the GC determination of urinary steroid profiles.

sample preparation procedure was established by counting 1-ml samples in 11 ml of the scintillation cocktail. Liquid scintillation counting was performed in a Packard TriCarb 2003 (Packard, Brussels, Belgium), or a Mark III (Searle Analytic, Des Plaines, Ill., U.S.A.) equipped with automatic quench correction, for 10 min or until 10,000 disintegrations had been counted.

Gas chromatography

Preparation of columns. Glass capillary columns were prepared according to the method described by Rutten and Luyten [20]. Columns (0.25 mm I.D. and 1.0 mm O.D.) were drawn from Pyrex glass tubes. The internal diameter was carefully kept constant during the drawing process. Columns were de-activated with benzyltriphenylphosphonium chloride (BTPPC; Aldrich) and coated with a solution of 0.25% (w/w) SE-30 (Merck) in hexane using the static procedure [41].

Instrumentation. GC was carried out using a Perkin-Elmer F 30 gas chromatograph, equipped with an all-glass solid injector [42] and modified for the use of glass capillary columns. A home-made gas chromatograph was also used, constructed with a Becker 1452 D air thermostat and Becker gas-flow regulators. Carrier gas (nitrogen) was controlled by a precision pressure controller (Wallace and Tiernan, Gunzburg, G.F.R.). The column was housed in an aluminium block to minimize the effect of temperature fluctuations in the oven. Samples were injected with a moving-needle injection system [42]. The flame ionisation detector and the amplifier were also home-built.

Identification of steroids

Kováts indices [43] were measured for 27 reference steroids on SE-30 at 250°. The inlet pressure of the carrier gas (nitrogen) was 1.0 kg/cm^2. The logarithmic plot of even-numbered *n*-alkanes between C_{24} and C_{32} was found to be linear. Indices were calculated by linear interpolation between the outer two *n*-alkanes. Times were measured by a chronograph and the appearance of the solvent was taken as inert gas time. Steroids in urine samples were identified by measuring Kováts indices under the same conditions as the reference steroids. When identification was only tentatively possible, because of small differences in the Kováts index, gas chromatography—mass spectrometry (GC—MS) was used to confirm the identity of the compound involved. Mass spectra of the 27 reference steroids as their MO-TMS derivatives were recorded by GC—MS using an AEI MS-12 mass spectrometer (trap-current 500 μA, source temperature 255°, accelerating voltage 4 kV, electron energy 70 eV, magnetic scan speed 2 sec/decade). GC was performed at 250° on SE-30. The gas chromatograph was directly coupled to the mass spectrometer [44]. No attempts were made in this study to establish the identity of minor compounds in the profile.

RESULTS AND DISCUSSION

Sample preparation

First, the accuracy and reproducibility of the XAD-2 extraction, hydrolysis/solvolysis and alkali wash were established in separate experiments. Subsequently, the overall recovery of the whole sample preparation was determined by carrying out the extraction, hydrolysis/solvolysis and alkali wash without establishing the recoveries of the individual steps. In this way calculated overall recoveries can be compared with values measured in separate experiments. The results are given in Table II.

TABLE II

ACCURACY AND REPRODUCIBILITY OF SAMPLE PREPARATION

Values are expressed as percentages ± standard deviation. NM: not measured.

	Steroid(s) administered						
	^{3}H-Labelled cortisol*	^{14}C-Labelled androstenedione** + ^{3}H-labelled DHEA		^{14}C-Labelled androstenedione** + ^{3}H-labelled estrone		^{14}C-Labelled DHEA—S** + ^{3}H-labelled DHEA	
	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H
XAD-2 Recovery	94 ± 5 (n = 73)	100 ± 3	94 ± 4	103 ± 3	94 ± 5	100 ± 1	96 ± 3
Hydrolysis and solvolysis	95 ± 8 (n = 43)	93 ± 4	87 ± 2	96 ± 3	91 ± 1	86 ± 3	83 ± 4
Alkali wash	NM	99 ± 4	97 ± 5	96 ± 3	84 ± 9	101 ± 3	101 ± 3
Overall recovery: calculated	89	92	79	95	72	87	80
Overall recovery: measured in separate experiments	88 ± 3 (n = 16)	92 ± 3	80 ± 5	96 ± 2	71 ± 7	87 ± 3	81 ± 2

*Seven different urine samples, including 13 series.
**Number of experiments for dual-labelled samples = 8.

XAD-2 procedure

Most of the experiments were carried out with urine samples of subjects to whom ^{3}H-labelled cortisol had been administered. Samples of seven different subjects were used; 13 series were carried out in two different laboratories by two technicians. No differences could be detected between the results obtained from either different urine samples or in different laboratories. For the extraction of steroid conjugates and steroids from urine, the batches of XAD-2 obtained from Serva and Rohm & Haas, gave equally good results.

We preferred to purify the resin using the method of Setchell et al. [2], since purification according to the method of Shackleton et al. [6] does not remove all impurities detectable by GC. Elution of steroids and steroid conjugates with 50 ml of methanol instead of 40 ml did not increase the recovery. The XAD-2 extraction resulted in a mean recovery of 94.5 ± 5.5% (n = 73). When 15 ml of urine instead of 10 ml were passed through the XAD-2 columns, significantly lower recoveries were found (87.5%, n = 20), while the standard deviation increased to 9.6%. Experiments with 10 ml of a urine sample from a patient suffering from Cushing's syndrome and excreting about ten times as much cortisol metabolites as normal subjects, yielded a XAD-2 recovery of 84.8 ± 3.4% (n = 8), 5% of the radioactivity being lost in the aqueous effluent. Only after a second elution with 15 ml of methanol could the remaining 10% of radioactivity be recovered from the columns. Care must therefore be taken to avoid overloading the column when urine samples of patients excreting abnormally large quantities of steroid conjugates are carried through the procedure. To avoid contamination of further experiments with the same column, we suggest that the columns are regenerated not only by washing with water, but also with 40 ml of methanol.

For the metabolites of androstenedione, estrone, DHEA and DHEA—S also, an almost quantitative recovery from the XAD-2 columns was obtained. Good agreement was found for urine samples from different normal subjects to whom identical steroids (androstenedione or DHEA) had been administered (Table II). The results, given above, agree well with those obtained by other workers [1, 2, 8, 23—26, 45].

Hydrolysis and solvolysis

For the quantitative analysis of steroid profiles, enzymatic hydrolysis must be followed by solvolysis [2,6,7,23,24], since the sulphatases present in *Helix pomatia* juice are not able to hydrolyse sulphate conjugates of a 3α-OH group in 5α-steroids [52], and of 17- and 20- hydroxyl groups [refs. 53 and 6, respectively]. In human urines these conjugates were found to be excreted in varying amounts. For cortisol metabolites the mean recovery of hydrolysis plus solvolysis was 95.0 ± 8.3% (n = 44). The high standard deviation is probably caused by small differences in enzyme activity, since several batches were used throughout this study. Hydrolysis and solvolysis temperatures could be varied between 35 and 50°, and 45 and 50°, respectively, without a significant change in the results. Maximum cleavage of steroid conjugates was achieved within 18 h for both hydrolysis and solvolysis.

These results also prove that of the quantitatively most important corticosteroid metabolites only a minor part is lost during the solvolysis procedure. It must be emphasized that solvolysis is not suitable for the analysis of corticosteroids for which artefact formation is known to occur, such as highly polar corticosteroids, normally present in almost negligible amounts in urine.

Hydrolysis and solvolysis of the metabolites of estrone, androstenedione, DHEA and DHEA—S also yielded satisfactory results. They are of the same order of magnitude as those reported by others [1, 3, 6, 7, 23—28, 45]. About 10% of the metabolites of DHEA and DHEA—S were lost with the aqueous phase after hydrolysis. Further experiments need to be carried out to find out whether these metabolites are not hydrolysed or are not extracted by ethyl acetate.

Alkali wash

Further purification of the sample (removal of acids) is achieved by washing the ethyl acetate fraction after solvolysis with aqueous sodium bicarbonate and water. Some highly polar steroids are not completely recovered or are altered during the alkali wash [6].

Alternative procedures using the anion exchanger Amberlyst A-26 suffer from the disadvantage that the recovery of some steroids is not quantitative. The use of diethylaminohydroxypropyl Sephadex LH-20 (DEAP-LH-20) seems to overcome these problems [39]. Since DEAP-LH-20 is not yet commercially available, we have used the alkali wash for purifying samples. Table II shows that almost no steroids are lost if this method of purification is used, with the exception of estrogens. About 15% of the metabolites of estrone are lost on the XAD-2 columns when the combined aqueous phases are passed through the columns and during the wash with distilled water. The reason for

this phenomenon is not yet fully understood, and further experiments will have to be carried out.

Overall recovery

From the data obtained for the individual steps, overall recoveries were calculated by multiplication. In separate experiments, the same urines were taken through the complete procedure without establishing the recoveries of the individual steps; only the resulting overall recovery was measured. From the results, presented in Table II, the excellent agreement between the calculated and measured recoveries is evident.

Preparation and purification of MO-TMS derivatives

MO-TMS derivatives were prepared according to the method described by Horning and co-workers [9—11, 13, 14] with small modifications. For persilylation the mixture BSA—TSIM—TMCS (3:3:2; v/v) [9] was used at 80° to shorten the reaction time. Optimum yield of derivatives was achieved in 4 h, except for cortols and cortolones. Full silylation of these substances required 24 h at 80°. No attempts were made to establish the accuracy of the derivatization procedure because of the absence of flame factors. The reproducibility of the method is given below. Direct injection of the reaction mixture using the moving-needle solid-injection system [42] is impossible owing to the low volatility of the silylating reagents, especially TSIM. Samples must therefore be purified before injection. For this reason the two methods described above were compared. Figs. 2 and 3 show urinary steroid profiles of a patient with Cushing's syndrome, obtained after purification of the MO-TMS derivatives by the two methods. It can be seen that even for minor compounds identical results were obtained. No specific loss of steroids or groups of steroids occurred with either of the methods. Purification of the sample with Lipidex-5000 according to the method described by Axelson and Sjövall [2, 37, 39] yielded a cleaner chromatogram, although differences were small. The method of De Jong [40] is less time-consuming and can

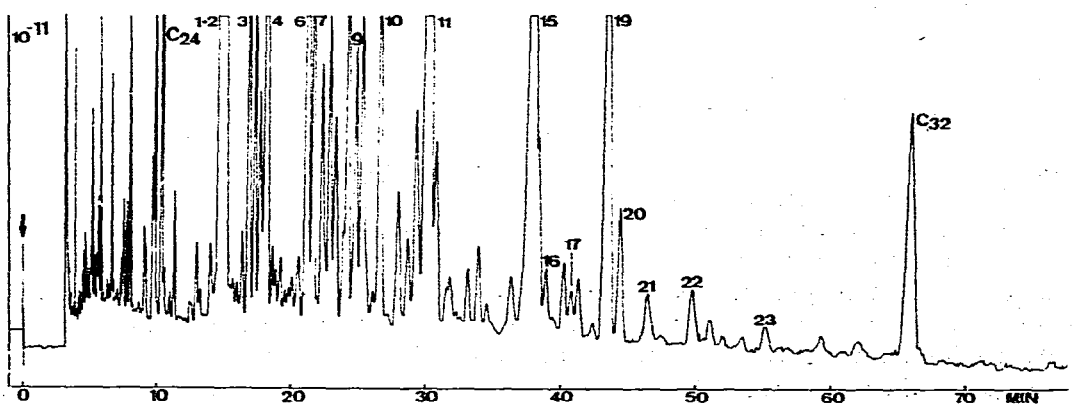

Fig. 2. Steroid profile of a patient with Cushing's syndrome. The sample was purified after derivatization following method A. GC conditions: 35 m × 0.25 mm WCOT SE-30 column; temperature, 250°; pressure of carrier gas (nitrogen), 1.0 kg/cm²; 1.0 μl of the sample was injected. The key to the numbering of the peaks is given in Table I.

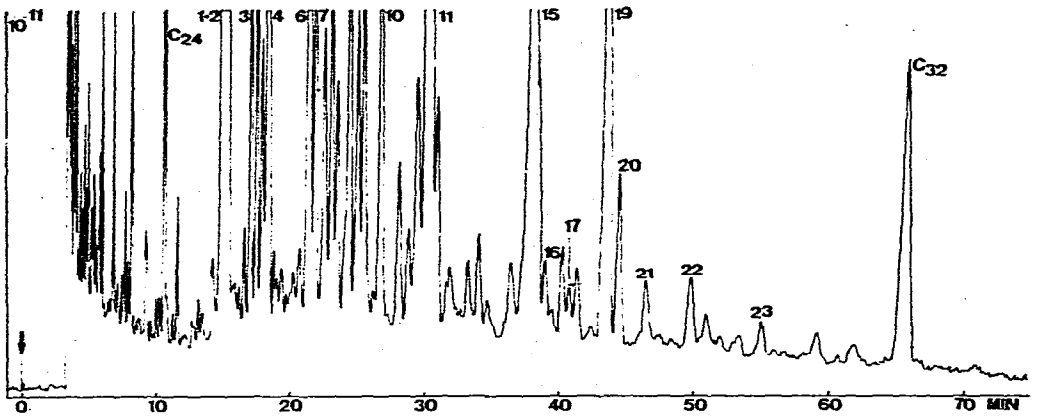

Fig. 3. Profile of the same sample as in Fig. 2, after purification of the MO-TMS derivatives following method B. GC conditions are the same as in Fig. 2.

therefore be recommended for routine analyses. If samples are to be analysed by GC—MS, purification over Lipidex-5000 is to be preferred.

Gas chromatographic analysis of MO-TMS derivatives

In most instances WCOT [20, 48] or SCOT [17—19, 49] columns, coated with the apolar phase SE-30, are used for the separation of steroid MO-TMS derivatives. The separation of 27 references steroids as their MO-TMS derivatives is shown in Fig. 4.

Kováts indices of the MO-TMS derivatives of the 27 references steroids were measured; the results are given in Table III. Using the home-made gas chromatograph, the reproducibility for all steroids was better than 0.5 index units ($n = 8$).

For quantitative analysis, flame factors or calibration constants must be available. However, they are difficult to establish because they depend not only on the choice of the reference compound(s), but also on the stationary phase [3], the purity of the steroids and reference compound(s), column conditions, variation of detector response and efficiency of the derivatisation [50]. Although studies on this subject are still in progress, some preliminary results can be given (assuming all steroids to be quantitatively derivatised). Most flame factors are in the range 0.8—1.0. A relatively poor response (50—70%) is given by some of the 17-desoxycorticosteroids (THDOC, THA, aTHB, aTHDOC) and also by THE. Also the peak obtained from 11-O-A and 11-O-E is rather small. These observations are in agreement with those reported by others [50, 51].

Reproducibilities (expressed as the coefficient of variation) have been measured for the GC analysis by repeated injection of the derivatized reference mixture. Peak areas were calculated relative to the sum of the areas of two co-injected *n*-alkanes (*n*-C_{24} and *n*-C_{32}) using the program described by Wijtvliet [52]. A DCC D 116 E laboratory computer was used for on-line data acquisition and a Burroughs B 7700 computer for off-line data handling. Values were obtained between 1.8 and 11.5% (except for THA: 18%). The highest values were obtained from the smallest peaks, indicating possible

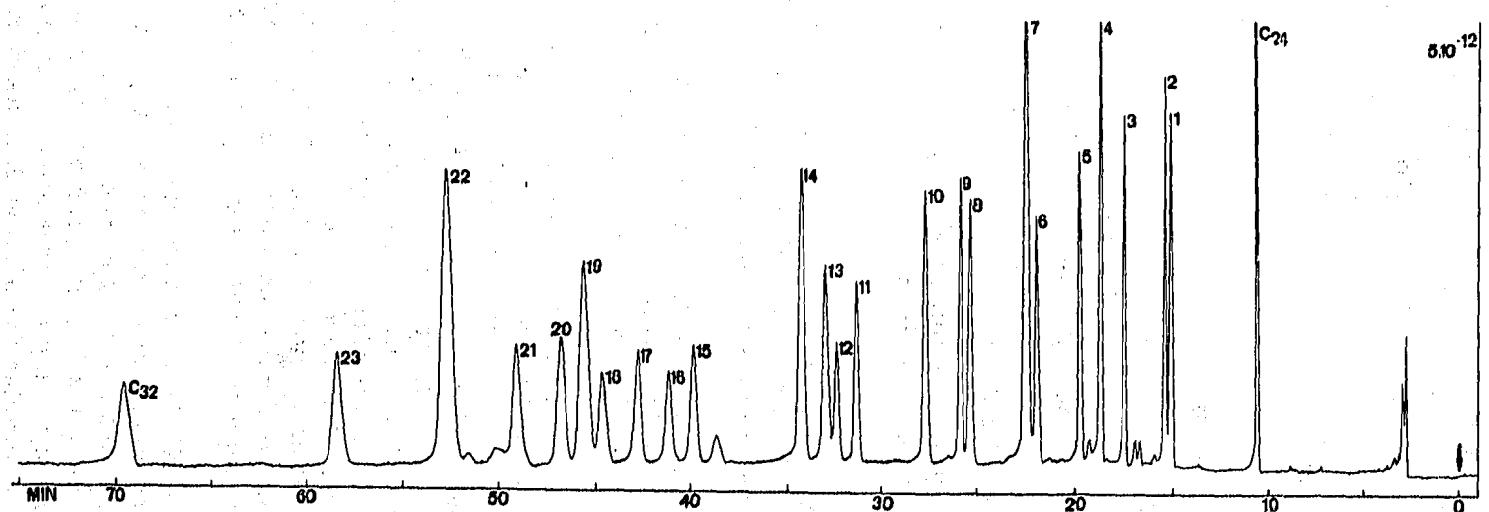

Fig. 4. Separation of the reference mixture on a 35 m × 0.25 mm WCOT SE-30 column. Column temperature, 250°; injection and detection temperatures, 300°; carrier gas (nitrogen) pressure, 1.0 kg/cm².

TABLE III

KOVÁTS INDICES OF MO-TMS DERIVATIVES OF STEROIDS

Kovâts indices were measured on SE-30 at 250°. Inlet pressure of carrier gas (nitrogen) 1.0 kg/cm².

Peak number	Compound	Kovâts index
1	Androsterone	2574
2	Etiocholanolone	2582
3	Dehydroepiandrosterone	2639
4	11-Keto-androsterone	2669
	11-Keto-etiocholanolone	2669
5	Estradiol	2694
6	11β-OH-Androsterone	2738
7	11β-OH-Etiocholanolone	2746
8	Allopregnanediol	2798
9	Pregnanediol	2807
10	Pregnanetriol	2835
11	THS	2884
12	THDOC	2897
13	Estriol	2906
14	aTHS + aTHDOC	2921
15	THE	2981
16	THA	2995
17	THB	3010
18	aTHB	3027
19	THF + aTHE	3036
20	aTHF	3045
21	α-Cortolone	3065
22	β-Cortol	3095
	β-Cortolone	3095
23	α-Cortol	3134

problems with establishing the true baseline. Also a urine sample from a normal male was injected seven times; peak areas ($A = h \times w_{1/2}$) were measured by hand relative to the sum of the peak areas of co-injected n-C_{24} and n-C_{32}. Variation coefficients of between 1.9 and 8.2% were found, the mean variation coefficient being slightly lower than that obtained for the reference mixture.

The reference mixture was derivatized six times in order to establish the reproducibility of the derivatization procedure. Analyses were carried out in the same way as described above. Values obtained ranged from 4.6 to 16.4% (with the exception of THA: 21.3%).

The reproducibility of the whole method, including sample preparation, was established for two different urine samples obtained from a normal adult and a patient with Cushing's syndrome, who was excreting abnormally large quantities of all metabolites. Peak areas were measured by hand. Variation coefficients of the individual steroids varied between 5.8 and 15.8%.

These values agree well with those obtained by others. Shackleton and Honour [50] recently described an automatic solid-injection device able to inject samples with variation coefficients between 1.8 and 8.1%. The re-

producibility of the whole method ranged from 2.4 to 16.6%. Setchell et al. [2], using a more elaborate method, reported variation coefficients of 3—23%. Others obtained variation coefficients of 11—20% [ref. 3] and 7—25% [ref. 22].

Application to human steroid GC profiles

To illustrate the possibilities of the method, GC steroid profiles of a normal male and some patients with various endocrine disorders were determined. Fig. 5 shows a profile of a normal male. Fig. 6 shows the profile of a patient with Cushing's syndrome caused by an adrenal carcinoma. Large quantities

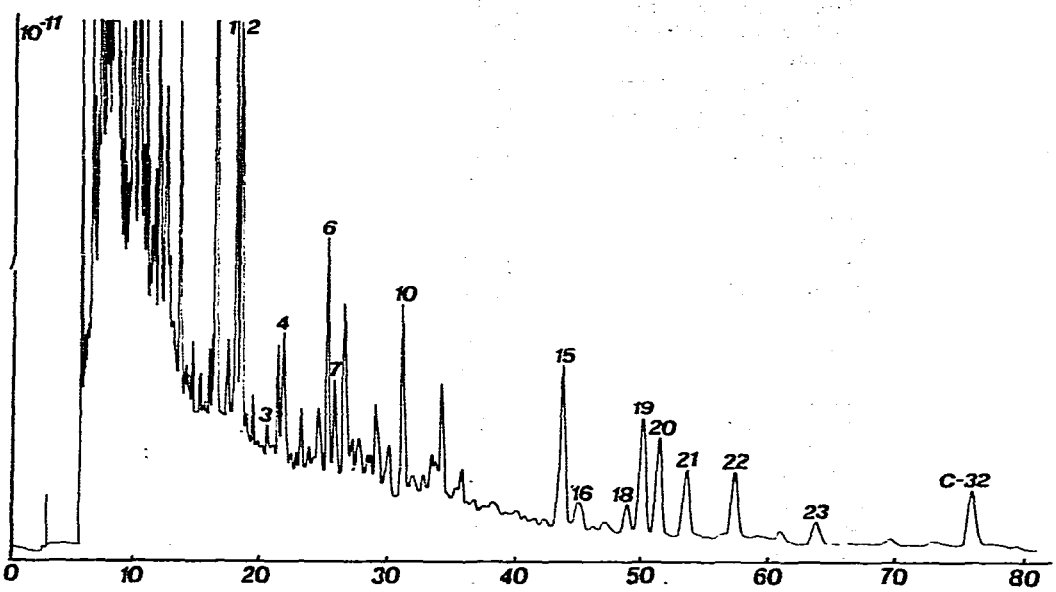

Fig. 5. Steroid profile of a normal male (age 26). GC conditions: 25 m × 0.25 mm WCOT SE-30 column: temperature, 250°; pressure of carrier gas (nitrogen), 1.5 kg/cm²; 1.0 μl of sample was injected.

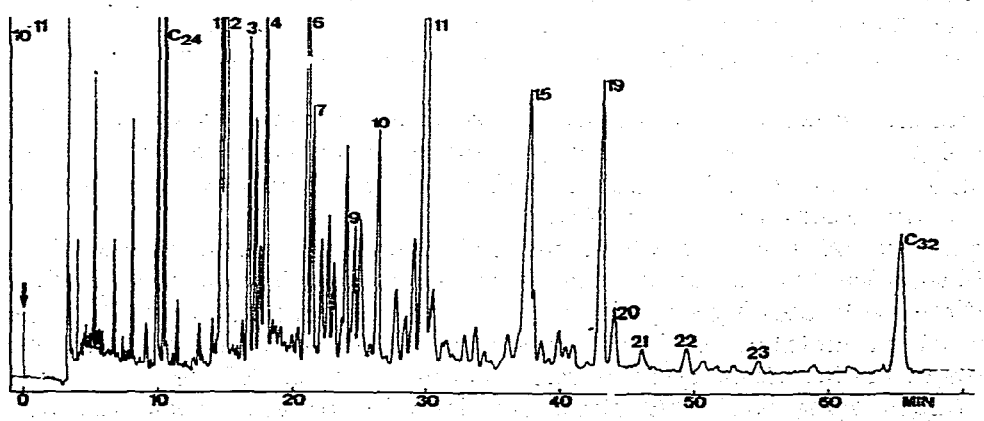

Fig. 6. Steroid profile of a patient with Cushing's syndrome caused by an adrenal carcinoma. GC conditions are the same as in Fig. 2, but only 0.3 μl of sample was injected.

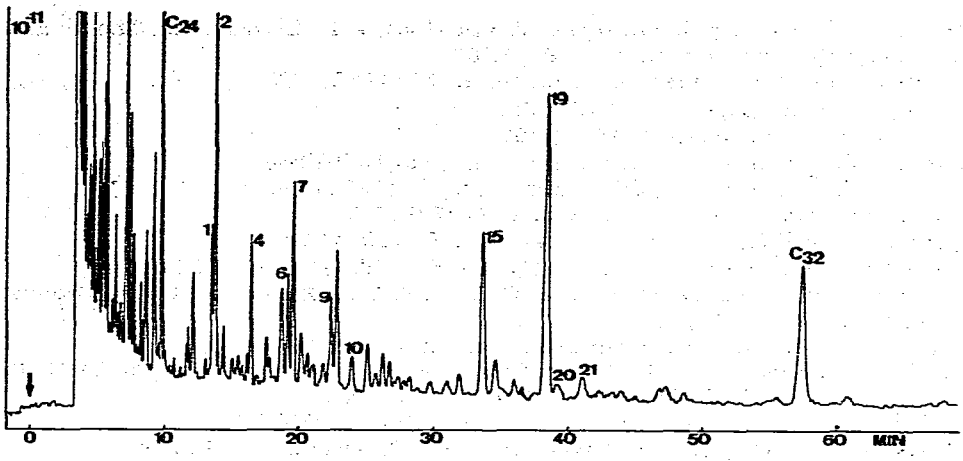

Fig. 7. Steroid profile of a patient with Cushing's syndrome caused by a pituitary tumour. GC conditions are the same as in Fig. 6.

of all steroids are excreted, but the very high excretion of THS is noteworthy. Touchstone et al. [53] and Lipsett et al. [54] had previously reported the production of large amounts of THS by adrenal carcinomas.

The excretion of DHEA is less elevated than is normally found in cases of adrenal carcinoma, although it is known [55] that not all adrenal carcinomas produce large quantities of DHEA. The steroid profile shown in Fig. 7 was obtained from a patient with Cushing's syndrome caused by a pituitary tumour. From this figure some changes in steroid metabolism typical of Cushing's syndrome can be seen. The ratio E/A, normally about 1.0, is increased [53].

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