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THE MULTICOMPONENT ANALYSIS OF CONJUGATES OF NEUTRAL STEROIDS IN URINE BY LIPOPHILIC ION EXCHANGE CHROMATOGRAPHY AND COMPUTERISED GAS CHROMATOGRAPHY–MASS SPECTROMETRY

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SUMMARY

A method for the separation and analysis of conjugates of neutral steroids in urine is described. Following Amberlite XAD-2 extraction of steroids, separation into neutral, glucuronide, monosulphate and disulphate conjugate groups was achieved by ion exchange chromatography on the lipophilic gel, diethylaminohydroxypropyl Sephadex LH-20 (DEAP-LH-20). Enzymatic and solvolytic procedures were used to hydrolyse the conjugate moiety and the steroids were analysed by gas-chromatography using open-tubular glass capillary columns after the preparation of the O-methyloxime-trimethylsilyl ether derivative. Characterization of the steroids was made by computerised GC–MS following repetitive magnetic scanning. Examples of the application of the method to the analysis of urine from normal male subjects, and a patient with Cushing's syndrome are presented.

INTRODUCTION

Over recent years gas–liquid chromatography (g.l.c) has been extensively applied to the multicomponent analysis of steroids in biological material. In spite of the improvement in gas chromatographic techniques by the introduction of open-tubular glass capillary columns [1–8], the inherent disadvantage with the majority of methods described is the inability to profile for all types of steroid groups. The methods which have been described generally involve the analysis of steroids recovered following hydrolysis with enzyme preparations, and while this type of procedure may be satisfactory in the routine screening of clinical disorders, in many cases more subtle changes in steroid levels may be difficult to evaluate from a profile of the total steroids excreted. Changes in specific steroid conjugate classes may prove more informative than the total steroid profile to the understanding of many pathological conditions. A technique is therefore required which will enable the simultaneous determination of several classes of steroids in biological material, with a high degree of accuracy and specificity.

The present paper describes a chromatographic procedure to fractionate steroids according to their class of conjugation utilising ion exchange chromatography on lipophilic ion exchange gels synthesised from Sephadex LH-20 [9, 10]. Qualitative and quantitative analysis of the steroids following liquid-gel chromatography was achieved by g.l.c. utilizing the high resolving power of open-tubular glass capillary columns coupled with computerized gas chromatography–mass spectrometry [GC–MS; 11, 12].

EXPERIMENTAL

Chemicals

All solvents were of reagent grade and were redistilled before use. Acidified ethyl acetate for solvolysis was prepared by saturating redistilled ethyl acetate (10 vol.) with 2 M sulphuric acid (1 vol.) in a separating funnel [13]. Hexamethyldisilazane (Applied Science Laboratories, State College, Pa., U.S.A.) was redistilled before use. Methoxyamine hydrochloride was obtained from Eastman Organic Chemicals (Rochester, N.Y., U.S.A.) and trimethylsilylimidazole from the Pierce Chemical Co. (Rockford, Ill., U.S.A.). The latter reagent contained an interfering impurity with a t_R of 0.56 (OV-1 column) giving an intense ion of mass 512. This impurity was not present in the product sold by Supelco (Bellefonte, Pa., U.S.A.).

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The following trivial names and abbreviations are used in this paper: dehydroepiandrosterone, 3β-hydroxy-5-androsten-17-one; pregnenolone, 3β-hydroxy-5-pregnen-20-one; stigmasterol, 5,22-stigmastadien-3β-ol. The terms pregnanolone, pregnanediol etc. indicate general structures and do not imply a specific stereochemistry. TMS, trimethylsilyl ether; MO, O-methyloxime; t_R, retention time relative to that of 5α-cholestane.

Sodium 2-bromoethane sulphonate was obtained from Fluka (Switzerland) while sodium 2-bromomethane sulphonate was kindly donated by Pharmacia Fine Chemicals (Uppsala, Sweden). *Helix pomatia* digestive juice was purchased from l'Industrie Biologique Francaise (Gennevilliers, France) and Ketodase[R] from Warner-Chilcott Laboratories (Morris Plains, U.S.A.).

Amberlite XAD-2 and Amberlyst A-15 were obtained from Rohm and Haas (Pa., U.S.A.). Amberlite XAD-2 200 g. was washed with 2 l each of 2 M sodium hydroxide, water, 2 M hydrochloric acid, water, acetone, ethanol and water, refluxed for 4 h in water and finally washed with distilled water prior to use. Fines were removed by decantation or by backwashing of columns. Amberlyst A-15 was prepared in the H^+ form by washing with 2 M hydrochloric acid in 72% ethanol, followed by 72% ethanol.

Sephadex LH-20 was obtained from Pharmacia Fine Chemicals. The material was sieved and the fractions 100–140 or 140–170 mesh were used. Lipidex 5000 was obtained from Packard Becker Ltd. (Groningen, Holland).

The solvents used in ion exchange chromatography were as follows:

(i) 72% ethanol; 280 ml of redistilled water was added to 720 ml of redistilled ethanol.

(ii) 0.25 M acetic acid in 72% ethanol; 15 g of acetic acid was added to 720 ml of ethanol and the volume made to 1 litre with water.

(iii) 0.25 M formic acid in 72% ethanol; 11.5 g of formic acid was added to 720 ml of ethanol and the vol. made to 1 litre with water.

(iv) 0.3 M acetic acid–potassium acetate solution pH 6.3; 18 g of acetic acid was added to 200 ml of water and 720 ml of ethanol, the apparent pH of this solution (measured with a glass electrode against an aqueous reference buffer) was adjusted to 6.3 by the addition of 10 g of potassium hydroxide and the volume then made to 1 litre with water.

(v) 0.5 M potassium acetate solution pH 10; 30 g of acetic acid was added to 200 ml of water and 720 ml ethanol and the pH of this solution was adjusted to 10.0 by the addition of concentrated potassium hydroxide solution, the volume then being made to 1 litre with water.

Steroids

Authentic unlabelled steroids were obtained from the MRC Reference Steroid Collection, curator Dr. D. N. Kirk, Westfield College, Hampstead, London. Labelled steroids were purchased from the Radiochemical Centre, Amersham, England: [1,2-^{3}H]-cortisol (50 Ci/mmol), [24-^{14}C]-cholic acid (55 mCi/mmol), 3β-hydroxy-5-[7-^{3}H]-pregnen-20-one (20 Ci/mmol), [7-^{3}H]-progesterone (5 Ci/mmol); and from NEN Chemicals, Dreieichenhain, W. Germany: 3β-hydroxy-5-[4-^{14}C]-androsten-17-one sulphate (55 mCi/mmol), 5β-[1,2-^{3}H]-pregnane-3α,20α-diol (50 Ci/mmol), [1,2-^{3}H]-testosterone glucuronide (50 Ci/mmol). The purity of all compounds was checked by chromatography on Sephadex LH-20 [14, 15]. The labelled pregnanediol was converted to a disulphate [16] and the product was purified by chromatography on Sephadex LH-20 [17].

Preparation of ion exchange gels

Sulphoethyl Sephadex LH-20 (SE-LH-20). Sephadex LH-20 (50 g) was suspended in 0.17 M sodium isopropoxide (1 litre) and sodium hydroxide (25 g), in a powdered form, was added. The mixture was stirred for 24 h under N_2 (avoiding the use of a magnetic stirrer which destroys the Sephadex beads). Sodium 2-bromoethane sulphonate (8.5 g) was then added five times at 8 h intervals with continued stirring under N_2. The product was filtered and washed with 1 litre each of 96% ethanol, 72% ethanol, 1 M sodium hydroxide in 72% ethanol, 72% ethanol (until neutral), 0.5 M hydrochloric acid in 72% ethanol and 72% ethanol (until neutral). About 1 g of this material was washed with ethanol and dried to constant weight for titration. The gel was suspended in 20 ml of 0.25 M sodium chloride in 72% ethanol and was titrated with 0.1 M sodium hydroxide in 72% ethanol. The capacity was usually between 0.15–0.30 mequiv g^{-1}.

A similar procedure was used to prepare sulphomethyl Sephadex LH-20 (SM-LH-20)

Carboxymethyl Sephadex LH-20 (CM-LH-20). Prepared essentially as described by Nyström [18]. The ion exchanger had a capacity of 1.5 mequiv g^{-1}.

Diethylaminohydroxypropyl Sephadex LH-20 (DEAP-LH-20). Sephadex LH-20 was used as the starting material to prepare chlorohydroxypropyl Sephadex LH-20 [9]. The product had a chlorohydroxypropyl content of 23.2% (w/w) and was reacted with diethylamine [10]. The resulting ion exchanger was washed with 50% aq. ethanol, 0.2 M acetic acid in 72% ethanol and 72% ethanol until neutral. The gel was stored in the acetate form in 72% ethanol. For determination of ion exchanging capacity, 1 g gel in OH^- form was suspended in 20 ml of 0.25 M sodium chloride in 72% ethanol and titrated with 0.1 M hydrochloric acid in 72% ethanol, after the addition of a known excess of sodium hydroxide solution. The gel used in this study had a capacity of 0.9 mequiv g^{-1} dry gel, and could bind about 0.5 mmol of cholic acid per g.

Diethylaminohydroxypropyl-hydroxyalkyl Sephadex LH-20 (DEAPHA-LH-20). Sephadex LH-20 was reacted with Nedox 1114 [19] and the hydroxyalkyl content of the product was 18.6% (w/w). This was then used as the starting material for the preparation of a chlorohydroxypropyl derivative (23.6% (w/w) substitution) which was then reacted with diethylamine [10] to yield the DEAPHA-LH-20 gel with a capacity of 0.6 mequiv g^{-1}.

Preparation of ion exchange columns

Glass columns, 200 × 4 mm, with a 50 ml reservoir and ground glass joint at the top were fitted with a Teflon end piece covered with Teflon gauze, 70 μm mesh size, to hold the column bed. The lipophilic Sephadex gels, 0.6 g, were allowed to swell in 72% ethanol for about 30 min and the column bed was packed with a pressure of 0.3 kg/cm^2, resulting in a bed height of 15–20 cm. During chromatography the pressure was adjusted after each solvent change to maintain a flow rate of approximately 25 ml/h.

Procedure for the analysis of steroids in urine

The general scheme of analysis is illustrated in Fig. 1. Urine (25 ml) was passed through a column of Amberlite XAD-2 resin (bed size, 25 cm × 1 cm.) [20]. After washing the column with water (50 ml), the steroids were recovered by elution with ethanol (180 ml). Distilled water (70 ml) was added to the ethanol extract and the sample passed through a column of SE-LH-20 cation exchanger (4 g; bed size 20 × 1 cm.), using a pressure of 0.5 kg/cm^2 to produce a flow of 30–50 ml/h. The effluent was taken to dryness, the residue redissolved in a small vol. of 72% ethanol and transferred to a column containing DEAP-LH-20 (0.6 g) in the acetate form in 72% ethanol. After application of the sample, pressure was applied to the column (0.3 kg/cm^2) to produce a flow rate of approximately 25 ml/h, and the *neutral* steroids were eluted from the column with 25 ml of 72% ethanol. The solvent was then changed to 0.25 M formic acid in 72% ethanol (25 ml) and the *glucuronide* conjugates were recovered. Steroid *monosulphates* were eluted with 25 ml of 0.3 M acetic acid–potassium acetate, pH 6.3, in 72% ethanol. Finally, steroid *disulphates* were recovered by elution with 25 ml of 0.5 M potassium acetate solution in 72% ethanol adjusted to pH 10.0 with potassium hydroxide.

Neutral fraction. The neutral fraction was taken to dryness, redissolved in 10 ml sodium acetate buffer (pH 4.5, 0.5 M) and incubated with 0.3 ml of *Helix pomatia* digestive juice for 16 h at 37°C. Following incubation the steroids were extracted on a column of Amberlite XAD-2 as described above.

The extract was dissolved in 72% ethanol (25 ml) and passed through a DEAP-LH-20 column (0.6 g) to recover the neutral compounds liberated by the enzyme preparation. The solvent was evaporated, the sample dissolved in ethanol (2 ml) and stored in small vials until required for analysis.

Glucuronide fraction. This was taken to dryness and redissolved in 10 ml of sodium acetate buffer (pH 4.5, 0.5 M). Ketodase^R (2 ml) was added and the sample incubated at 37°C for 48 h, a further 2 ml of Ketodase^R was added after the first 24 h. Following hydrolysis, the steroids were extracted on columns of Amberlite XAD-2 and the liberated steroids isolated by filtration through DEAP-LH-20 in an identical manner to that of the neutral steroid fraction.

Monosulphate and disulphate fractions. These fractions were taken to dryness, and distilled water added; 15 ml to the monosulphate fraction and 25 ml to the disulphate fraction to give a 0.5 M concentration of potassium acetate. The pH was adjusted to 4.5 by addition of hydrochloric acid, *Helix pomatia* digestive juice (0.3 ml) was added and the sample was incubated at 37°C for 16 h. Following hydrolysis, the steroids were extracted on Amberlite XAD-2 as described before. The ethanol extract was taken to dryness, 30 ml of acidified ethyl acetate was added and the sample was incubated at 39°C for 16 h. Following

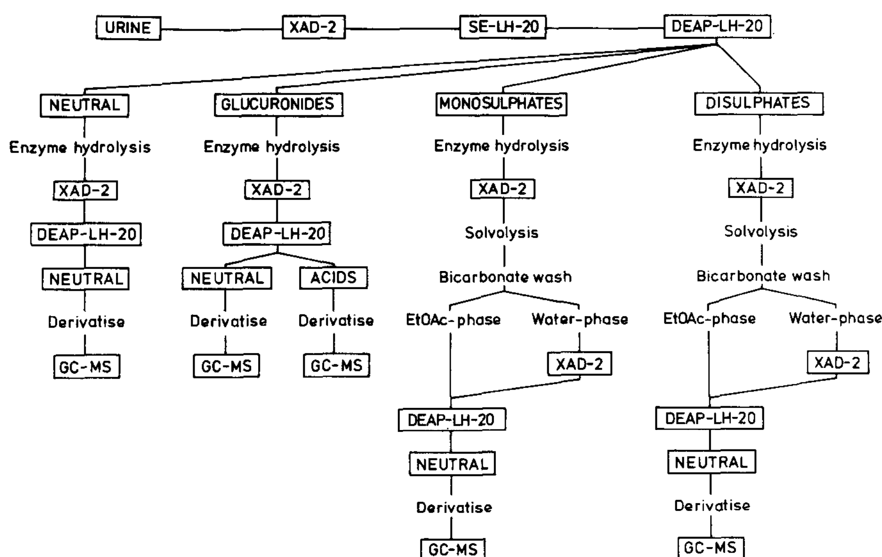

Fig. 1. General scheme for the analysis of urinary steroid conjugates.

this solvolysis, the ethyl acetate was washed to neutrality with sodium bicarbonate (8%) followed by water. The combined washings were passed through a column of Amberlite XAD-2 to recover any steroids extracted during the washing procedure and the XAD-2 extract was added to the ethyl acetate phase. The combined sample was then passed through a column of DEAP-LH-20 as before to recover the neutral steroids liberated during hydrolysis and solvolysis. The sample was stored in ethanol (2 ml) until required for analysis.

Preparation of derivatives for gas chromatography

The internal standards 5α-androstane-3α,17α-diol and stigmasterol (1–10 μg) were added to portions of the steroid extracts (1/2–1/10) and the sample taken to dryness in a stream of nitrogen. Methoxyamine hydrochloride (5 mg) and dry pyridine (50 μl) were added and the mixture incubated at 60°C for 15 min [21]. The sample was then dried over a stream of nitrogen, trimethylsilylimidazole (50 μl) added and the sample left to react for 2 h at 110°C [21]. After reaction, dipropylamine (100 μl) was added and the sample was transferred to a column of Lipidex 5000 (0.25 g) prepared in the solvent system hexane–pyridine–hexamethyldisilazane (98:1:1, by vol.), using 0.5 ml of this solvent. The sample tube was then washed with four successive 0.5 ml volumes of solvent which were then passed through the Lipidex 5000 column [22]. The effluent (2.5 ml) was collected in small stoppered glass centrifuge tubes. Prior to g.l.c. this solvent was evaporated and the derivatized sample redissolved in 10–200 μl of hexane.

Gas–liquid chromatography (g.l.c.)

G.l.c. was carried out on a Pye 104 or Becker 409 gas chromatograph equipped with a flame ionization detector and housing a 25 m open-tubular glass capillary column coated with OV-1 [23]. A glass solid injection system was used [24]. Nitrogen was the carrier gas at a pressure of approximately 0.5 kg/cm^2, giving a flow rate of approximately 1 ml/min. Both temperature programmed operation from 180° to 275°C at rates of 0.6–1.2°C/min, and isothermal conditions at temperatures varying between 210°–240°C were employed.

Computerized GC–MS

GC–MS was carried out using a modified LKB 9000 instrument. A 25 m open-tubular glass capillary column coated with OV-1 was connected to the ion source *via* a single stage adjustable jet separator. We are grateful to Dr. R. Ryhage for providing us with drawings of this separator. Other details of the connection between the capillary column and the ion source will be described elsewhere. Methods for computerized evaluation of the mass spectral data recorded on magnetic tape have been described previously [11, 12]. The operating conditions were as follows: temperature of column, either isothermal (250°C) or programmed from 235° to 275°C at 0.6 or 1.2°C/min; separator temperature 225°C; temperature of the ion source 290°C; energy of bombarding electrons 22.5 eV; ionizing current 60 μA; accelerating voltage 3.5 kV. Repetitive scanning (usually 10 scans/min) over the *m/e* range 0–800 was initiated after a suitable delay, when the more volatile non-steroidal compounds were removed through the separator pumps.

Identification and quantitation of steroids

The identification of a steroid was based upon the retention time, the complete mass spectrum and partial mass spectra obtained from fragment ion current (FIC) chromatograms constructed for characteristic ions given by the steroid derivatives. The *m/e* values employed in this study to detect derivatives of ster-

Table 1. Selected *m/e* values representative of TMS and MO-TMS derivatives of common steroids

Structure[a]	Molecular ion m/e	Fragment ions, m/e[b]
A-diolone[c]	450	360,345,306,270
A-olone	391	376,360,345,270
A-diolone	479	464,448,389,358,268,213
A$^{\Delta}$-olone	389	358,268,260,213
A$^{\Delta}$-diolone	477	446,387,356
A$^{\Delta}$-oldione[c]	405	390,315,300,284,269,261
A-diol	436	421,346,256,241
A-triol	524	434,344,254,215
A$^{\Delta}$-diol	434	344,254,215
A-tetrol	612	522,432,342,252,239,237
A$^{\Delta}$-triol	522	432,342,252,239,237
P-olone	419	404,388,298
P-diolone	507	476,417,402,386,296
P$^{\Delta}$-olone	417	402,386,296
P-triol	552	462,372
P$^{\Delta}$-diol	462	372
P-tetrol	640	550,460
P$^{\Delta}$-triol	550	460
P-triolone	595	564,492,474,402,384,312
P-dioldione[c]	521	490,431,400
P-trioldione[c]	609	578,506,488
P-tetrolone	683	652,562,472,382,292
P-tetrolone[c]	654	551
P-pentol	728	625,638,535
P-pentolone	771	681,650,591,560,470
P$^{\Delta}$-tetrolone	681	650,591,560,470

[a] A = androstane, P = pregnane, $^{\Delta}$ = double bond.
[b] For the sake of clarity *m/e* values are repeated. In practice, FIC chromatograms of these ions were plotted once only.
[c] Containing one underivatized keto group.

Table 2. Selected *m/e* values representative of common structural features in TMS and MO-TMS derivatives of steroids

Structure[a]	Fragment ions[b]
20-MO-21-deoxy	70, 87, 100
3-MO-Δ4	125,137,153
16,20-bis-TMS-21-deoxy	141,156,157
2,3-bis-TMS; 3-TMS-Δ4	142,143
TMS-ions[c]	103,117,129,191,217,243
P-ABCDΔ-20,21-bis-TMS[d]; P-ABCD2Δ-11-one[d]	449,359,269
P-diol[e]	449,359,269
P-ABCD2Δ-20,21-bis-TMS[d]; P-ABCD3Δ-11-one[d]	537,447,357,267
PΔ-diol[e]	447,357,267
P-ABCDΔ-11-one-20,21-bis-TMS[d]	463,373,283
P-(15,16,17,21)-TMS-20-MO[f]	175,188,276,201,289
P-ABCD-bis-TMS[d]	435,345,255
P-ABCD-tris-TMS[d]	523,433,253
P-ABCDΔ-tris-TMS[d]	521,431,341,251

[a] For abbreviations see Table 1. MO = O-methyloxime; TMS = trimethylsilyl ether.

[b] An excellent summary with references to original papers has been previously reported [51].

[c] Containing one, two and three carbons and one TMS group or one, three and five carbons and two TMS groups.

[d] ABCD = steroid ring skeleton; Δ = double bond(s) present in naturally occurring steroid or formed by loss of trimethylsilanol.

[e] Series of ions formed by loss of 15 and 90 mass units from molecular ion.

[f] Ions which may be formed from steroids having one or two TMS groups at C-15, 16, 17, or 21.

oids most likely to occur in urine from healthy men are listed in Tables 1 and 2.

An approximate estimation of the amounts of steroids present in the different fractions was obtained by addition of known amounts of two internal standards, 5α-androstane-3α,17α-diol and stigmasterol, to the samples prior to MO-TMS derivative formation. A line was drawn between the peak heights of the two standards and the heights of the steroid peaks were measured relative to this line [25]. Mixtures of known amounts of reference steroids were analysed in the same way and factors (between 0.76 and 1.70) were calculated to correct for differences in mass/peak height relationships. When pure reference compounds were not available the factor 1.0 was used.

RESULTS

Anion exchange chromatography

The labelled steroids progesterone, testosterone glucuronide, 3β-hydroxy-5-androsten-17-one sulphate and 5β-pregnane-3α,20α-diol disulphate were chosen to be representative of a neutral steroid, a glucuronide, a monosulphate, and a disulphate, respectively. These compounds were added to urine samples (25 ml) which were then extracted on Amberlite XAD-2 and passed through the cation exchanger using the procedure described. The steroid extracts were then subjected to chromatography on the two types of anion exchanging gels DEAP-LH-20 and DEAPHA-LH-20, in the acetate form. Different solvent systems were tested, and a satisfactory separation of the compounds was achieved using the solvent systems described in Fig. 2.

In addition, urine samples (25 ml) from two male subjects, one given orally [1,2-^{3}H]-cortisol (K.S., 20

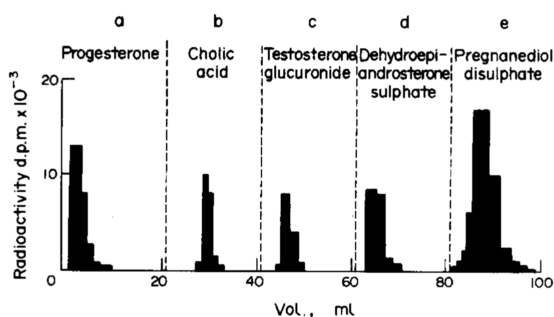

Fig. 2. Group separation of radioactive steroids added to urine samples. Ion exchange chromatography was performed on DEAP-LH-20 (0.6 g) in the acetate form. Stepwise elution of the steroids was achieved using the following solvents: (a) 72% ethanol, (b) 0.15 M acetic acid in 72% ethanol, (c) 0.25 M formic acid in 72% ethanol, (d) 0.3 M acetic acid-potassium acetate, pH 6.3, in 72% ethanol, (e) 0.5 M potassium acetate, pH 10, in 72% ethanol.

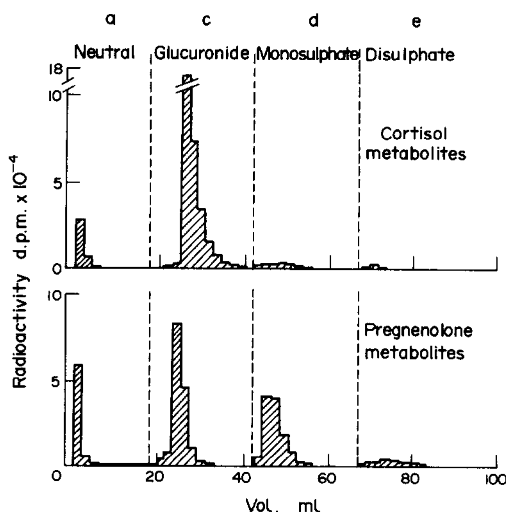

Fig. 3. Ion exchange separation of radioactive steroid metabolites isolated from the urine of subjects given an oral dose of [1,2-^{2}H]-cortisol or [7-^{3}H]-pregnenolone. Column and solvents were as in Fig. 2, with the exception that elution with acetic acid was omitted.

Table 3. Recovery of radioactive steroid metabolites from urine. Triplicate aliquots of urine from subjects administered ^{3}H-cortisol and ^{3}H-pregnenolone were processed through the method and the steroid conjugate metabolites separated on DEAP-LH-20

	Urine KS (^{3}H-Cortisol metabolites)			Urine BA (^{3}H-Pregnenolone metabolites)		
	1	2	3	1	2	3
Radioactivity in aliquot of urine (dpm)		301620			325189	
Following cation exchange chromatography (dpm)	295587	310668	298603	321900	328400	312181
Recovery %	98	103	99	99	101	96
DEAP-LH-20 Anion exchange chromatography						
Neutral fraction: dpm	34635	31572	30307	70111	75682	65099
(%)[a]	(12)	(10)	(9)	(22)	(24)	(22)
Glucuronide fraction: dpm	239885	257926	272561	141053	124050	132290
(%)[a]	(80)	(83)	(84)	(44)	(39)	(45)
Monosulphate fraction: dpm	19160	16593	16049	94691	92028	84426
(%)[a]	(6)	(5)	(5)	(30)	(29)	(29)
Disulphate fraction: dpm	4871	3633	5348	12830	24347	12166
(%)[a]	(2)	(2)	(2)	(4)	(8)	(4)
Total radioactivity (dpm)	298551	309724	324265	318685	316107	293981
Recovery %	99	103	108	98	97	90

[a] Per cent of the radioactivity recovered in this chromatography.

μCi) and the other 3β-hydroxy-5-[7-^{3}H]-pregnen-20-one (B.A., 10 μCi), were processed through the method. During chromatography on DEAP-LH-20, fractions (2 ml) were collected and the radioactivity measured. Fig. 3 shows the distribution of radioactivity in each conjugate fraction from both subjects. As expected, the major radioactive metabolites of ^{3}H-cortisol were recovered in the glucuronide fraction. Urine from the subject administered ^{3}H-pregnenolone contained a greater proportion of radioactive metabolites in the monosulphate and neutral fractions.

DEAP-LH-20 and its hydroxyalkyl derivative gave identical separations using the solvent systems and conditions described. Overlap of steroid groups was less than 1% even during continual elution with excessive volumes (100 ml) of each solvent, and compounds were quantitatively recovered in small volumes.

Cation exchange chromatography

When Amberlite XAD-2 extracts of urine were applied to DEAP-LH-20 columns without prior passage through a cation exchanger in the H$^+$ form, part of the anionic steroid conjugates appeared in the neutral fraction. Since adsorption of steroids to the commercially available cation exchange resin Amberlyst A-15 has sometimes been observed, the properties of cation exchangers synthesized from Sephadex LH-20 were studied.

Identical samples of urine (25 ml) from the subject administered 3β-hydroxy-5-[7-^{3}H]-pregnen-20-one were extracted on Amberlite XAD-2 and passed through columns (30 × 1 cm.) of Amberlyst A-15, SE-LH-20, SM-LH-20 and CM-LH-20 (4 g of each) in the H$^+$ form in 72% ethanol. The steroids were then separated into conjugate groups on DEAP-LH-20 columns. Fractions (2 ml) were collected and the radioactivity measured. The distribution of radioactivity for those samples passed through Amberlyst A-15, SE-LH-20 and SM-LH-20 was similar, while the sample passed through CM-LH-20 showed a greater proportion of radioactivity in the neutral fraction. Rechromatography of the latter fraction on a second column of DEAP-LH-20 showed that it was heterogenous, since radioactivity appeared in all conjugate fractions. In contrast, when the neutral fractions isolated from samples passed through Amberlyst A-15, SE-LH-20, and SM-LH-20 were rechromatographed, the radioactivity was quantitatively recovered in the neutral fraction.

Recovery of radioactive steroid metabolites

The reproducibility of the chromatography was assessed by analysing triplicate samples of urine obtained from the two subjects given [1,2-^{3}H]-cortisol and 3β-hydroxy-5-[7-^{3}H]-pregnen-20-one and determining the recovery of radioactivity at various stages (Table 3).

The mean recovery of radioactivity following cation exchange chromatography on SE-LH-20 was 99.3%. After anion exchange chromatography on DEAP-LH-20 a mean of 99.2% of the radioactivity was recovered in the fractions collected. The reproducibility of the separations on DEAP-LH-20 is evident from the results shown in Table 3.

Table 4. Steroids found in urine of normal male subjects.

No.	t_R [a]	Steroid structure	Conjugate fraction [b]		
1	0.51	3α-Hydroxy-androsten-17-one	G	M	-
2	0.53	3α-Hydroxy-5α-androstan-17-one	G	M	-
3	0.55	3α-Hydroxy-5β-androstan-17-one	G	M	-
4	0.55	5β-Androstane-3α,17β-diol	G	-	-
5	0.59	5-Androstene-3β,17α-diol	G	M	D
6	0.63	3β-Hydroxy-5-androsten-17-one	-	M	-
7	0.65	3β-Hydroxy-5α-androstan-17-one	-	M	-
8	0.65	Androstenediolone	G	-	-
9	0.66	5-Androstene-3β,17β-diol	-	M	D
10	0.67	3α-Hydroxy-5α-androstane-11,17-dione	G	-	-
11	0.68	Androstanediol	-	-	D
12	0.69	Androstanetriol	G	-	-
13	0.69	3α-Hydroxy-5β-androstane-11,17-dione	G	-	-
14	0.70	3β,7α-Dihydroxy-5-androsten-17-one	-	M	-
15	0.74	Androstanetriol	G	-	-
16	0.76	Androstanetriol	-	M	D
17	0.78	Androstanediolone	G	-	-
18	0.79	Androstanediolone	G	-	-
19	0.80	Androstenediolone	-	M	-
20	0.81	Androstanetriol	G	-	-
21	0.82	3α,11β-Dihydroxy-5α-androstan-17-one	G	M	-
22	0.84	3α,11β-Dihydroxy-5β-androstan-17-one	G	-	-
23	0.85	Pregnanolone	G	-	-
24	0.87	Pregnanediolone	G	-	-
25	0.89	Androstane-3,16,17-triol	G	-	-
26	0.89	Androstenetriol	G	-	-
27	0.89	5-Pregnenediol	G	-	-
28	0.91	3β,16α-Dihydroxy-5-androsten-17-one [c]	-	M	-
29	0.93	5-Androstenetriol	-	M	-
30	0.94	3β,16α-Dihydroxy-5-androsten-17-one [c]	-	M	-
31	0.94	5-Androstenetriol	-	M	-
32	0.95	5α-Pregnane-3α,20α-diol	G	-	-
33	0.95	Androstanetriol	G	M	-
34	0.97	Pregnanetriol	G	-	-
35	0.97	5β-Pregnane-3α,20α-diol	G	-	-
36	0.98	Androstanetriol	-	M	D
37	1.01	5-Androstene-3β,16β,17α-triol	G	M	D
38	1.01	Androstanediolone	G	-	-
39	1.05	5β-Pregnane-3α,17α,20α-triol	G	M	D
40	1.06	5-Pregnene-3α,17α,20α-triol [d]	G	M	D
41	1.08	5α-Pregnane-3α,17α,20α-triol [d]	G	M	D
42	1.12	Androstenediolone	G	-	-
43	1.13	5-Pregnene-3β,20α-diol	-	M	D
44	1.19	5-Androstene-3β,16α,17β-triol	-	M	D
45	1.22	Pregnanetriolone	G	-	-
46	1.24	Androstene-3,16,17-triol	-	M	D
47	1.26	5-Pregnene-3α,16α,20α-triol [d]	G	-	-
48	1.41	Pregnane-X,X,17,20-tetrol	-	M	-
49	1.44	Pregnane-X,X,17,20-tetrol	-	M	-
50	1.49	Pregnenediolone	-	M	-
51	1.60	5-Pregnene-3β,17α,20α-triol	G	M	D

Table 4 *continued*

No.	t_R [a]	Steroid structure	Conjugate fraction [b]		
52	1.63	3α,17α,21-Trihydroxy-5β-pregnane-11,20-dione	G	M	D
53	1.63	3α,21-Dihydroxy-5β-pregnane-11,20-dione	G	-	-
54	1.64	Pregnanetriol	-	M	-
55	1.67	Pregnenetetrolone	G	-	-
56	1.67	3α,21-Dihydroxy-5α-pregnane-11,20-dione	G	-	-
57	1.69	5-Pregnene-3β,16α,20α-triol	-	M	-
58	1.76	3,X,21-Trihydroxy-pregnan-20-one	-	-	D
59	1.78	3α,11β,21-Trihydroxy-5β-pregnan-20-one	G	-	-
60	1.82	Pregnanetetrol	G	-	-
61	1.86	3α,11β,21-Trihydroxy-5α-pregnan-20-one	G	-	-
62	1.88	3α,11β,21-Trihydroxy-5β-pregnan-20-on-18-al	G	M	-
63	1.92	3α,11β,17α,21-Tetrahydroxy-5β-pregnan-20-one	G	M	D
64	2.00	3α,11β,17α,21-Tetrahydroxy-5α-pregnan-20-one	G	M	D
65	2.07	3α,17α,20α,21-Tetrahydroxy-5β-pregnan-11-one	G	M	D
66	2.18	3β,11β,21-Trihydroxy-5α-pregnan-20-one	G	-	-
67	2.20	3,20,21-Trihydroxy-pregnan-11-one	G	-	-
68	2.24	5β-Pregnane-3α,11β,17α,20β,21-pentol	G	M	D
69	2.24	3α,17α,20β,21-Tetrahydroxy-5β-pregnan-11-one	G	M	D
70	2.28	Pregnenepentol	-	M	D
71	2.32	3,17,20,21-Tetrahydroxy-pregnan-11-one	G	M	-
72	2.53	5β-Pregnane-3α,11β,17α,20α,21-pentol	G	M	D
73	2.53	Pregnanetetrol-11-one	-	M	-
74	2.56	Pregnenetetrolone	-	M	-
75	2.80	Pregnenetetrolone	-	M	-
76	2.84	Pregnanetetrolone	-	M	-
77	2.87	1β,3α,17α,20β,21-Pentahydroxy-5β-pregnan-11-one	G	-	-

[a] Retention time of the TMS or MO-TMS derivative relative to that of 5α-cholestane on a 25 metre open-tubular glass capillary column at 230°C.
[b] G = glucuronide, M = monosulphate, D = disulphate.
[c] The MO-TMS derivative of this steroid gives two peaks.
[d] Tentative configurations.

Hydrolysis of steroid conjugates

Both enzymatic and solvolytic procedures were necessary to give a high yield of hydrolysed steroids. Ketodase[R] was used to hydrolyse the glucuronides, although *Helix pomatia* digestive juice gave similar results.

Complete conversion of radioactive metabolites in the glucuronide fraction to neutral steroids could not be achieved with either enzyme preparation, in spite of varying temperature, incubation time and amount of enzyme. Repeated hydrolysis did not significantly increase the yield of neutral compounds (range 75–90%). However, it was found that the radioactivity, thought to represent unhydrolysed glucuronides (10–25%) could be eluted from the DEAP-LH-20 column with 0.15 M acetic acid in 72% ethanol. Thus, it behaved like a steroid carboxylic acid and it is possible that it represented C_{21} steroid acids of the type recently shown to occur in human urine [26, 27].

GC–MS of urinary steroids

Daily urine collections were taken from normal male subjects (20–35 years) and aliquots processed through the method. A sample from a patient with Cushing's syndrome due to ectopic ACTH secretion was also analysed. Urine samples were immediately stored at −20°C on collection to inhibit possible bacterial degradation and artifact formation. The MO-TMS derivatives of each conjugate fraction were analysed by GC–MS. Chromatograms were constructed by the computer from the currents of selected *m/e* values and aided in the localisation of eluted steroid derivatives. The molecular ion (M^+) was always used, and fragment ions were generally selected among those resulting from loss of 15, 31, 90, 103, 117, 129 and 205 mass units and combinations of these, depending on the structure of the steroid. In addition a number of *m/e* values typical of specific structural configurations were included in the list. A

list of the conjugates of neutral steroids detected by this relatively unbiased analysis is given in Table 4. In cases where the exact stereochemical configuration was not ascertained, the retention time relative to 5α-cholestane is given.

Neutral fraction. Steroids could not be detected in this fraction from any of the samples from normal males. However, an abundance of corticosteroid metabolites was isolated and identified in the sample from the patient with Cushing's syndrome. Figure 4 shows fragment ion current chromatograms of *m/e* values typical of derivatives of polar corticosteroid metabolites isolated in this fraction. A comparison of the g.l.c. analysis of the individual conjugate fractions from the urine of this patient is shown in Figure 5. In addition to the excretion of large amounts of polar corticosteroid metabolites in the neutral and glucuronide fractions, considerable amounts were present as monosulphate conjugates.

Glucuronide fraction. As expected, the major part of steroids excreted in male urine was found in this fraction. Figure 6 illustrates fragment ion current chromatograms of *m/e* values characteristic of

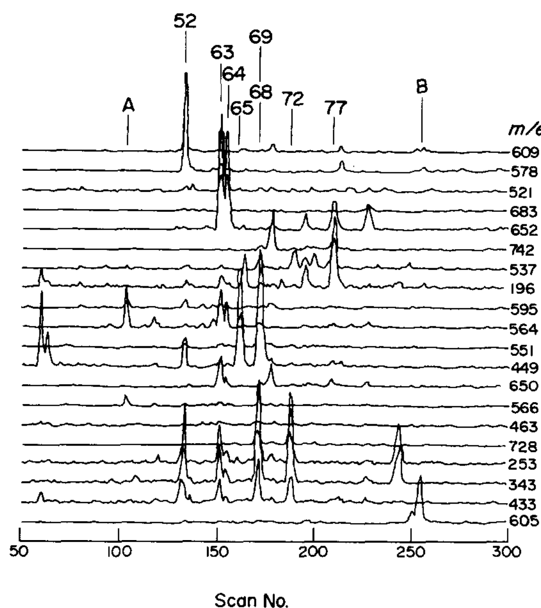

Fig. 4. Fragment ion current chromatograms of *m/e* values representative of TMS and MO-TMS derivatives of common polar steroids isolated from the neutral fraction of the urine from a patient with Cushing's syndrome. An aliquot corresponding to about 50 μl of urine was analyzed by GC-MS using a modified LKB 9000 mass spectrometer equipped with a 25 m open-tubular glass capillary column coated with OV-1. The column temperature was programmed at 1.2°C/min from 240° to 275°C. Spectra were taken by repetitive magnetic scanning, 10 spectra/min, starting 15 min after injection of the sample. The structures of the steroids indicated by the numbers can be obtained from Table 4. A = 3α,17α,21-trihydroxy-5β-pregnan-20-one; B = cortisol.

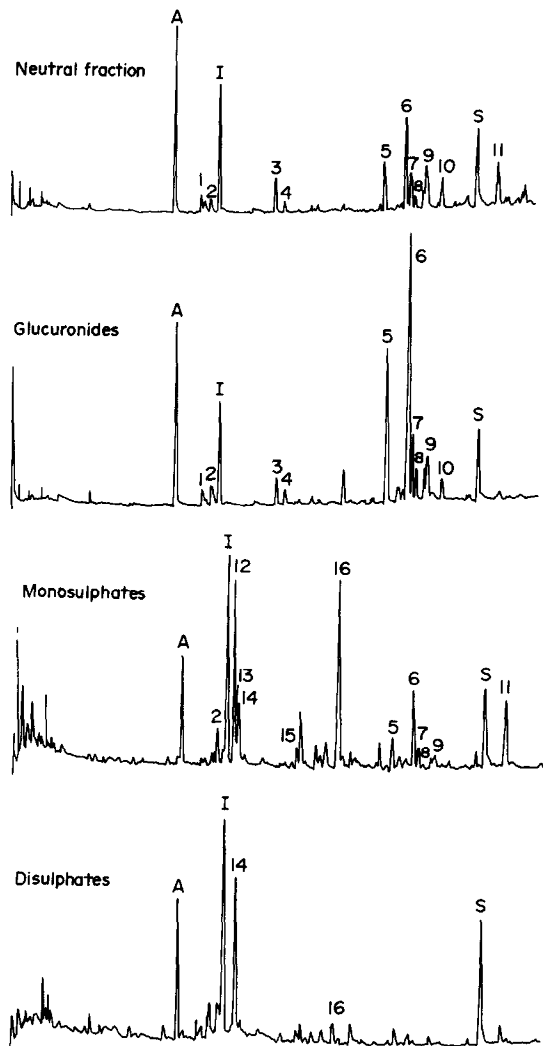

Fig. 5. Gas chromatographic analyses comparing the steroid conjugate fractions isolated from the urine of a patient with Cushing's syndrome. Aliquots equivalent to 1/3000 of the 24 h excretion were taken from the neutral and glucuronide fractions, and 5α-androstane-3α,17α-diol (A, 10 μg) and stigmasterol (S, 10 μg) were added. The same amount of stigmasterol and half the amount of the androstanediol were added to aliquots of the mono- and disulphate fractions (equivalent to 1/240 and 1/120 of the 24 h excretion, respectively). MO-TMS derivatives were prepared, and about 0.5% of the samples were analyzed on a Becker 409 gas chromatograph equipped with a 25 m open-tubular glass capillary column coated with OV-1. Column temperature was programmed at 1°C/min from 160° to 250°C. The following steroids are indicated: 1. 3α-hydroxy-5α-androstan-17-one, 2. 3α-hydroxy-5β-androstan-17-one, 3. 3α,11β-dihydroxy-5α-androstan-17-one, 4. 3α,11β-dihydroxy-5β-androstan-17-one, 5. 3α,17α,21-trihydroxy-5β-pregnane-11,20-dione, 6. 3α,11β,17α,21-tetrahydroxy-5β-pregnan-20-one, 7. 3α,11β,17α,21-tetrahydroxy-5α-pregnan-20-one, 8. 3α,17α,20α,21-tetrahydroxy-5β-pregnan-11-one, 9. 3α,17α,20β,21-tetrahydroxy-5β-pregnan-11-one and 5β-pregnane-3α,11β,17α,20β,21-pentol, 10. 5β-pregnane-3α,11β,17α,20α,21-pentol, 11. cortisol, 12. dehydroepiandrosterone, 13. 3β-hydroxy-5α-androstan-17-one, 14. 5-androstene-3β,17β-diol, 15. 3β,16α-dihydroxy-5-androsten-17-one (two peaks), 16. 5-androstene-3β,16α,17β-triol. I = impurity in trimethylsilylimidazole.

Table 5. Daily excretion of steroids isolated from the glucuronide fraction of urine from 10 healthy adult males aged 20–35 years

Steroid glucuronide	Excretion mg/24 h									
	1	2	3	4	5	6	7	8	9	10
3α-Hydroxy-5α-androstan-17-one	2.32	2.43	1.67	1.90	2.47	2.16	2.93	1.81	4.07	1.53
3α-Hydroxy-5β-androstan-17-one	1.90	1.82	2.48	2.29	3.33	1.58	2.68	1.16	2.30	1.07
3α-Hydroxy-5β-androstane-11,17-dione	0.51	0.34	0.58	0.26	1.05	0.36	0.61	0.68	-	0.53
3α,11β-Dihydroxy-5α-androstan-17-one	0.58	0.80	0.63	0.70	1.19	0.88	1.25	1.28	2.35	1.20
3α,11β-Dihydroxy-5β-androstan-17-one	0.32	0.11	0.46	0.14	0.80	0.28	0.76	1.05	0.28	0.52
5β-Pregnane-3α,20α-diol	0.49	0.37	0.57	0.32	0.70	0.43	0.90	0.71	0.75	0.80
5β-Pregnane-3α,17α,20α-triol	0.73	1.08	1.03	0.73	2.16	0.42	1.37	1.48	1.18	0.58
3α,17α,21-Trihydroxy-5β-pregnane-11,20-dione	2.25	4.21	3.06	2.92	4.58	2.03	4.24	4.49	5.45	1.93
3α,11β,21-Trihydroxy-5β-pregnan-20-one	0.14	0.12	0.15	0.15	0.17	0.11	0.19	0.25	0.19	0.13
3α,11β,21-Trihydroxy-5α-pregnan-20-one	0.22	0.46	0.20	0.12	0.21	0.26	0.40	0.66	0.76	0.33
3α,11β,17α,21-Tetrahydroxy-5β-pregnan-20-one	0.73	1.75	0.97	1.25	1.79	0.82	1.63	1.83	2.39	0.81
3α,11β,17α,21-Tetrahydroxy-5α-pregnan-20-one	0.59	1.32	0.55	0.38	0.87	0.47	1.06	1.63	2.93	0.63
3α,17α,20α,21-Tetrahydroxy-5β-pregnan-11-one	0.63	1.46	0.53	0.86	0.97	0.68	1.26	1.45	2.62	0.71
3α,17α,20β,21-Tetrahydroxy-5β-pregnan-11-one } 5β-Pregnane-3α,11β,17α,20β,21-pentol }	0.87	1.79	0.96	0.95	1.38	0.47	1.39	1.22	1.70	0.55
5β-Pregnane-3α,11β,17α,20α,21-pentol	0.19	0.36	0.18	0.24	0.30	0.14	0.31	0.29	0.69	0.18

MO-TMS derivatives of the common steroid structures. The daily excretion of sixteen major steroids identified in the glucuronide fraction from the urine of ten normal male subjects is given in Table 5.

Monosulphate fraction. The principal steroids characterized in this fraction are evident from the fragment ion current chromatograms shown in Figure 6. As expected, dehydroepiandrosterone is quantitatively the most important monosulphate excreted. The levels of some principal steroids found in this fraction are given in Table 6.

Disulphate fraction. The predominant steroids in

Table 6. Daily excretion of steroids isolated from the sulphate conjugate fractions of urine from 10 healthy adult males aged 20–35 years

Steroid sulphates	Excretion mg/24 h									
	1	2	3	4	5	6	7	8	9	10
Monosulphates[a]:										
3α-Hydroxy-5α-androstan-17-one	0.46	0.59	0.78	0.69	0.51	-	1.95	0.27	2.14	0.68
3α-Hydroxy-5β-androstan-17-one	0.14	0.26	0.48	0.35	0.51	-	0.86	0.38	0.39	0.14
3β-Hydroxy-5-androsten-17-one	0.65	1.74	1.77	1.57	1.09	-	7.89	1.24	5.83	1.47
3β-Hydroxy-5α-androstan-17-one	0.21	0.26	0.35	0.25	0.25	-	1.26	0.31	0.64	0.17
5-Androstene-3β,17β-diol	0.21	0.50	0.30	0.58	0.37	-	1.03	0.31	0.67	0.26
3β,16α-Dihydroxy-5-androsten-17-one	0.69	1.39	1.20	0.95	0.99	-	0.29	0.95	0.86	0.23
5-Androstene-3β,16α,17β-triol	0.78	0.89	0.72	0.34	0.74	-	0.68	0.44	1.24	0.29
5-Pregnene-3β,17α,20α-triol	0.29	0.37	0.60	0.82	0.29	-	0.88	0.54	0.94	0.28
Disulphates[b]:										
5-Androstene-3β,17α-diol	-	-	-	-	-	0.58	0.54	0.28	0.88	0.21
5-Androstene-3β,17β-diol	-	-	-	-	-	0.26	0.22	0.29	0.46	0.20
5-Androstene-3β,16β,17α-triol	-	-	-	-	-	0.08	0.08	0.11	0.06	0.06
5-Pregnene-3β,20α-diol	-	-	-	-	-	0.20	0.17	0.25	0.19	0.23
5-Androstene-3β,16α,17β-triol	-	-	-	-	-	0.16	0.18	0.20	0.04	0.17
5-Pregnene-3β,17α,20α-triol	-	-	-	-	-	0.27	0.06	0.15	0.48	0.20

[a] Not determined in subject 6.
[b] Not determined in subjects 1–5.

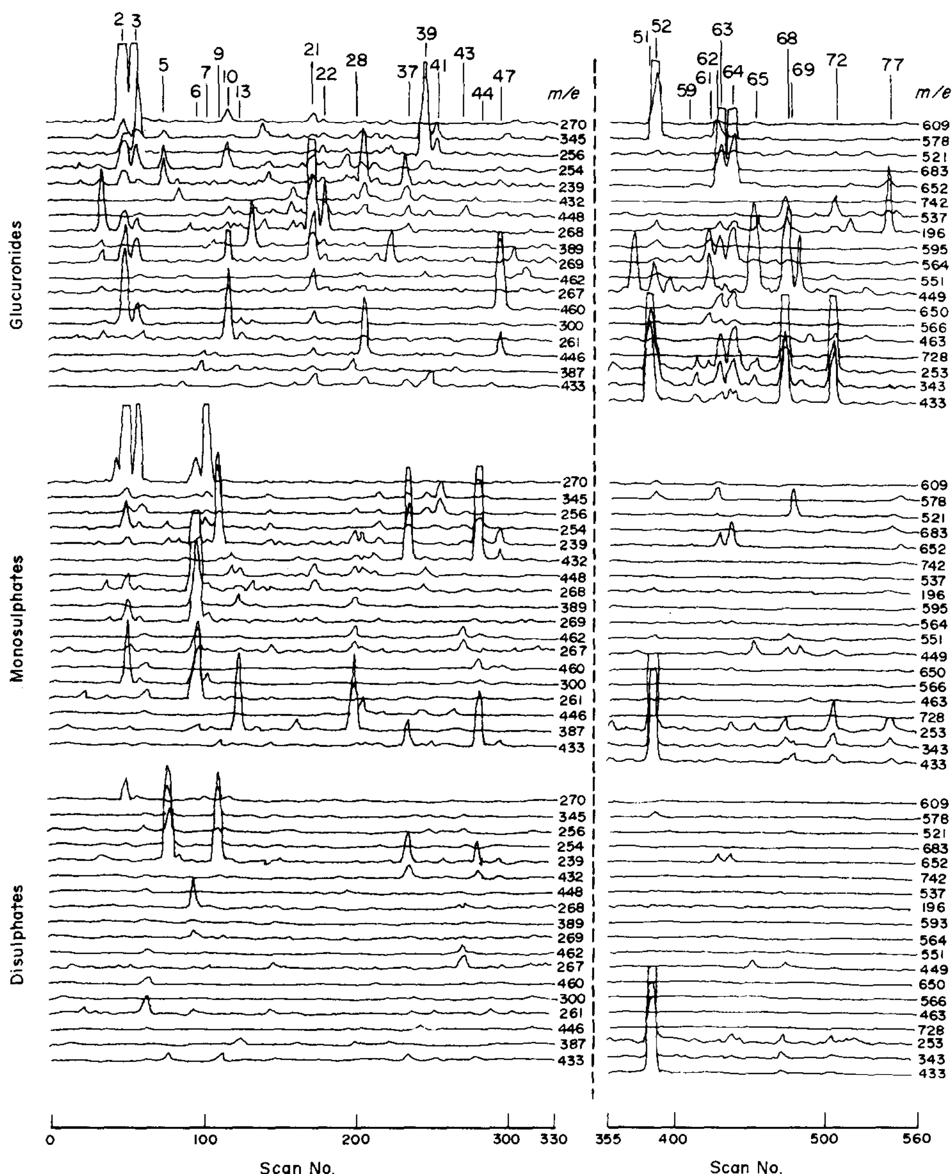

Fig. 6. Representative GC-MS analyses comparing the steroid profiles in different conjugate fractions isolated from urine of a healthy man. Steroid MO-TMS derivatives from aliquots of the glucuronide, monosulphate and disulphate fractions equivalent to 30, 40 and 80 μl of urine, respectively, were analyzed on a 25 m open-tubular glass capillary column coated with OV-1. The column was kept at 235°C for 23 min following injection of the sample. Repetitive magnetic scanning, 10 spectra/min, was then started, and the column temperature was programmed at 0.6°C/min to 275°C. Two series of fragment ion current chromatograms of *m/e* values characteristic of TMS and MO-TMS derivatives of common steroids having retention times of shorter than and longer than 1.5 that of 5α-cholestane are illustrated. Details of the structures indicated by *m/e* values can be obtained from Tables 1 and 2, and the principal steroids indicated by the numbers are listed in Table 4.

this fraction were isomers of 5-androstene-3,17-diol and 5-androstene-3,16,17-triol, 5-pregnene-3α,20α-diol and 5-pregnene-3α,17α,20α-triol (Fig. 6, Table 6).

Evaluation of the method

The precision of the method was determined by analysing 5 aliquots of a urine sample. The principal steroids in each conjugate fraction were measured. The individual levels, mean, standard deviation and coefficient of variations are given in Table 7.

DISCUSSION

The major pathway for the elimination of steroid hormones and their metabolites in man is excretion in the urine. Although a large number of steroids have

Table 7. Reproducibility of the method as determined by analysis of 5 aliquots of the same urine sample

Steroid	1	2	3	4	5	Excretion mg/day mean ± SD[a]	CV[a] %
Glucuronide conjugates:							
3α-Hydroxy-5α-androstan-17-one	2.29	2.44	2.28	2.29	2.33	2.33 ± 0.07	3
3α-Hydroxy-5β-androstan-17-one	1.76	1.96	1.87	1.98	1.91	1.90 ± 0.09	5
3α,11β-Dihydroxy-5α-androstan-17-one	0.55	0.63	0.49	0.57	0.66	0.58 ± 0.07	12
3α,11β-Dihydroxy-5β-androstan-17-one	0.31	0.35	0.28	0.32	0.33	0.32 ± 0.03	8
5β-Pregnane-3α,20α-diol	0.44	0.52	0.43	0.48	0.55	0.48 ± 0.05	11
5β-Pregnane-3α,17α,20α-triol	0.70	0.79	0.71	0.64	0.81	0.73 ± 0.07	10
3α,17α,21-Trihydroxy-5β-pregnane-11,20-dione	2.09	2.40	1.98	2.40	2.36	2.25 ± 0.20	9
3α,11β,17α,21-Tetrahydroxy-5β-pregnan-20-one	0.66	0.75	0.57	0.78	0.87	0.73 ± 0.12	16
3α,11β,17α,21-Tetrahydroxy-5α-pregnan-20-one	0.59	0.66	0.51	0.59	0.59	0.59 ± 0.05	9
3α,17α,20α,21-Tetrahydroxy-5β-pregnan-11-one	0.56	0.72	0.54	0.67	0.66	0.63 ± 0.08	12
3α,17α,20β,21-Tetrahydroxy-5β-pregnan-11-one + 5β-Pregnane-3α,11β,17α,20β,21-pentol	0.95	0.97	0.73	0.91	0.80	0.87 ± 0.10	12
5β-Pregnane-3α,11β,17α,20α,21-pentol	0.19	0.23	0.16	0.19	0.18	0.19 ± 0.03	13
Monosulphate conjugates:							
3α-Hydroxy-5α-androstan-17-one	0.31	0.26	0.23	0.29	0.28	0.27 ± 0.03	11
3α-Hydroxy-5β-androstan-17-one	0.37	0.39	0.36	0.41	0.37	0.38 ± 0.02	5
3β-Hydroxy-5-androsten-17-one	1.26	1.33	1.31	1.05	1.28	1.25 ± 0.11	9
3β-Hydroxy-5α-androstan-17-one	0.34	0.30	0.32	0.28	0.32	0.31 ± 0.02	7
5-Androstene-3β,17β-diol	0.32	0.28	0.35	0.31	0.31	0.31 ± 0.03	8
3β,16α-Dihydroxy-5-androsten-17-one	0.83	1.05	1.08	0.80	0.98	0.95 ± 0.13	13
5-Androstene-3β,16α,17β-triol	0.38	0.50	0.49	0.33	0.48	0.44 ± 0.08	18
5-Pregnene-3β,17α,20α-triol	0.49	0.57	0.56	0.50	0.56	0.54 ± 0.04	7
Disulphate conjugates:							
5-Androstene-3β,17α-diol	0.10	0.08	0.11	0.12	0.09	0.10 ± 0.02	16
5-Androstene-3β,17β-diol	0.21	0.18	0.20	0.29	0.24	0.22 ± 0.04	19
5-Pregnene-3β,20α-diol	0.39	0.25	0.42	0.45	0.49	0.40 ± 0.09	23
5-Androstene-3β,16α,17β-triol	0.28	0.24	0.22	0.19	0.23	0.23 ± 0.03	14
5-Pregnene-3β,17α,20α-triol	0.17	0.11	0.14	0.10	0.11	0.13 ± 0.03	23

[a] SD = standard deviation; CV = coefficient of variation.

been identified in human urine, and the nature of conjugation has been determined for several of the individual steroids, relatively few studies have been made where profiles of steroid metabolites in different conjugate classes have been analysed. This has in part been due to the lack of practical methods for quantitative group separation of steroid conjugates in urine.

The application of ion exchange chromatography to the separation of steroids is not new. Anion exchange resins have been used for the separation of steroid conjugates [29] but disadvantages with these resins are the adsorptive losses of material encountered and the large volumes of eluting solvents required. The former effect was largely overcome by the use of an anion exchanger based on Sephadex gels, DEAE-Sephadex, and its application to the separation of estrogen [30–32] and aldosterone [33] conjugates has been reported. DEAE-Sephadex, however, is too polar for use with organic solvents and in addition to the large column size and eluting volumes of aqueous solvents it would appear unsuitable for routine application to the separation of steriods in biological material.

The availability of a less polar lipophilic derivative of Sephadex, Sephadex LH-20 and more recently Lipidex 5000 (a hydrophobic hydroxyalkyl derivative) enabled steroid separations to be achieved in organic solvents by partition chromatography in both straight phase and reversed phase solvent systems [14, 15, 19, 34–37]. Due to the presence of the reactive hydroxyl group in the Sephadex matrix it is possible to attach ion exchanging groups to both Sephadex LH-20 and

Lipidex. The synthesis of these ion exchange gels and their potential in the separation of phospholipids and lipid soluble acids has recently been outlined [10, 38, 39]. Using these ion exchangers, a method was developed for the separation and analysis of conjugates of neutral steroids. A similar method for the separation of phenolic steroids is being developed and will be reported separately. Due to the high loading capacity of these gels, small column sizes (0.6 g) could be used, and highly reproducible, rapid separations with good recoveries could be obtained.

Prior to anion exchange chromatography it was found necessary to use a cation exchanging step. If this step was omitted, part of the acidic steroids appeared in the neutral fraction, possibly due to formation of ion pairs with organic cations present in the extracts of urine. Previously, a strong cation exchange resin, Amberlyst A-15, has been used to achieve a homogenous counter-ion composition prior to chromatography of steroid sulphates on Sephadex LH-20 [40]. Since losses of steroids due to adsorption has from time to time been experienced with this resin, cation exchanging derivatives were synthesised from Sephadex LH-20. The sulphoethyl derivative yielded satisfactory results while the carboxymethyl derivative proved to be too weak a cation exchanger. Due to the low ion exchange capacity of the sulphoethyl derivative, large column sizes were required and consequently this stage of the method has proved the most time-consuming. Work is at present in progress to synthesize this gel with a higher ion exchange capacity.

Although the chromatographic separation of steroids into their conjugate groups using DEAP-LH-20 is readily achieved in less than 4 h, the complete method described appears somewhat time-consuming and is presented principally as a research technique. The method, however, could readily be applied more routinely in situations where steroids of one conjugate class only require analysis. In its present form the method has only been tested with monoglucuronides, monosulphates and disulphates, and further studies are needed to establish the chromatographic behaviour of other conjugates, e.g. diglucuronides [41] or conjugates containing one glucuronic acid and one sulphate residue. If these or other conjugates are of particular interest it should be possible to modify the eluting buffers to achieve the necessary separations. In order to minimize losses, liquid–liquid partitioning steps have been eliminated as a means of extracting steroids, and since the gels are lipophilic, losses due to adsorption are not seen. The aqueous eluent from the Amberlite XAD-2 extraction is the only material discarded; even the solvolysis washings which contain considerable residual water soluble material are re-extracted using XAD-2. Finally, the gels can be washed, re-charged and used repeatedly.

Qualitative and semiquantitative analysis of the steroids identified in the conjugate groups was achieved using g.l.c. on open-tubular glass capillary columns and computerised GC–MS. Recent progress in the preparation of this type of column [1, 3, 5–7, 23, 42–45] has led to the demonstration of their potential in the multicomponent analysis of steroids from biological material [2, 4, 7, 8]. In fact, the mixture of steroids in urine is so complex that a satisfactory analysis cannot be performed using packed columns. An approximate estimation of the amounts of steroids present in the samples was obtained using flame ionisation detection after addition of two internal standards: 5α-androstane-3α,17α-diol, with a short retention time, and stigmasterol with a long retention time. Of internal standards previously employed, 5β-cholane-3α,24-diol was unsuitable because the TMS ethers of this compound and 5β-pregnane-3α,11β,17α,20α,21-pentol had the same retention times, whereas cholesteryl butyrate gave variable responses when the same amounts were repeatedly injected, indicating degradation or adsorption of this ester during solid injection or on the capillary column. In spite of the high resolving power of the capillary columns, quantification of several steroids cannot be achieved using a non-specific detector and work is in progress to use the mass spectrometer for an automated specific quantification [12]. However, a major difficulty in this procedure as well as in quantification using the flame ionization detector is the lack of pure reference steroids for establishment of accurate response factors.

When the same urine sample was analysed repeatedly and the major steroids in each group quantified in the manner described, the coefficients of variation were 3–23%. It was observed that the precision was less satisfactory for steroids in the disulphate fraction, possibly reflecting greater difficulties in quantitative hydrolysis of these conjugates.

Since GC–MS at the moment offers the highest degree of specificity in steroid analysis, this technique was chosen as the means of characterising the steroids isolated. Repetitive magnetic scanning over the entire mass range, 0–800 a.m.u. was carried out and due to the large number of scans recorded (usually 600–800) computerised evaluation of the data was necessary. This type of GC–MS analysis which is less frequently used in steroid research is much less sensitive than monitoring of a few pre-selected ions, but is ideal for the multicomponent analysis of complex mixtures.

In order to detect the majority of steroids, fragment ion current chromatograms were plotted by the computer using *m/e* values characteristic of substituted androstane and pregnane derivatives. In addition *m/e* values typical of specific structural features were chosen. Since the urine samples were from male subjects, ions given by estrogenic steroids were excluded from the search. By analyzing the samples in this way a relatively unbiased approach was achieved and the sensitivity of detection was of the order of 5 ng of

injected steroid. The potential of this method of analysis is apparent from the extensive list of steroids identified and from the general qualitative and quantitative agreement with previously reported data on urinary excretion of steroids in adults.

As expected, the major proportion of steroids were excreted as glucuronide conjugates. The distribution was generally related to structure. In the monosulphate and disulphate fractions the steroids generally possessed a 3β-hydroxy-5-ene structure while saturated steroids were predominantly excreted as glucuronides. The excretion of 3β-hydroxy-5α-androstan-17-one exclusively as a sulphate is a notable exception. In general these observations are consistent with previously reported data [46, 47]. The failure to detect steroids in the neutral fraction from urine of healthy men requires further study, especially since this fraction was subjected to enzyme hydrolysis in order to detect the possible occurrence of steroids conjugated with neutral sugar derivatives [48]. In contrast, the major part of the steroids present in urine from baboons have been found in this fraction [49], and large amounts of polar corticosteroid metabolites were also present in the neutral fraction from the patient with Cushing's syndrome. Another striking feature in the steroid pattern of this patient was the presence of large amounts of corticosteroid metabolites in the monosulphate fraction. The urine samples from healthy men yielded only trace amounts of corticosteroids in the sulphate fractions, a finding which corroborates the previously reported existence of sulphated corticosteroid metabolites in urine from humans [50].

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