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ANALYSIS OF PROFILES OF CONJUGATED STEROIDS IN URINE BY ION-EXCHANGE SEPARATION AND GAS CHROMATOGRAPHY—MASS SPECTROMETRY

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SUMMARY

A simplified, flexible method for the analysis of metabolic profiles of steroids in urine is described. Solid extraction with Amberlite XAD-2 or Sep-Pak C_{18} cartridges is followed by group fractionation of unconjugated neutral and phenolic steroids, monoglucuronides, monosulphates and disulphates on the lipophilic strong anion exchanger triethylaminohydroxypropyl Sephadex LH-20 (TEAP-LH-20). Following brief enzymatic hydrolysis or solvolysis the steroids are purified on TEAP-LH-20. O-Methyloxime and trimethylsilyl ether derivatives are prepared and purified by filtration through Lipidex 5000, and are then analyzed by glass capillary column gas—liquid chromatography and gas chromatography—mass spectrometry.

Between 2 and 5 ml of urine are used for a comprehensive analysis. Unconjugated neutral and phenolic steroids are isolated in half a day, corresponding steroids in the conjugate fractions in two days. No fraction containing steroids is discarded, but the analysis can be limited to a selected fraction.

INTRODUCTION

Many methods have been described for analysis of metabolic profiles of steroids in urine [1—8]. In most cases, hydrolysis of conjugated steroids is the first step in the analysis. Since the state of conjugation depends on the structure and metabolic origin of the steroid metabolites, important information about metabolic pathways may be lost by the use of such procedures. A method based on ion-exchange separation of groups of conjugates prior to hydrolysis and analysis by gas—liquid chromatography (GLC) and gas chromatography—mass spectrometry (GC—MS) has been developed in this laboratory [9]. However, this method is time-consuming and less suitable for routine analyses. Various modifications have therefore been tried in order

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to simplify and increase the speed of all steps in this procedure. This paper describes a method which is the result of these studies. The steroid conjugates are separated on a strong anion exchanger which yields cleaner fractions. Less time-consuming methods for extractions, hydrolysis and solvolysis are employed. The method has now been in use for about two years.

EXPERIMENTAL

Chemicals and glassware

All solvents were of reagent grade and were redistilled in all-glass apparatus twice before use. Glassware was cleaned in an ultrasonic bath. Hexamethyldisilazane and trimethylchlorosilane (Applied Science Labs., State College, PA, U.S.A.) were redistilled and trimethylsilylimidazole (Supelco, Bellefonte, PA, U.S.A.) was used as supplied. Methoxyamine hydrochloride (Eastman Organic Chemicals, Rochester, NY, U.S.A.) was recrystallized from methanol. Tetrahydrofuran was refluxed and distilled over lithium aluminium hydride. It could be stored for one week. Triethylamine sulphate was prepared as a 0.5 *M* solution in water, pH 7.2.

Helix pomatia digestive juice was from Pharmindustrie (Clichy, France). Immediately before use, 0.3 ml were added to 5 ml of 0.2 *M* sodium acetate buffer, pH 4.5, and the solution was passed through a 80 × 4 mm column of Amberlite XAD-2 at a rate of 0.4 ml min^{-1} [10].

Amberlite XAD-2 (Rohm and Haas, Philadelphia, PA, U.S.A.) was extensively washed with acid, base, water and solvents and was then stored in ethanol [9]. Sep-Pak C_{18} cartridges (Waters Assoc., Milford, MA, U.S.A.) were used as supplied. Lipidex 5000 (Packard Instrument, Downers Grove, IL, U.S.A.) was washed with 20% and 50% aqueous ethanol and ethanol at 70°C. It was stored in methanol at 4°C. SP-Sephadex C-25 (Pharmacia Fine Chemicals, Uppsala, Sweden) was converted into the sodium form, washed in the same way as Lipidex 5000 and stored in ethanol at 4°C. Prior to use it was converted into the H^+ form with 0.5 *M* hydrochloric acid, washed with water until neutral, and suspended in 72% methanol. SP-Sephadex was stable in this form at 4°C for a month. Triethylaminohydroxypropyl Sephadex LH-20 (TEAP-LH-20) was prepared as follows [11]. Sephadex LH-20, 50 g, was converted into a chlorohydroxypropyl (23%, w/w) derivative [12]. After washing with 85% aqueous ethanol, ethanol, chloroform and ethanol, the derivative was dried at room temperature and used for the synthesis of TEAP-LH-20 essentially as described for TEAPHA-LH-20 [13]. The gel, about 65 g, was allowed to swell at room temperature in 270 ml 50% aqueous methanol under continuous stirring for 30 min. Sodium hydroxide (6.5 g) in 500 ml of 50% aqueous methanol was then added, followed by 500 ml of triethylamine (50 ml min^{-1}). The temperature was increased to 55°C and the mixture was stirred for 3.5 h. The product was washed on a Buchner funnel with 1 l of the following series of solutions: 50% aqueous ethanol, 0.3 *M* sodium hydroxide in 72% ethanol, water (until neutral) and 72% ethanol. The ion-exchanging capacity was 0.8—1.0 mequiv. g^{-1}. The gel was converted into the Cl^- form with 0.3 *M* hydrochloric acid in 72% ethanol, washed until neutral with water and extracted at 70°C as described for Lipidex. It was then converted into

the acetate form via the OH^- form and stored dry at —20°C. Immediately before use the appropriate amount was washed with 0.3 *M* sodium hydroxide in 72% methanol, water and 72% methanol.

Chromatography columns were 200 × 4 mm with a reservoir of 10 ml. PTFE gauze, 70 μm, and a PTFE tube with stopcock were inserted at the bottom end. Sep-Pak C_{18} cartridges were connected to the glass columns via the PTFE tube. When necessary, appropriate flow-rates were obtained by application of nitrogen (carbon dioxide, see below) pressure.

Steroids

Unlabelled steroids were those used in previous studies [9]. Radioactively labelled steroids were from the Radiochemical Centre (Amersham, Great Britain) and NEN Chemicals (Dreieich, G.F.R.). [6,7-^{3}H]Estrone sulphate and [6,9-^{3}H]estriol-16-glucuronide were kindly donated by Prof. H. Adlercreutz, Helsinki, Finland. The disulphate of 5α-[1,2-^{3}H]androstane-3β,17β-diol was a gift from Dr. U. Rudqvist at this department.

Analytical procedure

A flow scheme of the method is shown in Fig. 1.

Extraction. Urine, 2—5 ml, is diluted with 1—3 ml of water and passed at a flow-rate 0.4 ml min^{-1} through a column of XAD-2 (80 × 4 mm, packed in ethanol and washed with 15 ml of water). The column is washed with

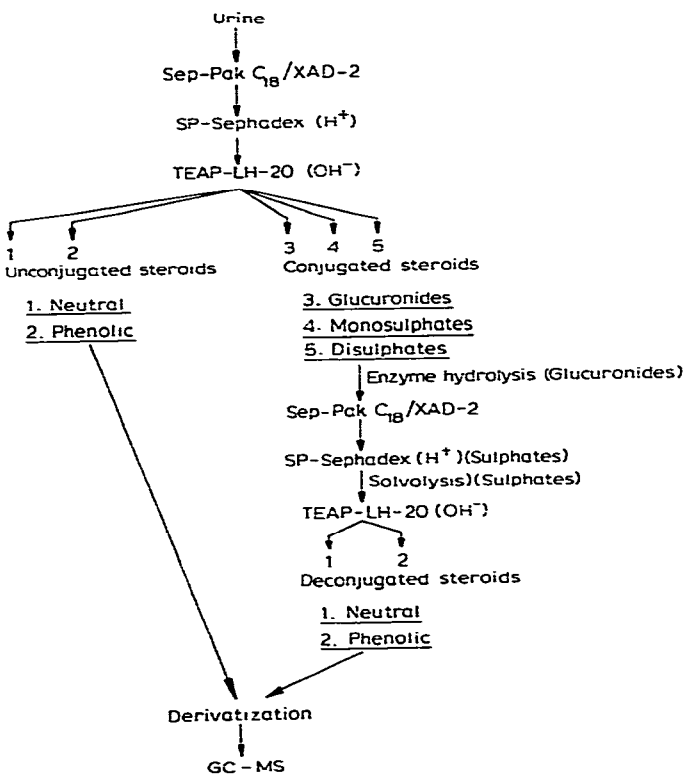

Fig. 1. General flow scheme for analysis of steroids in urine.

3 ml of water, 2 ml of 0.5 *M* triethylamine sulphate and 3 ml of water. Steroids are eluted with 8 ml of methanol.

Following the description of an extraction method based on Sep-Pak C_{18} cartridges [14] this method has been used as a faster alternative to the XAD-2 procedure. The urine is directly filtered through the cartridge (washed with 5 ml each of methanol and water prior to use) followed by 5 ml of water (flow-rate 1 ml min^{-1}). Steroids are eluted with 8 ml of methanol.

Cation exchange. Water, 3 ml, is added to the eluate from the extraction step, and the solution is filtered (flow-rate 0.7 ml min^{-1}) through SP-Sephadex in H^+ form (40 × 4 mm, packed in 72% methanol) followed by a rinse with 3 ml of 72% methanol.

Anion exchange, group separation. The entire effluent from SP-Sephadex is passed through TEAP-LH-20 in OH^- form (40 × 4 mm, packed in 72% methanol, flow-rate 0.5 ml min^{-1}). The effluent is collected together with a 4-ml rinse with 72% methanol. Neutral (uncharged) steroids are present in this fraction. Unconjugated phenolic steroids are then eluted with 8 ml of 72% methanol saturated with carbon dioxide, the flow-rate being maintained by a pressure of carbon dioxide. Monoglucuronides are then eluted with 10 ml of 0.4 *M* formic acid, monosulphates with 10 ml of 0.3 *M* acetic acid—potassium acetate, apparent pH 6.5, and disulphates with 15 ml of potassium acetate—potassium hydroxide, 0.5 *M* in acetate with an apparent pH of 10.0, all solutions being made with 72% methanol.

Glucuronide fraction. The fraction is taken to dryness in vacuo and the residue is dissolved in about 5 ml of the purified enzyme solution. Following incubation for 1 h at 62°C [15] the reaction mixture is extracted with Sep-Pak C_{18} or Amberlite XAD-2 as above, the triethylamine sulphate wash being omitted in the XAD-2 extraction. The methanol eluate is directly passed through a column of TEAP-LH-20 in OH^- form (40 × 4 mm, packed in methanol). The effluent and a rinse with 4 ml of methanol contain the liberated neutral steroids. Phenolic steroids are eluted with 8 ml of methanol saturated with carbon dioxide as above. The fractions are stored in methanol at 4°C until analyzed.

Sulphate fractions. The fractions are concentrated in vacuo to about 2 ml (monosulphates) and 3 ml (disulphates). Water, 5 ml, is added and pH is adjusted to 4.5 with concentrated hydrochloric acid (about 20 µl to the mono- and 200 µl to the disulphate fractions, respectively). If desired, enzymatic hydrolysis may be performed at this stage. However, in the present method, the fractions are extracted with Sep-Pak C_{18} or XAD-2 as described for urine, an additional wash of XAD-2 with 6 ml water being added to remove triethylamine sulphate more completely. Water, 1 ml, is added to the methanol eluate and the solution is filtered and washed through SP-Sephadex as described for the extract of urine. The combined effluent is concentrated in vacuo at 25°C to about 100 µl. Distilled tetrahydrofuran, 5 ml, acidified with 5 µl of 4 *M* aqueous sulphuric acid is added [16]. Following incubation for 1 h at 50°C, 1 ml of methanol is added and the solution is passed (0.5 ml min^{-1}) through TEAP-LH-20 in OH^- form, the column (80 × 4 mm) being packed in methanol and washed with 3 ml of tetrahydrofuran—methanol (5:1, v/v), prior to application of the sample. The column is washed with 6

ml of the latter solvent. The liberated neutral steroids are recovered in the combined effluent. Phenolic steroids are eluted with the same solvent saturated with carbon dioxide (under carbon dioxide pressure). Solvents are removed in vacuo and the fractions are stored in methanol at 4°C until analyzed.

GLC and GC—MS analyses. One microgram of 7-ketocholesterol is added as internal standard to all neutral steroid fractions. O-Methyloxime-trimethylsilyl ether derivatives are then prepared [17] and purified as described [13]. Estrogens are converted into trimethylsilyl ethers after addition of a suitable amount of 5β-cholestan-3α-ol as internal standard [13].

GLC was carried out using a Pye 104 gas chromatograph equipped with a 25 m × 0.3 mm open tubular glass capillary column coated with SE-30 [2]. Nitrogen was used as the carrier gas at an inlet pressure of 50 kPa, giving a flow-rate of about 1 ml min^{-1}. The oven temperature was programmed from 230°C to 265°C at a rate of 1°Ç min^{-1}.

GC—MS was carried out using a modified LKB 9000 and a glass capillary column (SE-30) connected via a single stage jet separator [13]. Column temperature was programmed from 225°C to 270°C at a rate of 1.2°C min^{-1}. Conditions for repetitive scanning mass spectrometry and computer evaluation were as described previously [18]. The identification of a steroid was based upon the retention time, the complete mass spectrum and partial mass spectra obtained from fragment ion current chromatograms constructed for characteristic ions given by the steroid derivatives. Details have been given in ref. 9.

Relative amounts of steroids were estimated by comparing the GLC peak areas given by the steroids with that of the internal standard. Since a true quantification was not the aim of this part of the study, a mass response factor of 1.0 was used.

RESULTS

The group fractionation of steroids on TEAP-LH-20 was tested with radioactively labelled steroids and steroid conjugates and with extracts of urine from two men given [^{3}H]cortisol and [^{3}H]pregnenolone, respectively. Fig. 2 shows the separation of synthetic steroids added to extracts of urine. A complete and wide separation was achieved of unconjugated neutral and phenolic steroids, glucuronides, monosulphates and disulphates. Table I shows the distribution of radioactivity between different fractions in the separation of metabolites in urine from the two men given the labelled steroids. As expected, cortisol metabolites appeared mainly in the glucuronide fraction while pregnenolone metabolites were excreted mostly as monosulphates. The same pattern was previously obtained using DEAP-LH-20 [9].

The glucuronide fraction was hydrolyzed with Helix pomatia intestinal juice at 62°C for 1 h. This rapid procedure was compared with the method employed in a previous study [9] (48 h, 37°C), using urine from the subject given [^{3}H]cortisol. No significant differences in recovery of radioactive metabolites or GLC profiles were noted. About 85—90% of the radioactivity was recovered as neutral steroids. Less than 5% appeared in the glucuronide fraction. In spite of the good recoveries of [^{3}H]cortisol metabolites, the

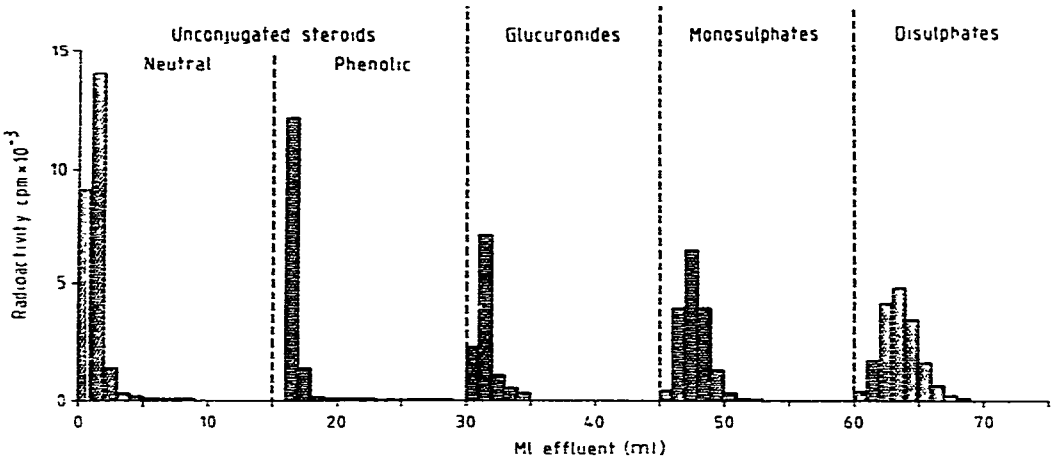

Fig. 2. Separation on a column (40 × 4 mm) of TEAP-LH-20 in OH^- form of radioactive steroids added to an extract of urine. Unconjugated neutral steroids (progesterone and cortisol) were eluted with 72% aqueous methanol, and estrogens (estrone, estradiol and estriol) with the same solvent, saturated with carbon dioxide. Glucuronides (testosterone glucuronide and estriol-16-glucuronide), monosulphates (3β-hydroxy-5-androsten-17-one sulphate) and disulphates (5α-androstane-3β,17β-diol disulphate) were then stepwise eluted with solvents described in Experimental.

TABLE I

PERCENTAGE DISTRIBUTION OF RADIOACTIVE METABOLITES IN URINE FROM TWO MEN GIVEN [^{3}H]CORTISOL AND [^{3}H]PREGNENOLONE, RESPECTIVELY
Six aliquots of each urine sample were extracted and fractionated on TEAP-LH-20.

Steroid fraction	[^{3}H]Cortisol metabolites (% ± S.D.)	[^{3}H]Pregnenolone metabolites (% ± S.D.)
Neutral	10 ± 3	12 ± 2
Glucuronide	86 ± 9	36 ± 2
Monosulphate	4 ± 1	44 ± 3
Disulphate	<1	8 ± 4

precision in the GLC analyses of ring A-reduced metabolites of cortisol and cortisone was poorer than for other steroids (see below).

The sulphate fractions were subjected to rapid solvolysis in tetrahydrofuran—sulphuric acid since enzyme hydrolysis liberated only about 40% of the sulphated metabolites of [^{3}H]pregnenolone. The presence of sulphate ions derived from triethylamine sulphate may contribute to the poor yield.

Fig. 3. GC—MS analyses of steroids in different conjugate fractions isolated from urine of a healthy woman during follicular phase. Fragment ion current chromatograms constructed by the computer are shown for ions characteristic of trimethylsilyl and O-methyloxime-trimethylsilyl derivatives of metabolites of androgens (*m/z* 270—248), progestins (*m/z* 269—253) and corticosteroids (*m/z* 253—431). The principal steroids indicated by the numbers are listed in Table III. A 25-m glass capillary column coated with SE-30 was used, and the oven temperature was programmed at 1.2°C min^{-1} from 225°C to 270°C.

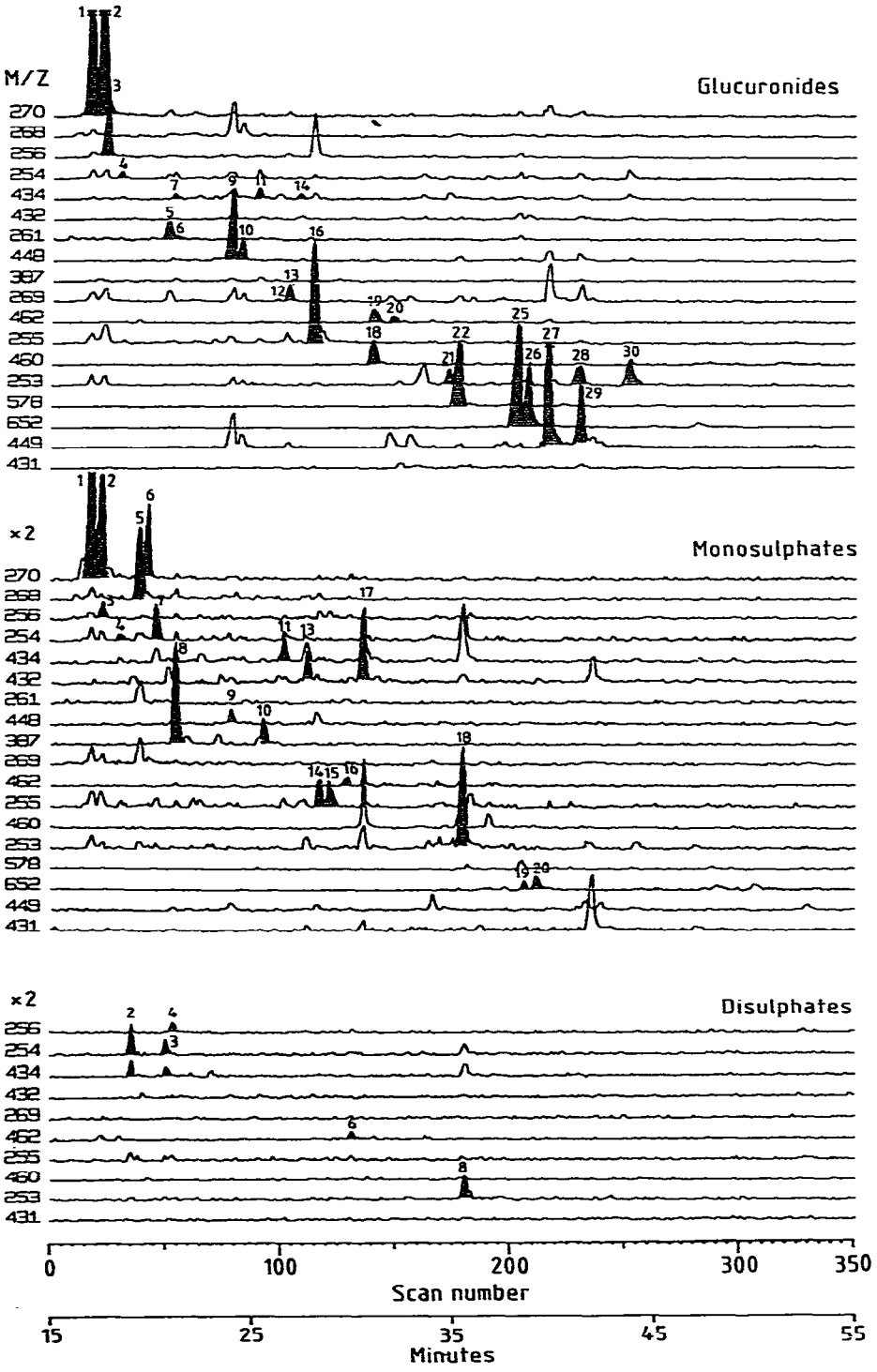
M/Z
Glucuronides
270
268
256
254
434
432
261
448
387
269
462
255
460
253
578
652
449
431
×2
Monosulphates
×2
Disulphates
0
100
200
300
350
Scan number
15
25
35
45
55
Minutes

TABLE II

RECOVERIES THROUGH THE METHOD OF RADIOACTIVELY LABELLED STEROIDS ADDED TO URINE (2—5 ml) AND OF LABELLED STEROID METABOLITES IN URINE AFTER ORAL ADMINISTRATION OF [^{3}H]CORTISOL OR [^{3}H]PREGNENOLONE

Steroid conjugate(s)	Amount added (cpm)	Recovery from columns (%)					
		Extraction and group separation	*n*	Hydrolysis/ solvolysis and purification	*n*	Total*	*n*
[^{3}H]Testosterone glucuronide	7500	94 ± 5	6	79 ± 5	5	76 ± 5	9
[^{3}H]Estriol 16-glucuronide	8000	92 ± 7	2	91 ± 1	2	84 ± 7	2
[^{14}C]3β-Hydroxy-5-androsten-17-one sulphate	8200	100 ± 2	10	94 ± 4	12	94 ± 4	19
[^{3}H]Estrone sulphate	16000	98 ± 3	3	78	1	73 ± 4	3
[^{3}H]Androstane-3β,17β-diol disulphate	7600	79 ± 4	8	88 ± 12	8	73 ± 6	8
[^{3}H]Cortisol metabolites		92 ± 8	6	86 ± 4**	3		
[^{3}H]Pregnenolone metabolites		97 ± 5	6	81 ± 3***	4		

*Filtration of methyloxime-trimethylsilyl ether derivatives through Lipidex 5000 not included
**Glucuronide fraction.
***Monosulphate fraction.

Solvolysis liberated about 80% of these metabolites. Similar results were obtained with ethyl acetate—sulphuric acid (39°C, 16 h), used in a previous study [9].

The recoveries of radiolabelled steroids added to urine or obtained as metabolites of cortisol and pregnenolone are shown in Table II. In most cases the recoveries were better than 90% after extraction on Amberlite XAD-2, filtration through SP-Sephadex and group separation. The 5α-androstane-3β,17β-diol disulphate constituted an exception (see Discussion). Hydrolysis and solvolysis resulted in losses of 6—21% and the recoveries throughout the method varied between 73 and 94%.

The precision of the method was determined by triplicate analyses of urine samples from the follicular (day 4) and luteal (day 23) phases of a healthy woman. The principal steroids in each fraction were identified by GC—MS (Fig. 3) and were quantified from the GLC analyses (Fig. 4). The results are summarized in Table III which also gives the coefficients of variation. It should be pointed out that a mass response factor of 1 was used and that the values in Table III are not true absolute excretion rates.

The method was used to study the changes of the steroid profile during a 24-h period in a healthy woman (day 19 of the cycle). The results are summarized in Table IV. The diurnal variation in the excretion of 11-oxygenated adrenal steroid metabolites is clearly seen. However, if 24-h portions of urine are difficult to obtain, a representative metabolic profile of steroids may be obtained from a single sample, frozen immediately and preferably collected in the morning. Incomplete collection or bacterial conversions during a 24-h

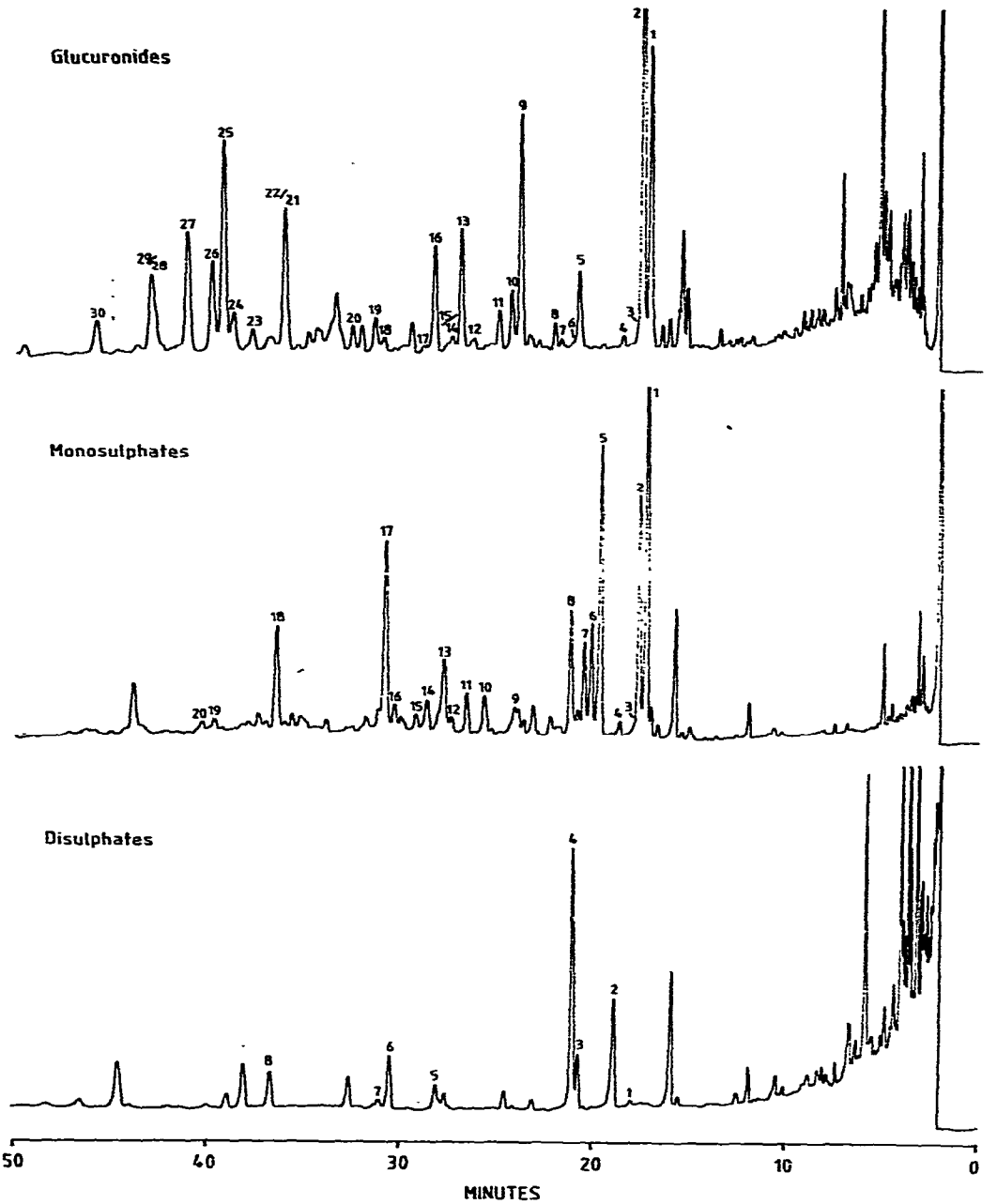

Fig. 4. GLC analyses of steroid profiles in different conjugate fractions isolated from urine of a healthy woman during follicular phase. Aliquots equivalent to 7 μl and 20 μl of urine were injected from the glucuronide and sulphate fractions, respectively. The principal steroids indicated by the numbers are listed in Table III. A 25-m glass capillary column coated with SE-30 was used, and the oven temperature programmed at 1°C min^{-1} from 230°C to 265°C.

TABLE III

DAILY EXCRETION OF STEROID CONJUGATES IN URINE FROM A 32-YEAR OLD WOMAN DURING FOLLICULAR AND LUTEAL PHASES

The samples were analyzed in triplicate.

No.	t_R*	RI**	Steroid conjugates***	Excretion (mg per 24 h)§			
				Cycle day 4		Cycle day 23	
				Mean	C.V.§§ (%)	Mean	C.V. (%)
			Glucuronides				
1	0.50	2593	5α-A-3α-ol-17-one	2.47	2	3.10	2
2	0.51	2600	5β-A-3α-ol-17-one	4.06	6	3.41	1
3	0.51	2602	5β-A-3α,17β-ol				
4	0.55	2630	A^5-3β,17α-ol				
5	0.64	2687	5α-A-3α-ol-11,17-one	0.85	12	0.72	2
6	0.65	2693	5β-A-3α-ol-11,17-one				
7	0.65	2693	A-triol				
8	0.68	2703	A-triol				
9	0.76	2753	5α-A-3α,11β-ol-17-one	2.48	12	2.55	2
10	0.78	2760	5β-A-3α,11β-ol-17-one	0.80	17	0.81	10
11	0.80	2770	A-triol				
12	0.90	2815	5α-P-3α,20α-ol				
13	0.91	2821	5β-P-3α,20α-ol	1.39	11	5.25	3
14	0.92	2825	A-triol				
15	0.92	2825	A^{Δ}-triol				
16	0.98	2850	5β-P-3α,17α,20α-ol	1.12	5	1.30	3
17	0.99	2853	5α-P-3α,17α,20α-ol				
18	1.12	2900	P^5-3α,16α,20α-triol				
19	1.14	2908	P-3,16,20-ol				
20	1.21	2930	P-3,16,20-ol				
21	1.42	2990	P^5-3β,17α,20α-ol	1.70	25	1.30	10
22	1.43	2993	5β-P-3α,17α,21-ol-11,20-one				
23	1.52	3018	5β-P-3α,11β,21-ol-20-one	0.23	9	0.24	4
24	1.60	3038	5α-P-3α,11β,21-ol-20-one	0.42	7	0.56	7
25	1.63	3047	5β-P-3α,11β,17α,21-ol-20-one	2.50	25	2.00	25
26	1.66	3053	5α-P-3α,11β,17α,21-ol-20-one	1.10	25	0.90	25
27	1.76	3078	5β-P-3α,17α,20α,21-ol-11-one	1.80	3	1.75	1
28	1.88	3103	5β-P-3α,11β,17α,20β,21-ol	1.22	7	1.26	3
29	1.89	3105	5β-P-3α,17α,20β,21-ol-11-one				
30	2.09	3145	5β-P-3α,11β,17α,20α,21-ol	0.61	7	0.71	1
			Monosulphates				
1	0.50	2593	5α-A-3α-ol-17-one	1.39	1	1.05	4
2	0.51	2600	5β-A-3α-ol-17-one	0.87	5	0.70	6
3	0.51	2602	5β-A-3α,17β-ol				
4	0.55	2630	A^5-3β,17α-ol				
5	0.59	2650	A^5-3β-ol-17-one	0.91	13	0.36	3
6	0.61	2670	5α-A-3β-ol-17-one	0.33	20	0.13	9
7	0.62	2675	A^5-3β,17β-ol	0.34	3	0.16	6
8	0.64	2687	A^5-3β,7α-ol-17-one				
9	0.75	2748	5α-A-3α,11β-ol-17-one				
10	0.83	2788	A^{Δ}-diolone				
11	0.87	2803	A-triol				
12	0.89	2812	5β-P-3α,20α-ol				
13	0.93	2830	A^5-3β,16β,17α-ol	0.34	6	0.30	7

TABLE III (continued)

No.	t_R*	RI**	Steroid conjugates***	Excretion (mg per 24 h)§			
				Cycle day 4		Cycle day 23	
				Mean	C.V.§§(%)	Mean	C.V. (%)
14	0.98	2850	5β-P-3α,17α,20α-ol	0.12	14	0.10	7
15	1.01	2860	5α-P-3α,17α,20α-ol				
16	1.06	2880	P^5-3β,20α-ol	0.14	11	0.17	20
17	1.09	2890	A^5-3β,16α,17β-ol	0.82	6	1.01	1
18	1.42	2990	P^5-3β,17α,20α-ol	0.32	20	0.18	15
19	1.63	3047	5β-P-3α,11β,17α,21-ol-20-one				
20	1.66	3053	5α-P-3α,11β,17α,21-ol-20-one				
			Disulphates				
1	0.51	2602	5β-A-3α,17β-ol				
2	0.55	2630	A^5-3β,17α-ol	0.31	12	<0.1	
3	0.59	2675	A^5-3β,17β-ol	0.21	15	<0.1	
4	0.63	2680	5α-A-3β,17β-ol	0.91	13	1.20	12
5	0.93	2830	A^5-3β,16β,17α-ol				
6	1.06	2880	P^5-3β,20α-ol	0.15	16	<0.1	
7	1.09	2890	5α-P-3β,20α-ol				
8	1.42	2990	P^5-3β,17α,20α-ol	0.14	5	<0.1	

*Retention time of the trimethylsilyl ether and methyloxime-trimethylsilyl ether derivative relative to that of 5α-cholestane on a glass capillary column, SE-30, 250°C.
**Retention index [19].
***A and P = androstane and pregnane, superscript indicates position of double bond, greek letters denote configuration of hydroxyl groups.
§Values calculated using a mass response factor of 1.00. Absence of a value indicates difficulties in quantitation by GLC due to small amounts or presence of contaminants (see Fig. 3).
§§C.V. = coefficient of variation.

period can probably result in larger variations of the steroid profiles than seen in Table IV.

DISCUSSION

The method for isolation of steroids from urine follows the same general principles as described in previous publications from this laboratory [9, 13, 20]. Thus, a solid extraction method is used followed by purification and group separation on lipophilic ion exchangers. Three column materials are used in this process and have been selected in order to increase simplicity and speed of analysis. In the present form the method permits extraction, separation, hydrolysis and purification of each group of steroids in two days. Unconjugated neutral or phenolic steroids are isolated in a few hours.

Extractions are performed at three stages: as the initial step, for desalting prior to solvolysis, and after enzymatic hydrolysis. Losses of steroid sulphates in extraction with Amberlite XAD-2 have been reported by several workers [21—23]. In our experience, recoveries of steroid sulphates from urine are usually high, whereas large losses occur when the conjugates are extracted

TABLE IV

DAILY EXCRETION OF STEROID CONJUGATES IN URINE FROM A 39-YEAR OLD WOMAN DURING THE LUTEAL PHASE (DAY 19)

Steroid conjugates*	Excretion (mg/24 h)						Mean ± S.D.**	C.V. (%)**	24-h collection
	Calculated from values for urine collected between								
	7.15—9.35	9.35—13.00	13.00—18.35	18.35—22.10	22.10—2.00	2.00—7.15			
Glucuronides									
5α-A-3α-ol-17-one	5.48	4.85	4.26	4.66	4.34	4.07	4.61 ± 0.51	11	4.58
5β-A-3α-ol-17-one	7.17	5.96	5.15	5.26	5.35	5.91	5.80 ± 0.75	13	5.80
5α-A-3α-ol-11,17-one	1.71	1.65	1.55	1.57	1.35	1.13	1.49 ± 0.22	15	1.48
5α-A-3α,11β-ol-17-one	6.38	4.96	3.76	3.03	2.05	3.44	3.94 ± 1.53	39	3.81
5β-A-3α,11β-ol-17-one	1.68	1.54	1.57	1.55	1.24	1.07	1.44 ± 0.23	16	1.44
5β-P-3α,20α-ol	9.13	10.66	8.49	8.31	7.55	7.18	8.55 ± 1.24	15	8.55
5β-P-3α,17α,20α-ol	3.28	3.50	3.31	3.06	2.92	2.52	3.10 ± 0.35	11	3.12
P^5-3β,17α,20α-ol 5β-P-3α,17α,21-ol-11,20-one	1.28	0.77	0.38	1.57	0.59	1.82	1.07 ± 0.57	53	1.09
5β-P-3α,11β,21-ol-20-one	0.43	0.25	0.18	0.40	0.16	0.44	0.31 ± 0.13	42	0.31
5α-P-3α,11β,21-ol-20-one	1.20	0.87	0.55	0.64	0.31	1.02	0.77 ± 0.33	43	0.76
5β-P-3α,11β,17α,21-ol-20-one	3.21	2.28	1.04	2.09	1.18	2.91	2.12 ± 0.88	42	2.08
5α-P-3α,11β,17α,21-ol-20-one	1.57	1.43	0.88	1.57	0.91	1.39	1.29 ± 0.32	25	1.28
5β-P-3α,17α,20α,21-ol-11-one	3.01	3.25	2.28	1.82	1.30	1.83	2.25 ± 0.75	33	2.20
5β-P-3α,11β,17α,20β,21-ol 5β-P-3α,17α,20β,21-ol-11-one	1.77	2.37	1.66	1.62	0.88	1.11	1.57 ± 0.53	34	1.54
5β-P-3α,11β,17α,20α,21-ol	0.93	1.09	0.78	0.76	0.44	0.61	0.77 ± 0.23	30	0.76

Monosulphates									
5α-A-3α-ol-17-one	2.31	1.74	1.93	2.18	1.37	1.53	1.84 ± 0.37	20	1.83
5β-A-3α-ol-17-one	1.70	1.10	1.57	1.83	0.90	1.09	1.37 ± 0.38	28	1.37
A^5-3β-ol-17-one	1.23	1.57	0.88	0.62	0.57	0.78	0.94 ± 0.39	41	0.92
5α-A-3β-ol-17-one	0.27	0.46	0.25	0.29	0.31	0.37	0.33 ± 0.08	24	0.31
A^5-3β,17β-ol	0.14	0.28	0.17	0.17	0.18	0.23	0.20 ± 0.05	25	0.19
A^5-3β,16β,17α-ol	0.64	0.71	0.74	0.48	0.41	0.38	0.56 ± 0.16	29	0.56
A^5-3β,16α,17β-ol	0.96	0.78	1.21	0.80	1.00	0.86	0.94 ± 0.16	17	0.97
P^5-3β,17α,20α-ol	0.09	0.21	0.12	—	—	0.13	0.09	—	0.10
Disulphates									
5β-A-3α,17β-ol	0.09	0.15	0.09	0.17	—	0.13	0.11	—	0.11
A^5-3β,17α-ol	0.07	0.27	0.06	0.30	0.10	0.44	0.21 ± 0.15	71	0.23
5α-A-3β,17β-ol	1.49	1.56	1.18	1.18	1.40	1.37	1.47 ± 0.21	14	1.46
Creatinin (mmol per 24 h)	11.28	12.41	21.10	12.19	11.23	11.11	11.72 ± 0.57	5	11.96

*For abbreviations, see Table III.
**S.D. = standard deviation; C.V. = coefficient of variation.

at low ionic strength. The problem is circumvented if the column is washed with triethylamine sulphate in water prior to elution of steroids with methanol [21]. Triethylamine has been assumed to form ion pairs with the steroid conjugates, thus facilitating elution [21]. However, we have found that the strong adsorption of steroid sulphates is due to ion exchange, Amberlite XAD-2 acting as a weak anion exchanger [24]. The need for a wash with triethylamine sulphate increases the time of extraction, and small amounts of the compound will contaminate the steroid extract. This has an adverse effect on enzyme hydrolysis and solvolysis (see below). Recent studies indicate that sodium sulphate can replace triethylamine sulphate thereby simplifying the procedure [24].

When the present work was in the final stages, extraction of steroids with Sep-Pak C_{18} cartridges was described [14]. Since this method is superior with respect to speed, simplicity and recovery, it has largely replaced the Amberlite XAD-2 extraction steps.

Ion-exchange chromatography

The extract of urine is filtered through SP-Sephadex in the H^+ form. Ion exchange occurs in aqueous methanol and the high capacity permits use of a small column bed. This step may not be necessary for quantitative sorption of steroid conjugates on TEAP-LH-20, as is the case with the weak anion exchanger DEAP-LH-20 (or Lipidex-DEAP). However, it removes substances which affect derivatization and GLC of phenolic steroids. Furthermore, solvolysis does not occur if sulphate fractions extracted with Amberlite XAD-2 are not passed through SP-Sephadex. This is due to the presence of triethylamine sulphate acting as buffer. SP-Sephadex removes triethylamine (provided that water is added to the methanol eluate) and generates sulphuric acid. To avoid decomposition of sulphated steroids, the eluate must not be taken to complete dryness. Filtration through SP-Sephadex may not be needed prior to solvolysis when triethylamine sulphate is omitted, e.g. after extractions with Sep-Pak C_{18}.

The base form of a strong anion exchanger, TEAP-LH-20, is used instead of the acetate form of the weaker DEAP-LH-20 previously employed for group separation of steroid conjugates [9]. The purity of the neutral steroid fraction is higher since weak organic acids are more efficiently sorbed. Furthermore, phenolic steroids may be separately isolated [13]. Glucuronides of neutral steroids and A-ring glucuronides of phenolic steroids can be eluted prior to estrogen glucuronides with a free phenolic hydroxyl group. A similar separation was previously obtained using DEAP-LH-20 [25], and further subfractionation of glucuronides of aromatic steroids has been achieved using DEAE-Sephadex in methanol [26]. However, these methods are more time-consuming. A disadvantage with the present method is that the basic nature of the ion exchanger may affect recoveries of alkali-labile steroids.

TEAP-LH-20 permits ion exchange in a variety of solvents. Thus, the solvolysis mixture can be filtered through the ion exchanger after addition of methanol. This permits direct isolation of neutral and phenolic steroids in two separate fractions simultaneously with the removal of sulphuric acid and acids liberated by the solvolysis. It should be noted that the separations

on TEAP-LH-20 are not achieved in tetrahydrofuran or tetrahydrofuran—water alone.

Hydrolysis of steroid conjugates

In most methods, hydrolysis and solvolysis constitute rate-limiting steps and result in uncontrolled losses of steroids. We have attempted to use as rapid procedures as possible but it is evident that selective losses of steroids may occur, e.g. due to unknown enzyme selectivities and differential solubilities in the solvolysis mixture. Comparison with commonly used slower procedures showed no differences. Some factors affecting reproducibility may be pointed out. The enzyme solution must be purified to remove substances which interfere in the GLC analysis. The method used does not decrease enzyme activity [10]. In the solvolysis, it is important that tetrahydrofuran is freshly distilled and that the flasks are well stoppered. Finally, if mixed conjugates, diglucuronides or unknown types of conjugates are present they will probably escape detection unless additional means of hydrolysis are used. It is an advantage of the method that such compounds can be recovered from the second chromatography on TEAP-LH-20.

Quantitative aspects

The general pattern of steroids obtained with the present method is essentially as expected from previous studies [1—9, 27, 28]. The finding of 5α-androstane-3β,17β-diol as the major steroid in the disulphate fraction seems to be new. This steroid conjugate appears to be particularly sensitive to solvolytic conditions, and is readily lost upon evaporation of acidic solutions. There are other types of steroids which are not quantitatively recovered with the present procedure. Catechol estrogens are partly destroyed on TEAP-LH-20 [29, 30] and the conditions used for formation of O-methyloximes are not suitable for metabolites of aldosterone and 18-hydroxylated corticosteroids [31, 32]. Thus, individual steps in the general method may have to be modified when labile steroids are of particular interest. Depending on the problem to be studied it may also be more suitable to select other types of derivatives for the GLC and GC—MS analyses. In the present study O-methyloxime and trimethylsilyl ether derivatives were selected since they are the most generally applicable ones. Irrespective of the type of derivative selected it is necessary to determine a mass response factor for each steroid to be quantitated. The accuracy of the analyses will then depend mainly on the purity of the fractions isolated, and on the resolving power, dynamic range and sensitivity of the GLC or GC—MS systems.

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