The Indirect Analysis of 17-Oxosteroid Glucosiduronates and Sulphates in Human Urine

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1. An analytical procedure has been developed for the determination of urinary 17-oxosteroid sulphates and 11-deoxy-17-oxosteroid glucosiduronates. 2. By submitting urine to the following operations: (a) extraction of the 17-oxosteroid conjugates into chloroform (in the presence of pyridinium sulphate), (b) solvolysis (in the absence of acid) of the steroid sulphates, (c) separation of the steroid glucosiduronates, (d) hydrolysis of the glucosiduronates, (e) oxidation with *tert.*-butyl chromate of the liberated steroids and (f) analysis of the final extracts by gas-liquid chromatography, the 17-oxosteroid conjugates are converted into and measured as androstanediones and androstane-(ene)triones. 3. Values for the daily excretion of 17-oxosteroid glucosiduronates and sulphates in normal men and women are given.

Direct and indirect evidence has conclusively shown that the principal constituents of the C₁₉, 17-oxygenated steroids fraction of human urine, androsterone, aetiocholanolone, their 11-oxygenated derivatives and dehydroepiandrosterone are excreted conjugated with either sulphuric acid or glucopyranosiduronic (glucosiduronic) acid (Lieberman, Mond & Smyles, 1954; Kellie & Wade, 1957; Schneider & Lewbart, 1959; Foggitt & Kellie, 1964).

During the past few years, work by the groups of Baulieu, Diczfalussy and Lieberman (Baulieu, 1965; Diczfalussy, 1965; Gurrpide, Mann, Sandberg & Lieberman, 1965; and references there cited) has changed the concept that steroid sulphates and glucosiduronates are only the end-products of the metabolic transformations of the steroid hormones and it has shown that some conjugates are, in fact, the preferred substrates in certain intermediate enzymic reactions involved in steroid biosynthesis. In the light of these findings, it would appear that separate determination of these two classes of conjugates may contribute to a fuller understanding of steroid metabolism as a result of the study of urinary excretion.

McKenna & Norymberski (1960) showed that in the presence of pyridinium ion, steroid sulphates can be quantitatively extracted from aqueous solutions into chloroform, in the form of pyridinium salts. Application of this procedure to the extraction of urinary 17-oxosteroid sulphates (McKenna & Norymberski, 1960; McKenna, Menini & Norymberski, 1961) afforded extracts almost free

from the accompanying pigments. 17-Oxosteroid conjugates other than the sulphates can also be extracted into chloroform in high yields by increasing the concentration of pyridinium sulphate in the aqueous phase (McKenna & Rippon, 1965).

Menini & Norymberski (1962a) proposed a scheme for the systematic analysis of urinary steroids. The application of this approach to the analysis of urine has been recently reported (Menini & Norymberski, 1965). In this scheme after an initial reduction of the steroids with potassium tetrahydroborate (borohydride) followed by the cleavage of the glycols with sodium metaperiodate (Few, 1961), the compounds derived from the glucosiduronates of the C₁₉, 17-oxygenated steroids (and pregnanediol) are separated from the other urinary steroids and they are oxidized with *tert.*-butyl chromate to steroidal polyketones. The sequence of reactions and operations of the procedure converts the glucosiduronates of androsterone, aetiocholanolone and their 11-oxygenated derivatives into 5 α -androstane-3,17-dione, 5 β -androstane-3,17-dione, 5 α -androstane-3,11,17-trione and 5 β -androstane-3,11,17-trione respectively, which are resolved and measured by gas-liquid chromatography in a non-selective phase (SE-30).

Application of the same analytical scheme to extracts containing the urinary steroid sulphates resulted in large destruction of dehydroepiandrosterone, one of the major constituents of this fraction.

The present work deals with a procedure evolved from the following analytical principles: (1)

extraction of the urinary 17-oxosteroid conjugates into chloroform in the presence of pyridinium ion; (2) solvolysis of the steroid sulphates and separation of the steroid glucosiduronates; (3) enzymic hydrolysis of the glucosiduronates; (4) oxidation of the liberated steroids with *tert.*-butyl chromate; (5) gas-liquid chromatography of the formed androstanediones and androstanetriones. The method aims to supplement the results provided by the systematic analysis of urinary steroids with a similar type of information on the 17-oxosteroid sulphate fraction. Concurrently, the glucosiduronates of androsterone aetiocholanolone and dehydroepiandrosterone can be also estimated.

MATERIALS AND METHODS

Reagents and solvents. The aqueous solution of pyridinium sulphate was prepared according to McKenna & Rippon (1965). Other reagents and solvents were prepared or purified as described by Menini & Norymberski (1962b; 1965).

Paper chromatography. Systems A [light petroleum-methanol-water (5:4:1, by vol.)], B₃ [light petroleum-benzene-methanol-water (67:33:80:20, by vol.) (Bush, 1952)] and E₄ [(2,2,4-trimethylpentane-2-methylpropan-2-ol-methanol-water (20:9:9:2, by vol.) (Eberlein & Bongiovanni, 1955)] were used on Whatman no. 2 paper, by the descending technique, at 22°. Oxosteroids were detected by the Zimmermann test and steroidal 4-ene-3,6-diones by the alkali test. Experimental details were reported previously (Menini & Norymberski, 1962b).

Reference compounds. Sodium aetiocholanolone sulphate, sodium androsterone sulphate, sodium dehydroepiandrosterone sulphate, aetiocholanolone glucosiduronic acid and androsterone glucosiduronic acid were obtained from the M.R.C. Reference Steroid Collection (Prof. W. Klyne), dehydroepiandrosterone glucosiduronic acid and epiandrosterone glucosiduronic acid were a gift of Dr A. E. Kellie to Mrs P. A. Sadler, androst-4-ene-3,6,17-trione, pregn-4-ene-3,6,20-trione and cholest-4-ene-3,6-dione were prepared by the method of Menini & Norymberski (1962b), pregnanediol glucosiduronic acid was purchased from Sigma Co. Ltd. (London), 5 α -androstane-3,17-dione, 5 β -androstane-3,17-dione, 5 α -androstane-3,11,17-trione, 5 β -androstane-3,11,17-trione, 5 α -pregnane-3,20-dione and 5 β -pregnane-3,20-dione were commercial products obtained from Zori Pharmaceutical Ltd. (Tel Aviv, Israel). Other steroids were purchased from Koch-Light Laboratories (Colnbrook, Bucks.). Solutions of the steroid conjugates in ethanol were stored at 4° and were used before any spontaneous hydrolysis could be detected by paper chromatography (system A).

Gas-liquid chromatography. The instrument used was a Panchromatograph (Pye Scientific Instruments, Cambridge) equipped with an argon ionization detector with a ⁹⁰Sr radioactive source. The injection port and the upper part of the chromatographic columns were modified to allow the introduction of solid analytical samples deposited on Dixon gauze rings as described by Menini & Norymberski (1965). In some cases the transfer of the analytical samples in ethylene dichloride solutions (0.2-0.3 ml.) to the Dixon

rings was speeded by placing the polytetrafluoroethylene plate under an infrared lamp.

Analytical columns were glass tubes, 3.5 mm. i.d. They were treated with dimethyldichlorosilane in toluene and were packed with acid-washed, silanized Gas-Chrom P (mesh 100-120) or Gas-Chrom Q (mesh 80-100) coated with 0.8-1% of stationary phase, SE-30 or QF-1. Experimental details were as described by Brooks & Hananeh (1963). Columns containing SE-30 were operated at 190° and those containing QF-1 at 210°. The flow of argon was 30 ml./min. and the voltage applied to the detector was 1000 v.

Quantitative analyses of the final urinary extracts were performed in 150 cm.-long, W-shaped columns containing SE-30 as stationary phase; these columns had efficiencies of about 3000 theoretical plates for 3 α , 20 α -diacetoxy-5 β -pregnane. Purity of the unconjugated reference compounds and further characterization (and in some cases quantitative estimation, see the Results section) of some of the end products in the final urinary extracts were carried out in 40 cm.-long, straight columns containing QF-1 as stationary phase. These columns had efficiencies of 500-600 theoretical plates for androst-4-ene-3,6,17-trione.

Collection of urine. Twenty-four-hour specimens were collected from normal subjects and were stored at 4°. Usually urines were analysed within 2 weeks of collection.

Determination of total 17-oxosteroids. A modification of the method of Dreker *et al.* (1952) as described by Norymberski, Stubbs & West (1953) was used for the estimation of total 17-oxosteroids. Zimmermann chromogens were measured according to the method of Corker, Norymberski & Thow (1962).

Standard analytical procedure

Extraction of unconjugated steroids. Unconjugated steroids were removed from urine by two extractions with an equal volume of chloroform.

Extraction of 17-oxosteroid conjugates. Urine freed from unconjugated steroids (or aqueous solutions of 17-oxosteroid conjugates; 20 ml.) was diluted with 10 ml. of an aqueous solution of pyridinium sulphate (3M) and extracted with chloroform (30 ml.). Emulsions, encountered very rarely, were dispersed by centrifugation. The top aqueous layer was removed by suction and a portion of the chloroform (25 ml.), free from water, was transferred to a test tube and evaporated to dryness in a water bath at 60-70°. Towards the end of the evaporation traces of pyridine were displaced from the test tube by a slow stream of nitrogen.

Solvolysis of the 17-oxosteroid sulphates and separation of the 17-oxosteroid glucosiduronates from the solvolysed material. Solvolysis of the 17-oxosteroid sulphates was carried out according to McKenna & Rippon (1965). The dry residue containing the 17-oxosteroid conjugates was dissolved in dioxan (2 ml.), kept at 40° for 4 hr. and then left overnight at room temperature. Chloroform (10 ml.) was then added and the organic phase extracted successively with 2 ml. portions of water, 2N-NaOH and water. The combined aqueous extracts contained the 17-oxosteroid glucosiduronates. The dioxan-chloroform mixture contained the 17-oxosteroids liberated from the 17-oxosteroid sulphates. A portion of the organic phase (10 ml.) was concentrated to about 1 ml. on a water bath at 60-70°

and the remaining dioxan was evaporated off in an evacuated desiccator (2-3 hr.).

Enzymic hydrolysis of the 17-oxosteroid glucosiduronates. A portion (5 ml.) of the alkaline, aqueous solution containing the 17-oxosteroid glucosiduronates was diluted with water (5 ml.) and the pH adjusted to 5.0 by addition of aqueous 50% acetic acid. β -Glucuronidase (Ketodase, Warner-Chilcott Laboratories, New York, U.S.A.) was added to give a concentration of 1500 Fishman units/ml. of final solution. After 48 hr. at 38° the mixture was extracted with two 10 ml. portions of chloroform.

Emulsions, when formed, were dispersed by centrifugation. The chloroform extract was washed with 2N-NaOH (4 ml.) and water (4 ml.) and was then evaporated to dryness on a water bath at 60-70°.

Oxidation with tert.-butyl chromate. This was performed as described by Menini & Norymberski (1965). The dry residue containing the enzyme-liberated 17-oxosteroids from the glucosiduronate fraction was dissolved in 0.25 ml. of pyridine and treated with 0.25 ml. of tert.-butyl chromate reagent overnight at room temperature. The reaction mixture was diluted with a freshly prepared saturated ethanolic solution of citric acid (0.5 ml.) and shaken for 15 min. It was then diluted with N-H₂SO₄ (2.5 ml.) and extracted with ethylene dichloride (5 ml.). The top aqueous layer was removed by suction and the extract washed successively with water (1 ml.), aqueous 10% (w/v) solution of sodium metabisulphite (2 ml.) and water (1 ml.). The dry residue containing the steroids liberated by solvolysis was dissolved in 0.15 ml. of pyridine and treated with 0.25 ml. of tert.-butyl chromate reagent (see the Results section). Subsequent operations were as described above.

Portions of the extracts containing the products of the oxidation were analysed for Zimmermann chromogens and suitable fractions were admixed with an internal standard and submitted to gas-liquid chromatography.

RESULTS

Transfer of pure steroid conjugates from aqueous solutions of pyridinium sulphate into chloroform. Table 1 shows the effect of the concentration of

pyridinium sulphate on the transfer of several 17-oxosteroid conjugates and pregnanediol glucosiduronate from aqueous solutions into chloroform. Paper chromatography (system A) of part of the chloroform containing the extracted conjugates did not show any hydrolysis of the glucosiduronates during extraction.

Transfer of steroid conjugates from urine into chloroform in the presence of pyridinium sulphate. Three portions from each of two urine samples (urines 1 and 10, from a normal male and a normal female respectively) were made 0.5, 1.0 and 1.5 M in pyridinium sulphate and processed in duplicate. Aetiocholanolone glucosiduronate was added to the duplicate portions of the first urine (4.1 μ g./ml.) to evaluate the effect of the concentration of pyridinium sulphate on the recovery of this conjugate. The samples were submitted to the standard procedure of analysis (see the Materials and Methods section). A portion of the aqueous alkaline phase containing the glucosiduronates extracted from the dioxan-chloroform mixture after solvolysis was hydrolysed with hydrochloric acid (final normality 1.2; 20 min. at 100°) and the liberated 17-oxosteroids were assayed by the Zimmermann reaction. Aetiocholanolone was recovered in yields of 90, 87 and 94% from the samples initially made 0.5, 1.0 and 1.5 M in pyridinium sulphate respectively, indicating that recovery of the added material was independent of the concentration of the salt over the range tested. Recoveries of aetiocholanolone glucosiduronate, calculated from the increase of the gas-liquid chromatographic peak corresponding to 5 β -androstene-3,17-dione in the glucosiduronate end-fractions, were 87, 84 and 90% in the order of increasing salt concentration.

The results of the analysis of the samples carried

Table 1. *Effect of the concentration of pyridinium sulphate on the transfer of some 17-oxosteroid conjugates and pregnanediol glucosiduronate from aqueous solutions into chloroform*

The ratio of volumes was 1. Results are given as percentage of the conjugate found in the chloroform, determined by the Zimmermann reaction. Number of experiments (in duplicate) is indicated in parenthesis.

Concn. of pyridinium sulphate.....	Percentage of conjugate transferred to chloroform		
	0.5 M	1.0 M	1.5 M
Sodium dehydroepiandrosterone sulphate	96 (3)		
Sodium androsterone sulphate	98 (4)		
Aetiocholanolone sulphate	89 (3)		
Dehydroepiandrosterone glucosiduronic acid	70 (4)	88 (3)	88 (3)
Androsterone glucosiduronic acid	73 (3)	87 (2)	84 (2)
Aetiocholanolone glucosiduronic acid	72 (3)	94 (2)	84 (2)
Epiandrosterone glucosiduronic acid	77 (3)	92 (2)	
Pregnanediol glucosiduronic acid*	54 (2)	65 (4)	67 (2)

* Determined by the sulphuric acid reaction (Klopper, Michie & Brown, 1955).

through the entire procedure are included in Tables 8 and 9 (urines 1a, 1b, 1c, 10a, 10b and 10c). As expected, an increase in the concentration of pyridinium sulphate in the urine had no effect on the values obtained for the constituents of the sulphate fraction. Examination of the glucosiduronate fraction shows that although the salt concentration did not influence the values of the 11-deoxy-17-oxosteroids, there was an increase (especially marked in urine 1) in the values of the 11-oxygenated, 17-oxosteroids with increasing concentration of the pyridinium sulphate.

These results are easily explained considering that the glucosiduronates of the 11-oxygenated, 17-oxosteroids are the most polar of the compounds under investigation and consequently the most difficult to transfer into a relatively non-polar solvent such as chloroform. An indirect evaluation of the extent to which these more polar conjugates are transferred into chloroform from urine containing pyridinium sulphate was carried out by submitting to the procedure for the systematic analysis of steroids (Menini & Norymberski, 1965) samples from five urines previously analysed by the present method. Table 2 shows the comparison of the results given by the two procedures for the derivatives of the 17-oxosteroid glucosiduronates and pregnanediol glucosiduronate (which is estimated simultaneously). Whilst the two methods gave values for the 11-deoxy compounds which agreed reasonably well (with one exception), the procedure for the systematic analysis furnished values for the 11-oxygenated compounds and pregnanediol significantly higher than those given by the present method. Since even at the highest concentration of pyridinium sulphate (1.5M, in urine 1), the amounts of the 11-oxygenated com-

pounds and pregnanediol are not more than 55% of the amounts found by the other method, no attempt was made to modify the procedure to allow the determination of the more polar glucosiduronates.

Solvolysis of steroid sulphates and separation and hydrolysis of steroid glucosiduronates. In agreement with previous work (Cohen & Oneson, 1953, McKenna & Norymberski, 1957) it has been found that pyridinium salts of the hydrogen sulphates of dehydroepiandrosterone, androsterone and aetiocholanolone obtained by chloroform extraction of aqueous solutions of their sodium salts in the presence of pyridinium sulphate were completely solvolysed by treatment with dioxan (Table 3). Cohen & Oneson (1953) found that the glucosiduronates of pregnanediol and oestriol were completely stable to dioxan treatment and McKenna & Rippon (1965) could not detect any hydrolysis of dehydroepiandrosterone glucosiduronate and dehydroepiandrosterone phosphate upon incubation of these compounds with dioxan. In a series of experiments the glucosiduronates of androsterone, dehydroepiandrosterone and epiandrosterone were transferred from aqueous 1M-pyridinium sulphate into chloroform. After evaporation of the chloroform the residues were treated with dioxan under the conditions used for the solvolysis of the sulphates (see Materials and Methods). The dioxan was diluted with chloroform and the mixture extracted with 2ml. portions of water, 2N-sodium hydroxide and water. The combined aqueous extracts were hydrolysed with mineral acid and the liberated 17-oxosteroids assayed by the Zimmermann reaction. Table 3 shows that androsterone, dehydroepiandrosterone and epiandrosterone were recovered in high yields

Table 2. *Comparison of the values for the derivatives of the urinary 17-oxosteroid glucosiduronates and pregnanediol glucosiduronate obtained by the procedure for the systematic analysis of steroids and the present procedure*

Experimental details are given in the text. Results are expressed as mg./24 hr. Method A: present procedure; method B: systematic analysis of steroids (Menini & Norymberski, 1965).

Urine.....	1*		3		4		5		14	
Method	A B		A B		A B		A B		A B	
Compound†	A	B	A	B	A	B	A	B	A	B
5β-A-3,17	2.24	2.64	1.22	0.93	0.92	0.96	1.30	1.13	2.36	2.05
5α-A-3,17	3.16	3.42	1.98	1.84	2.16	1.40	3.35	2.87	4.06	3.70
5β-A-3,11,17	0.48	1.12	0.36	0.94	0.10	0.58	0.09	0.66	0.30	0.75
5α-A-3,11,17	0.52	0.92	0.24	0.64	0.13	0.60	0.15	0.38	0.40	1.04
5β-P-3,20	0.59	1.71	0.36	0.57	0.16	0.57	0.23	0.43	0.64	1.95

* Urine no. 1 was made 1.5M in pyridinium sulphate, before extraction of the conjugates into chloroform.

† Compounds are abbreviated as follows: 5β-androstane-3,17-dione, 5β-A-3,17; 5α-androstane-3,17-dione, 5α-A-3,17; 5β-androstane-3,11,17-trione, 5β-A-3,11,17; 5α-androstane-3,11,17-trione, 5α-A-3,11,17; 5β-pregnane-3,20-dione, 5β-P-3,20.

Table 3. *Solvolysis of some 17-oxosteroid sulphates and recovery of some 17-oxosteroid glucosiduronates from the dioxan-chloroform solvent mixture*

Experimental details are given in the text. A portion (10–25 μ g./ml. of dioxan) of each compound was used for each experiment. Results are expressed as percentage of the conjugate found in the appropriate phase, determined by the Zimmermann reaction. Number of experiments (in duplicate) is indicated in parenthesis.

	Percentage recovery of liberated steroids	
	From dioxan-chloroform (1:5; v/v) after solvolysis	From aqueous extract after acid hydrolysis
Sodium dehydroepiandrosterone sulphate	97 (5)	
Sodium androsterone sulphate	91 (3)	
Sodium aetiocholanolone sulphate	92 (3)	
Dehydroepiandrosterone glucosiduronic acid	0 (4)	85 (4)
Androsterone glucosiduronic acid	0 (3)	78 (3)
Epiandrosterone glucosiduronic acid	0 (2)	96 (2)

Table 4. *Oxidation of dehydroepiandrosterone with tert.-butyl chromate in the presence of pyridine*

The *tert.*-butyl chromate reagent and the pyridine were in the ratio 5:3 (v/v). Other experimental details are given in the text.

Dehydro-epiandrosterone oxidized (μ g.)	Percentage conversion into androst-4-ene-3,6,17-trione	
	By Zimmermann reaction	By gas-liquid chromatography
11.4	98	94
28.5	78	82
57.0	76	71
85.5	80	81
114.0	78	73
	Mean: 82	80

from the alkaline, aqueous extracts. In all cases the dioxan-chloroform mixture after extraction of the glucosiduronates was evaporated to dryness and assayed by means of the Zimmermann reaction. Values indistinguishable from the reagent blanks were consistently obtained.

Oxidation of fractions containing steroids liberated by β -glucuronidase or solvolysis. The products of the glucuronidase hydrolysis were oxidized with *tert.*-butyl chromate reagent (see Materials and Methods). Under these conditions dehydroepiandrosterone, usually one of the major components of the fraction containing the urinary steroid sulphates, is oxidized to androst-4-ene-3,6,17-trione in a yield of 70% (Menini & Norymberski, 1962b). In an attempt to increase the yield of this reaction, dehydroepiandrosterone was oxidized with *tert.*-butyl chromate in the presence of various amounts of pyridine. Maximum yield of androst-4-ene-

3,6,17-trione (81%) was obtained by oxidizing solutions of dehydroepiandrosterone in pyridine with *tert.*-butyl chromate reagent in the ratio 3:5 (v/v). Subsequent preparation of the reaction mixture was as described in the Materials and Methods section. Table 4 shows the results of the oxidation of different quantities of dehydroepiandrosterone in 0.06 ml. of pyridine with 0.1 ml. of the *tert.*-butyl chromate reagent. Androst-4-ene-3,6,17-trione was measured in the end fractions by the Zimmermann reaction and by gas-liquid chromatography on a column containing SE-30. Portions of the end fractions were also analysed by gas-liquid chromatography on a 40 cm.-long column containing QF-1 and by paper chromatography. In both cases gas-liquid chromatography showed only one peak with the same retention time as authentic androst-4-ene-3,6,17-trione. Paper chromatography in the system B₃ and development of the chromatogram by the Zimmermann test and by the alkali test, for oxosteroids and for 6-oxygenated 4-ene-3-oxo compounds respectively, revealed the presence of a compound with the mobility of authentic androst-4-ene-3,6,17-trione and a more polar, minor compound, probably its enol form (Menini & Norymberski, 1962b).

Gas-liquid chromatography. Gas-liquid chromatography was performed according to the methods of Horning, VandenHeuvel & Creech (1963). Analytical samples were introduced into the columns in the solid state by the technique of Menini & Norymberski (1965). Quantitative evaluation of the gas-chromatograms was carried out by measuring the peak heights of the compounds, having the expected retention times, relative to the height of the peak corresponding to a known amount of an internal standard (3 α ,20 α -diacetoxy-5 β -pregnane

in columns containing SE-30) and using appropriate calibration curves (Menini & Norymberski, 1965). By using this method the error in the determination of any single compound in a mixture of steroidal di- and tri-ketones (Fig. 1) likely to be

Fig. 1. Gas-liquid chromatographic separation of a mixture of pure steroids on a 150 cm. long column containing SE-30, at 190°. 1, 5 β -Androstane-3,17-dione (0.55 μ g.); 2, 5 α -androstane-3,17-dione (0.66 μ g.); 3, 5 β -androstane-3,11,17-trione (1.74 μ g.); 4, 5 α -androstane-3,11,17-trione (0.80 μ g.); 5, 5 β -pregnane-3,20-dione (0.87 μ g.); 6, 5 α -pregnane-3,20-dione (1.12 μ g.); 7, 3 α ,20 α -diacetoxy-5 β -pregnane (internal standard, 1.80 μ g.).

produced by the present procedure was less than 7% (Table 5). Portions of the final urinary extracts containing 1–2 μ g. of steroidal material, as estimated by the Zimmermann reaction, were mixed with a known amount of the internal standard and analysed on a 150 cm.-long column containing SE-30 as the stationary phase. When the heights of the peaks corresponding to the minor components of the mixture were too small to be measured accurately (less than 10 mm., which under the conditions used corresponded to 0.1–0.15 μ g. of steroid), the analysis was repeated on a larger portion and the minor constituents were estimated from the second run. In most cases a portion of the final urinary extract was chromatographed also on a 40 cm.-long column containing QF-1 as the stationary phase. A typical separation on one of these columns of some of the compounds expected in the end fractions is shown in Fig. 2. With these columns, androstane-3,17-dione, pregnane-3,20-dione, androstane-3,11,17-trione (of the 5 α or the 5 β series), androst-4-ene-3,6,17-trione and pregn-4-ene-3,6,20-trione (or cholest-4-ene-3,6-dione) are completely separated; however, 5 α -androstane-3,17-dione and 5 α -pregnane-3,20-dione are not separated from their 5 β -epimers, pregn-4-ene-3,6,20-trione is not separated from cholest-4-ene-3,6-dione and 5 α - and 5 β -androstane-3,11,17-trione are only partially resolved.

Chromatography on columns containing QF-1, of urinary extracts corresponding to steroid sulphate fractions, was performed to ensure that the androst-4-ene-3,6,17-trione (the expected derivative of dehydroepiandrosterone) measured with columns containing SE-30 was indeed this compound and not a mixture of androst-4-ene-3,6,17-

Table 5. *Measurement of the individual constituents of a mixture of pure polyoxosteroids after their gas-liquid chromatographic separation (Fig. 1)*

Experimental details are given in the text. Results are given as mean values of 14 determinations performed on different days.

Compound*	Separation factor	Present in the mixture (μ g.)	Determined mean $\pm$ s.d. (μ g.)
5 β -A-3,17	1.11	0.55	0.58 $\pm$ 0.03
5 α -A-3,17	1.14	0.66	0.67 $\pm$ 0.02
5 β -A-3,11,17	1.16	1.74	1.80 $\pm$ 0.06
5 α -A-3,11,17	1.18	0.80	0.86 $\pm$ 0.03
5 β -P-3,20	1.12	0.87	0.93 $\pm$ 0.01
5 α -P-3,20		1.12	1.16 $\pm$ 0.03

* Compounds are abbreviated as in Table 2.

Fig. 2. Gas-liquid chromatographic separation (at 212°) of a mixture of pure steroids on a 40cm. long column containing QF-1. 1, 3β -Hydroxypregn-5-en-20-one (internal standard, $0.35\mu\text{g.}$); 2, 5α -androstane-3,17-dione ($0.62\mu\text{g.}$); 3, 5α -pregnane-3,20-dione ($0.48\mu\text{g.}$); 4, 5α -androstane-3,11,17-trione ($0.85\mu\text{g.}$); 5, androst-4-ene-3,6,17-trione ($1.80\mu\text{g.}$); 6, pregn-4-ene-3,6,20-trione ($2.3\mu\text{g.}$).

trione and 5α -pregnane-3,20-dione (separation factor on SE-30, 1.04). Extracts derived from the glucosiduronate fraction of urine usually contained small quantities of 5α -pregnanedione. In this case the columns containing QF-1 permitted the separation of this compound from androst-4-ene-3,6,17-trione and the quantitative estimation of the latter. Chromatography on the short columns provided additional evidence on the identity of some of the compounds measured with columns containing SE-30.

Examination of the fractions containing 17-oxo-steroid glucosiduronates. The possibility was considered that some C_{21} steroid glucosiduronates (the major constituents of the urinary steroid glucosiduronate fraction) might be partially transferred into chloroform from urine containing pyridinium sulphate. In such event, these compounds, after enzymic hydrolysis and oxidation with *tert.*-butyl chromate, will yield androstanediones and androstane-3,17-triones (Menini & Norymberski, 1962b) and will contribute to the compounds derived from the C_{19} steroid glucosiduronates. The possible contribution of compounds derived from C_{21} steroid glucosiduronates to the end products derived from the C_{19} steroid glucosiduronates was evaluated by submitting a number of urine samples to the standard analytical procedure and measuring the 17α -hydroxycorticosteroids in portions of the extracts containing the steroids released by β -glucuronidase. Nine extracts, equivalent to

20ml. of urine (from five males and four females), containing the products of β -glucuronidase hydrolysis, were each halved. One portion was oxidized directly with *tert.*-butyl chromate in pyridine. The other was dissolved in 5ml. of water (with the aid of 0.1ml. of ethanol), reduced with potassium borohydride and oxidized with sodium metaperiodate (Few, 1961) as described by Menini & Norymberski (1965). Residues obtained by the latter treatment were divided in two equal parts. One part was used for the determination of 17-oxosteroids (an indirect evaluation of the 17α -hydroxycorticosteroids; Appleby, Gibson, Norymberski & Stubbs, 1955) by the Zimmermann reaction, and gave in all cases values indistinguishable from the reagent blanks (Table 6), which indicates the absence of 17α -hydroxycorticosteroids. The other part was dissolved in pyridine and oxidized with *tert.*-butyl chromate. Determination of the 17-oxosteroids by the Zimmermann reaction gave virtually the same values as direct oxidation (*tert.*-butyl chromate) of the products of β -glucuronidase hydrolysis (Table 6).

Norymberski & Sermin (1953) showed that oxidation of steroid glucosiduronates with sodium bismuthate converted these compounds into a mixture of the parent steroid alcohols and their formic acid esters. Thus oxidative degradation of the glucosiduronate moiety affords an alternative approach to the hydrolysis of this class of conjugates. A similar type of action is to be expected from *tert.*-butyl chromate and accordingly the following experiment was carried out. A portion (20ml.) of one sample of urine (no. 10 from a female) was submitted to the standard procedure of analysis. After solvolysis of the sulphates, 5ml. of the alkaline extract containing the steroid glucosiduronates was diluted with 10ml. of water and acidified with 3N-sulphuric acid to pH 5-6. Pyridinium sulphate was added to the aqueous phase to give a molarity of 1 and then the steroid glucosiduronates were re-extracted with chloroform (25ml.). The organic phase was evaporated to dryness and the residue dissolved in 0.15ml. of pyridine and oxidized with 0.25ml. of *tert.*-butyl chromate. The subsequent treatment of the reaction mixture was as described in the Materials and Methods section. Portions of the extract containing the products of the oxidation were submitted to paper and gas-liquid chromatography. Paper chromatography in system E_4 and development of the chromatogram by the Zimmermann test revealed one large spot with the same mobility as 5α - or 5β -androstane-3,17-dione (not resolved in this system) or both, the expected derivatives of androsterone glucosiduronate and aetiocholanolone glucosiduronate, and a minor less polar constituent (visually estimated as 20-30% of the

Table 6. 17-Oxosteroids derived from the glucosiduronate fraction of some urines submitted to various treatments

Experimental details are given in the text. Results are expressed as $\mu\text{g.}$ of Zimmermann chromogens, in terms of dehydroepiandrosterone, per 20ml. of urine.

Urine no.	Total 17-oxosteroids								
	1	2	3	4	5	6	10	12	14
Treatment									
(a) Butyl chromate	46.8	119.0	31.2	43.9	61.2	111.0	103.0	15.0	83.9
(a) KBH_4									
(b) NaIO_4	0	0	0	0	0	0	0	0	0
(a) KBH_4									
(b) NaIO_4									
(c) Butyl chromate	45.0	104.0	27.0	42.9	65.2	108.0	98.7	15.3	88.0

total material), presumably the formate of androsterone or aetiocholanolone or of both. Gas-liquid chromatography of a portion of the extract on a column containing SE-30 showed the presence of two compounds with the same retention times as 5α -androsterone-3,17-dione and 5β -androsterone-3,17-dione and another peak with a retention time of 1.15 relative to 5α -androsterone-3,17-dione, identical with the relative retention time of authentic androsterone formate.

When pure androsterone glucosiduronate was oxidized with *tert.*-butyl chromate, under identical conditions, paper chromatography and gas-liquid chromatography showed that the oxidation products consisted of a major compound with the same mobility and retention time as 5α -androsterone-3,17-dione and a second substance with the same mobility and retention time as the compound observed in the urinary extract, and, by analogy, assumed to be androsterone formate.

Analysis of urines. Nine urines from normal men and six urines from normal women were analysed. Gas-liquid chromatography on columns containing SE-30 of the extracts derived from the glucosiduronate fractions showed in all cases the expected patterns (Fig. 3): the two largest peaks had the same retention times as 5α -androsterone-3,17-dione, and 5β -androsterone-3,17-dione, the expected derivatives of androsterone and aetiocholanolone; two smaller peaks had the retention times of 5α -androsterone-3,11,17-trione and 5β -androsterone-3,11,17-trione. A compound with the same retention time as 5β -pregnane-3,20-dione was present in all the female urines, usually in rather large quantities, and in most of the male urines, in small amounts. The peak corresponding to this compound was accompanied by another small peak, or sometimes two ill-resolved peaks (peak 6, Fig. 3), with the

Fig. 3. Gas-liquid chromatography on SE-30 at 190° , of the 17-oxosteroids derived from the glucosiduronate fraction of the urine of a normal woman (urine no. 15, Table 9). The separated steroids have the same retention time as: 1, 5β -androsterone-3,17-dione; 2, 5α -androsterone-3,17-dione; 3, 5β -androsterone-3,11,17-trione; 4, 5α -androsterone-3,11,17-trione; 5, 5β -pregnane-3,20-dione; 6, 5α -pregnane-3,20-dione or androst-4-ene-3,6,17-trione or both; S, $3\alpha,20$ -diacetyloxyprogesterone (internal standard). The extract chromatographed is equivalent to 1.0 ml. of urine. Peaks 3 and 4 were evaluated from the analysis of a larger portion ($\times 3$) of the extract.

same retention time as 5α -pregnane-3,20-dione or androst-4-ene-3,6,17-trione (the expected derivative of dehydroepiandrosterone). A compound with the retention time of 5β -pregnane-3,11,20-trione was present in trace amounts in most urines. Other occasional minor components were ignored. Among the minor constituents androst-4-ene-3,6,17-trione was estimated quantitatively by analysing a portion of the final extract (equivalent to 3–5 ml. of urine) on a column containing QF-1 (Fig. 4). Using this column 5α - and 5β -androsterone-3,17-

Fig. 4. Gas-liquid chromatography on QF-1 at 212°, of the 17-oxosteroids derived from the glucosiduronate fraction of the urine of a normal woman (urine no. 10b, Table 9). The separated peaks correspond to compounds with the same retention time as: 1, androstane-3,17-dione ($5\alpha+5\beta$); 2, unidentified; 3, 5α -androstane-3,11,17-trione; 4, androst-4-ene-3,6,17-trione. Sensitivity was increased ($\times 3$) just before the emergence of androst-4-ene-3,6,17-trione. The extract chromatographed is equivalent to 5 ml. of urine.

dione emerged under a single peak (5β -pregnane-3,20-dione was separated from the former compounds only if present in relatively large amounts) while 5α - and 5β -androstane-3,11,17-trione appeared as two partially resolved peaks. A compound with the same retention time as androst-4-ene-3,6,17-trione was seen in most chromatograms and, when present, emerged as a discrete readily measurable peak (peak 4, Fig. 4). Process blanks, obtained by submitting water to all the operations of the analytical procedure, gave only insignificant peaks with the retention times of the steroids under investigation.

Recovery experiments were carried out by adding pure dehydroepiandrosterone glucosiduronate and aetiocholanolone glucosiduronate to urine and measuring the increase of the gas-chromatographic peaks corresponding to androst-4-ene-3,6,17-trione and 5β -androstane-3,17-dione respectively (Table 7). Each conjugate gave rise to an increase of only the expected compound. The results of the analyses of urine from nine normal men and five women are given in Tables 8 and 9. Also included are the results of the determination of the total 17-oxosteroids, after hot acid hydrolysis of urine, in the final extracts, and,

Table 7. Recovery of steroid conjugates added to urine

Each conjugate was added to urine in quantities varying from 2 to 4 $\mu\text{g./ml.}$ Results were calculated from the increase of the expected gas-chromatographic peak in the appropriate fraction. Number of experiments is indicated in parenthesis.

	Percentage recovery $\pm$ S.D.
Sodium dehydroepiandrosterone sulphate	73 ± 6 (8)
Sodium androsterone sulphate	78 ± 7 (5)
Sodium aetiocholanolone sulphate	88 ± 6 (4)
Dehydroepiandrosterone glucosiduronic acid	68 ± 4 (5)
Aetiocholanolone glucosiduronic acid	83 ± 3 (4)

in some cases, after the enzymic or solvolytic hydrolysis of the conjugates. The recorded values for the 11-oxygenated compounds and pregnane-dione, for the reasons considered above, have no quantitative significance. In seven cases, urines were analysed in duplicate. The coefficients of variation between duplicate estimates for the major components were 6% for 5α -androstane-3,17-dione and 4% for 5β -androstane-3,17-dione.

Extracts containing the steroids derived from the sulphate fraction of urine were analysed by gas-liquid chromatography as described for the glucosiduronate fraction. Chromatograms obtained with columns containing SE-30 (Fig. 5) showed in all cases two large peaks with the same retention time as 5α - and 5β -androstane-3,17-dione, two smaller peaks corresponding to 5α - and 5β -androstane-3,11,17-trione and another peak with the retention time of androst-4-ene-3,6,17-trione. A small peak with the retention time of 5β -pregnane-3,20-dione was observed occasionally. A portion of the extracts derived from the steroid sulphates fraction was also analysed on short columns containing QF-1 to exclude the possibility that the gas-chromatographic fraction corresponding to androst-4-ene-3,6,17-trione (from the analysis on the SE-30 phase) had been contaminated with 5α -pregnane-3,20-dione (separation factor on the SE-30 phase 1.04), an unlikely but possible constituent of these extracts. The gas-liquid chromatograms showed one large peak corresponding to a mixture of 5α - and 5β -androstane-3,17-dione, two smaller, only partially resolved peaks with the retention times of 5α - and 5β -androstane-3,11,17-trione and one peak corresponding to androst-4-ene-3,6,17-trione. Some of the chromatograms (see Tables 8 and 9) showed the presence of one peak with a retention time 1.46 relative to androst-4-ene-3,6,17-trione, identical with the retention time of cholest-4-ene-3,6-dione (the product expected

Table 8. *Analysis of urines of healthy men*

Results are expressed in mg./24 hr. Experimental details are given in the text. Compounds marked (+) were present but their quantity was not determined.

Urine no.	1a*		1b*		1c*		2		3		4		5		6		7		8		9		Mean (with range)			
Compound**	G†	S†	G	S	G	S	G	S	G	S	G	S	G	S	G	S	G	S	G	S	G	S	G	S		
5β-A-3,17	2.04	0.83	2.10	0.88	2.24	0.88	2.73	0.16	1.22	0.71	0.92	0.33	1.30	0.71	3.46	0.32	2.30	0.16	1.43	0.37	3.61	0.69	2.11	(0.92-3.61)	0.48	(0.16-0.88)
5α-A-3,17	2.97	2.70	2.84	3.24	3.16	2.68	3.56	0.47	1.98	0.90	2.16	1.13	3.35	1.02	3.56	1.54	1.91	1.12	1.85	1.32	1.98	2.25	2.80	(1.91-3.56)	1.33	(0.47-3.24)
5β-A-3,11,17	0.22	0.51	0.25	0.53	0.48	0.54	0.27	0.16	0.36	0.12	0.10	0.33	0.09	0.30	0.56	0.25	0.13	0.16	0.40	0.16	0.28	0.24	0.25	(0.09-0.56)	0.25	(0.12-0.53)
5α-A-3,11,17	0.28	0.22	0.32	0.25	0.52	0.26	0.23	0.14	0.24	0.10	0.13	0.28	0.15	0.10	0.26	0.15	0.45	0.16	0.19	0.20	0.38	0.14	0.26	(0.13-0.32)	0.17	(0.10-0.23)
5β-P-3,20	0.50	0.15	0.50	0.15	0.59	0.14	0.64	0.14	0.36	+	0.18	0.23	0.23	0.10	0.80	0.38	+	0.10	0.47	+	0.41	0.16	0.41	(0.16-0.80)	0.15	(0.10-0.23)
Δ ⁴ -A-3,6,17	+	4.05	0.21	3.98	+	4.14	0.11	0.79	+	+	0.08	1.10	+	0.89	0.13	2.55	+	0.47	+	4.55	+	3.06	0.13	(0.08-0.21)	2.29	(0.47-4.55)
Total	5.51	8.31	5.72	8.68	6.40	8.50	6.90	1.72	3.80	1.81	3.39	3.17	4.89	3.02	7.97	4.81	4.79	2.07	3.97	6.60	6.25	6.38	5.44	(3.39-7.97)	4.25	(1.72-8.68)
Total 17-OS (after oxidation)§	5.2	8.0	6.2	8.6	6.3	8.6	7.1	2.0	4.6	1.7	2.6	3.0	3.9	3.4	8.6	4.5	5.2	3.1	4.8	7.9	7.4	9.10	5.5	(2.6-8.6)	4.8	(1.7-9.1)
Total 17-OS (before oxidation)	5.7	6.9	6.6	7.1	7.5	6.8	6.8	1.6							8.9	3.4	4.7		5.3	7.3	7.7	7.9				
Total 17-OS (after acid hydrolysis)††	12.6	12.6	12.6	12.6	10.2	10.2	6.8	5.6							11.0	7.2	8.6		12.2	13.5	9.7	9.7				
Δ ⁴ -Ch-3,6††																										

* Urines 1a, 1b and 1c were made initially 0.5, 1.0 and 1.5 M in pyridinium sulphate respectively.

† G, Glucosiduronate fraction.

‡ S, Sulphate fraction.

§ 17-OS denotes 17-oxosteroids; they were measured (after oxidation by *tert*-butyl chromate) by the Zimmermann reaction and they are expressed in terms of dehydroepiandrosterone (DHA).

|| 17-OS were measured after hydrolysis (G) or solvolysis (S) by the Zimmermann reaction and they are expressed in terms of DHA.

** Δ⁴-A-3,6,17 denotes androst-4-ene-3,6,17-trione; Δ⁴-Ch-3,6 denotes cholest-4-ene-3,6-dione; other compounds are abbreviated as in Table 2.

†† 17-OS were measured by the Zimmermann reaction and they are expressed in terms of DHA.

‡‡ Detected by paper chromatography and gas-liquid chromatography (see text).

Table 9. *Analysis of urines of healthy women*

Results are expressed in mg./24 hr. Experimental details are given in the text. Compounds marked (+) were present, but their quantity was not determined.

Urine no.	10a*		10b*		10c*		11		12		13**		14		15		Mean (with range)		
Compound¶	G†	S†	G	S	G	S	G	S	G	S	G	S	G	S	G	S	G	S	
5β-A-3,17	1.69	0.55	1.83	0.62	1.69	0.60	1.45	1.40	0.30	0	3.63	2.36	0.29	0.89	0.31	1.62	(0.89-2.36)	0.59	(0.29-1.45)
5α-A-3,17	2.63	0.60	2.63	0.65	2.52	0.62	1.05	0.92	0.47	0	1.02	4.06	1.31	0.63	0.40	2.06	(0.63-4.06)	0.77	(0.40-1.31)
5β-A-3,11,17	0.09	0.12	0.09	0.10	0.10	0.10	1.05	0.14	0.16	0	2.24	0.30	0.15	0.12	0.11	0.16	(0.09-0.30)	0.31	(0.10-1.05)
5α-A-3,11,17	0.25	0.10	0.37	0.10	0.40	0.11	0.54	0.18	+	0	0.82	0.40	0.14	0.14	0.07	0.27	(0.14-0.40)	0.17	(0.07-0.54)
5β-P-3,20	0.17	+	0.21	+	0.19	+	0.33	1.14	0.85	0	0.76	0.64	0.12	0.87	+	0.71	(0.19-1.14)	0.10	(+ -0.33)
Δ ⁴ -A-3,6,17	+	0.26	0.18	0.28	+	0.28	1.98	0.09	0.85	0	+	0.11	0.96	0.12	0.41	0.12	(0.09-0.18)	0.89	(0.28-1.98)
Total	4.66	1.63	5.10	1.75	4.71	1.71	6.07	2.73	1.78	0	7.71	7.23	2.85	1.90	1.30	4.25	(1.90-7.23)	2.75	(1.30-6.07)
Total 17-OS (after oxidation)§	5.6	2.2	5.3	2.4	5.3	2.7	5.1	2.4	1.5	0	9.0	6.2	3.3	2.0	1.6	3.8	(2.0-6.2)	2.8	(1.5-5.1)
Total 17-OS (before oxidation)§	5.9	1.6	6.3	1.7	6.1	1.7	4.1	2.3	0.9	+	7.0	10.3	4.3	+	+	7.6	(4.3-10.9)	+	+
Total 17-OS (after acid hydrolysis)§	8.3	+	8.3	+	10.9	+	10.9	10.3	4.5	+	+	+	+	+	+	+	+	+	+
Δ ⁴ -Ch-3,6																			

* Urines 10a, 10b and 10c were initially made 0.5, 1.0 and 1.5 M in pyridinium sulphate respectively.

† G, Glucosiduronate fraction.

‡ S, Sulphate fraction.

§ Zimmermann chromogens expressed as DHA (see Table 8).

|| Detected by paper chromatography and gas-liquid chromatography (see text).

¶ Abbreviations for compounds as in Table 8.

** Values for urine 13 are not included in the mean (range) see text.

Fig. 5. Gas-liquid chromatography on SE-30 at 190°, of the 17-oxosteroids derived from the sulphate fraction of the urine of a normal man (urine no. 1b, Table 8). The separated steroids have the same retention times as: 1, 5 β -androsterone-3,17-dione; 2, 5 α -androsterone-3,17-dione; 3, 5 β -androsterone-3,11,17-trione; 4, 5 α -androsterone-3,11,17-trione; 5, 5 β -pregnane-3,20-dione; 6, androst-4-ene-3,6,17-trione; S, 3 α ,20 α -diacetoxyprogesterone (internal standard). The extract chromatographed is equivalent to 1.0 ml. of urine.

from the oxidation of cholesterol (Menini & Norymberski, 1962b). As cholest-4-ene-3,6-dione and pregn-4-ene-3,6,20-trione (the expected product from the oxidation of 3 β -hydroxypregn-5-en-20-one) are not separated on short columns containing QF-1 (separation factor 1.04), portions of the extracts in which the compound with the retention time of cholest-4-ene-3,6-dione (or pregn-4-ene-3,6,20-trione) had been detected were analysed by paper chromatography in the system E₄, where the R_F values for pregn-4-ene-3,6,20-trione and cholest-4-ene-3,6-dione are 0.21 and 0.78 respectively. All the paper chromatograms revealed the presence of a compound with the same mobility as cholest-4-ene-3,6-dione, detected by the yellow colour and orange fluorescence given on treatment with cold alkali, characteristic of the 4-ene-3,6-dione system (Menini & Norymberski, 1962b).

When sodium dehydroepiandrosterone sulphate, sodium androsterone sulphate and sodium aetiocholanolone sulphate were added to urine their expected derivatives were recovered in the final fractions in high yields (Table 7). The results of the analyses of the sulphate fractions corresponding to the urines of nine normal men and six normal women are summarized in Tables 8 and 9. Nine of

these urines were analysed in duplicate. The coefficients of variation between duplicate determinations of the components of this fraction were 6% for 5 α -androsterone-3,17-dione, 5% for 5 β -androsterone-3,17-dione, 8% for 5 α -androsterone-3,11,17-trione, 6% for 5 β -androsterone-3,11,17-trione and 7% for androst-4-ene-3,6,17-trione.

DISCUSSION

The procedure for the systematic analysis of urinary steroids of Menini & Norymberski (1965) provides quantitative information about the presumptive secretory precursors of the urinary steroid metabolites excreted as glucosiduronates. Whilst the bulk of the urinary 17-hydroxycorticosteroids are excreted in this form, the 17-oxosteroids are excreted both as glucosiduronates and sulphates (Kellie & Wade, 1957), and dehydroepiandrosterone mainly as sulphate (Munson, Gallagher & Koch, 1944). Attempts to incorporate the 17-oxosteroid sulphates into the groups of steroids measured by the procedure for the systematic analysis of steroids were unsuccessful because of the deleterious action of the glycol-cleaving agents on compounds with the 5-en-3 β -ol grouping.

The present method provides a simple and reliable procedure for the indirect determination of the urinary 17-oxosteroid sulphates and 11-deoxy-17-oxosteroid glucosiduronates in the same sample of urine. The separation of the steroids derived from the sulphates from the other conjugated steroids is based on the preferential solvolysis (in the absence of acid) of the former group of compounds. The sequence of the operations and reactions of the method converts the urinary C₁₉ steroids with two or three non-vicinal oxygen functions into a mixture of di- and tri-ketones of the androstane series. Thus androsterone, aetiocholanolone, their 11-oxygenated derivatives and dehydroepiandrosterone are converted, by oxidation with *tert.*-butyl chromate, into 5 α -androsterone-3,17-dione, 5 β -androsterone-3,17-dione, 5 α -androsterone-3,11,17-trione, 5 β -androsterone-3,11,17-trione and androst-4-ene-3,6,17-trione which are separated and measured by submitting the final extracts to gas-liquid chromatography.

In the interpretation of the results furnished by the present method the same considerations which apply to the results given by the systematic analysis of urinary steroids must be taken into account. Thus it must be borne in mind that by oxidizing the secondary alcohols to the corresponding ketones, differentiation of the oxygen functions attached to the steroid nucleus (oxo, α -hydroxy or β -hydroxy groups) becomes no longer possible. For instance, although urinary androsterone is the most likely precursor of 5 α -androsterone-3,17-dione measured in

the final extracts, this compound could equally well derive from any urinary 5α -androstane disubstituted at C-3 and C-17. However, since carbonyl and secondary hydroxyl groups are metabolically interconvertible and since no information is lost about the number and position of the oxygen functions and the stereochemistry of the steroid nucleus, the chemical transformation does not affect the correlation of the end products with their presumptive secretory precursors. The comparison of the determination of the total 17-oxosteroids before and after *tert.*-butyl chromate oxidation (Tables 8 and 9) indicates, in agreement with previous results (Menini & Norymberski 1965), that in normal urines the contribution of 17-hydroxy-androstanes to the final products is insignificant.

Evidence that 5α -androstane-3,17-dione, 5β -androstane-3,17-dione, their corresponding 11-oxo derivatives and androst-4-ene-3,6,17-trione in the end fractions, are largely, if not entirely, derived from the urinary androsterone, aetiocholanolone, their 11-oxygenated derivatives and dehydroepiandrosterone respectively is provided by the comparison of the determinations of the total 17-oxosteroids after acid hydrolysis of urine and the sum of the 17-oxosteroids in the final extracts corresponding to the glucosiduronate and sulphate fractions. On average the total 17-oxosteroids found in the urine after acid hydrolysis accounted for 112% in females and 102% in males of the 17-oxosteroids measured by gas-liquid chromatography (uncorrected for losses).

In the Results section, evidence has been presented that the method, in its present form, grossly underestimates the 11-oxygenated, 17-oxosteroid glucosiduronates and that therefore the quantitative significance of the constituents of this fraction must be restricted to the 11-deoxy-17-oxosteroids. Similarly, the pregnanediol excreted as glucosiduronate is not quantitatively recovered in the final fractions owing to incomplete extraction of the conjugate from aqueous solutions, as has been shown by experiments with the model compound.

The use of short gas-liquid-chromatographic columns containing QF-1 as stationary phase has made it possible to estimate the daily excretion of dehydroepiandrosterone glucosiduronate, by measuring its expected derivative androst-4-ene-3,6,17-trione. Here again, because of the loss of information on the nature of the oxygen functions, accompanying the oxidation with *tert.*-butyl chromate, correlation of the latter compound with the former rests on the assumption that the amounts of other possible urinary precursors (6α - or 6β -hydroxyandrost-4-ene-3,17-dione, for instance) are negligible. With this proviso, the amount of dehydroepiandrosterone excreted as glucosiduron-

ate per day has been estimated as $125\mu\text{g.}$ in men (mean of four determinations) and $119\mu\text{g.}$ in women (mean of four determinations), uncorrected for losses. Siiteri, Vande Wiele & Lieberman (1963), after administration of tritiated dehydroepiandrosterone to one normal male subject, calculated a minimum daily excretion of $105\mu\text{g.}$ of dehydroepiandrosterone glucosiduronate.

The great majority of values found in the literature for the excretion of individual 17-oxosteroids comprise both the fraction excreted as glucosiduronate and as sulphate and therefore are not comparable with the present results. Kellie & Wade (1957) determined the individual urinary 17-oxosteroids in the two groups of conjugates. In their procedure glucosiduronates were hydrolysed with enzyme and after extraction of the liberated steroids the sulphates were cleaved by continuous ether extraction at pH 1. They separated the individual 17-oxosteroids, isolated from urine, by gradient elution on an alumina adsorption column.

Comparison of the results given by their procedure and by the present method offers some points of interest. Kellie & Wade (1957) found that 79% of the total androsterone and 90% of the total aetiocholanolone were excreted conjugated with glucosiduronic acid. By the present procedure, 70% of the total androsterone and 79% of the total aetiocholanolone are found in the glucosiduronate fraction, the rest being recovered in the sulphate fraction. These results are at variance with those of Segal, Segal & Nes (1960), who could detect only trace amounts of aetiocholanolone and androsterone after acid-catalysed solvolysis of urinary extracts. The presence of sulphatases in the β -glucuronidase preparation used by Kellie & Wade (1957) could easily account for the slightly lower proportion of steroid sulphates found by these authors.

Tables 8 and 9 show that dehydroepiandrosterone is predominantly excreted as its sulphate ester. The same results were found by Kellie & Wade (1957) when sulphatase activity in their enzyme preparation was inhibited by potassium dihydrogen phosphate. In the present study, men excreted considerably higher amounts of dehydroepiandrosterone than women: on average, two to three times as much. Similar findings have been reported by other investigators (Johnsen, 1956; Brooks, 1958; Brooksbanks & Salokangas, 1959; Beas, Zurbrugg, Cara & Gardner, 1962; Vestergaard & Claussen, 1962; Hamman & Martin, 1964; Lim, Fesler & Dingman, 1964), who found ratios varying from 1.3 to 6.0 for the dehydroepiandrosterone excreted by men and women.

Examination of the values found in the literature for the excretion of total androsterone and aetiocholanolone shows that men excrete slightly higher amounts of androsterone in relation to aetio-

cholanolone than women. The ratios reported for the mean excretion of androsterone and aetiocholanolone vary from 0.8 to 1.5 for male subjects and from 0.7 to 1.2 for females (Rubin, Dorfman & Pincus, 1954; Kappas & Gallagher, 1955; Johnsen, 1956; Kellie & Wade, 1957; Brooks, 1958; Brooksbanks & Salokangas, 1959; Jailer, Vande Wiele, Christy & Lieberman, 1959; James, 1961; Beas *et al.* 1962; Vestergaard & Claussen, 1962; Hamman & Martin, 1964; Lim, Fesler & Dingman, 1964), which compare favourably with the values calculated from the present results, namely 1.6 for men and 1.3 for women.

It is of interest that in one urine (no. 13) from a woman with no apparent signs of endocrine disorders, all the 17-oxosteroids (about 8mg./24hr.) were conjugated with sulphuric acid. Operator's error can be ruled out, as the analysis was repeated on three occasions by different persons, with the same results. Other special features of this urine were the very high amounts (2.24mg./24hr., which, corrected for losses, probably represent 4–5mg./24hr.) of 5β -androstane-3,11,17-trione, most likely derived from 11β -hydroxy or 11-oxoaetiocholanolone, and the absence of androst-4-ene-3,6,17-trione (the derivative of dehydroepiandrosterone). The 17-oxosteroid values for this urine have been excluded from the calculated means and ranges given in Table 9.

17-Oxosteroid glucosiduronates have been hydrolysed enzymically by β -glucuronidase. However, one experiment, in which these compounds were oxidized directly with *tert.*-butyl chromate, showed that this reagent is capable of degrading the glucosiduronic acid moiety of these conjugates, producing a mixture of steroidal ketones and 3α -formoxy derivatives.

The small volumes of urine (20ml.), and the simplicity of the operations involved in the present method, make this procedure well suited to serial analysis of urine samples.

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