An Approach to the Systematic Analysis of Urinary Steroids

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1. Human urine, its extracts, extracts of urine pretreated with enzyme preparations containing β-glucuronidase and steroid sulphatase or β-glucuronidase alone, and products derived from the specific solvolysis of urinary steroid sulphates, were submitted to the following sequence of operations: reduction with borohydride; oxidation with a glycol-cleaving agent (bismuthate or periodate); separation of the products into ketones and others; oxidation of each fraction with *tert.*-butyl chromate, resolution of the end products by means of paper chromatography or gas–liquid chromatography or both. 2. Qualitative experiments indicated the kind of information the method and some of its modifications can provide. Quantitative experiments were restricted to the direct treatment of urine by the basic procedure outlined. It was partly shown and partly argued that the quantitative results were probably as informative about the composition of the major neutral urinary steroids (and certainly about their presumptive secretory precursors) as those obtained by a number of established analytical procedures. 3. A possible extension of the scope of the reported method was indicated. 4. A simple technique was introduced for the quantitative deposition of a solid sample on to a gas–liquid-chromatographic column.

Systematic analysis of urinary steroids by their resolution into individual compounds presents formidable difficulties owing to the complexity of the analytical sample. Menini & Norymberski (1962*a*) proposed a scheme aiming to simplify the analytical task by reducing the complexity of the sample through suitable chemical transformations leading to a much less complex and more readily resolvable mixture of products. The proposed scheme involves the following operations: reduction with borohydride followed by the cleavage of glycols with sodium bismuthate (Appleby, Gibson, Norymberski & Stubbs, 1955); separation of the reaction products into a ketonic fraction (K) and a non-ketonic fraction (A) according to the method of Girard & Sandulesco (1936); oxidation of each fraction with *tert.*-butyl chromate according to the method of Menini & Norymberski (1962*b*) to yield fractions KK and KA respectively, the latter having been freed from any non-ketonic material; resolution of fractions KK and KA into their respective constituents. The outcome of these operations may be predicted from the modes of action of the reagents employed: (1) unless steroid sulphates had been hydrolysed, only steroid glucosiduronic acids and unconjugated steroids will be estimated; (2) oestrogens will be removed by the alkaline wash after glycol fission and hence escape detection; (3) compounds containing in the side chain two vicinal oxygen functions, one of which is a tertiary hydroxyl group, will yield ketones found in fraction KK, and so would any di-tertiary ring glycols (8,9- and 8,14-diols in the steroid series); (4) other compounds with two vicinal oxygen functions will yield carboxylic acids removed from fraction KK by an alkaline wash; (5) aldehydes, primary alcohols and compounds with two oxygen functions in β-position will give acidic products removed from fraction KA by an alkaline wash; (6) all other compounds containing at least one keto group or one secondary hydroxyl group will give rise to ketones found in fraction KA; (7) the homoallylic 5-en-3β-ols will yield 4-ene-3,6-diones; (8) common structural features other than those mentioned will remain unchanged. Special structural features may change the predicted outcome: thus the outlined sequence of reactions converts aldosterone into the neutral lactone of 18-hydroxy-3,11-dioxochol-4-enic acid (J. K. Norymberski, unpublished work). From what is known about the composition of urinary steroids it may be further expected that fraction KK will

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contain predominantly di- and tri-ketones of the androstane series derived from urinary 17-hydroxy corticosteroids, and fraction KA di- and tri-ketones of the androstane and pregnane series derived from compounds lacking vicinal oxygen functions.

The proposed scheme was qualitatively evaluated by submitting fractions KK and KA derived from a pregnancy urine to paper chromatography and to gas–liquid chromatography, whereby both methods of resolution gave the expected simple chromatographic patterns (Menini & Norymberski, 1962*a*). Brooks & Hanaineh (1963) confirmed these findings by gas–liquid chromatography of similar fractions prepared by the present authors from another urine specimen. Bailey (1964) applied gas–liquid chromatography to the analysis of the oxidation products of paper-chromatographically purified fractions of unconjugated urinary steroids. The present investigation aimed to extend our initial observation (Menini & Norymberski, 1962*a*) and to assess the practicability of the proposed analytical scheme.

MATERIALS AND METHODS

Reagents and solvents

Ethylene dichloride was shaken repeatedly with conc. H_2SO_4 until no more pigments formed, washed successively with water, alkali and water, then dried over anhydrous Na_2SO_4, refluxed over P_2O_5 and distilled. Ether and dioxan were refluxed over CaH_2 and distilled. Chloroform was refluxed over anhydrous K_2CO_3 and distilled. Methanol was distilled. Other solvents (analytical grade) were used as supplied. The *tert.*-butyl chromate reagent was prepared as described by Menini & Norymberski (1962*b*). The Girard T reagent was crystallized from ethanol.

Paper chromatography

The systems B_3 (Bush, 1952) [light petroleum–benzene–methanol–water (67:33:80:20, by vol.)] and E_4 (Eberlein & Bongiovanni, 1955) [2,2,4-trimethylpentane–2-methylpropan-2-ol–methanol–water (20:9:9:2, by vol.)] were used. A mixture of androst-4-ene-3,11,17-trione, 5β-androstane-3,11,17-trione, 5α-androstane-3,17-dione and 5β-pregnane-3,20-dione was chromatographed in a lane parallel to that of the analytical sample. Chromatograms were inspected under ultraviolet light (approx. 250mμ) and then treated with the Zimmermann reagent. Details were as reported by Menini & Norymberski (1962*b*).

Gas–liquid chromatography

The Pye Argon Chromatograph with a ^{90}Sr argon ionization detector was used. Columns were glass tubings (length 120cm., internal diam. 3·5–4·0mm.) widening into an evaporation chamber (length 10cm., internal diam. 12mm.) and ending with a B14 ground-glass socket. The evaporation chamber fitted into the central hole of a cylindrical aluminium block secured to the top of the Chromatograph housing and heated with a 450w Knuckle Refractory Heater (Hedin Ltd., London) controlled by a variable transformer. A perforated glass tube (external diam. 8–9mm., perforations of 1–2mm. diam.) fitted into the evaporation chamber by means of a B14 cone. The upper part of the cone connected to two side arms: one for argon supply and the other (3cm. higher and 9cm. long) for storage of analytical samples (usually 12) deposited on small pieces of stainless-steel gauze (see below). The upper side arm was closed with a silicone rubber stopper and, when operating conditions were reached, one sample at a time was moved with a magnet towards the vertical tubing and allowed to drop into the perforated tube.

The columns were packed with silicone-treated Gas-Chrom P (mesh 100–120) coated with 1% of SE-30 stationary phase; they were primed by heating to 260° for 24hr. in a slow stream of argon. For details of these operations see Brooks & Hanaineh (1963). Operating conditions were: 30ml./min. argon flow; column at 220°; evaporation chamber at 245–250°; 1250v detector voltage (nominal); $\times 10$ attenuation. The efficiency of the column was about 1300 theoretical plates for 5α-androstane-3,17-dione.

Quantitative transfer of an analytical sample on to the chromatographic column was brought about as follows. A stainless-steel gauze ring (Dixon Gauze Rings, $\frac{1}{16}$in.$\times$ $\frac{1}{16}$in; Griffin and George Ltd., Manchester), washed with chloroform, was placed in the indentation of a polytetrafluoroethylene spotting plate and the analytical sample added in not more than 0·1ml. of chloroform. On evaporation of the solvent the solute was deposited on the gauze. When necessary, the vessel originally containing the sample was washed with one or more portions of chloroform and any solute present deposited on the same gauze as before. The extinction at 240mμ of several Δ^4-3-oxo steroids deposited on gauzes in this manner accounted for 95–99% of the material used. A smooth surface of the spotting plate (essential for the success of the operation) was obtained by pressing the bottom of a hot (250°) test tube against the indentations. A chloroform solution of Sudan Red placed on a smooth and clean plate should leave, on evaporation of the solvent, the dye concentrated in a pin point. In most instances analytical samples were admixed with a known quantity of 3α,20α-diacetoxy-5β-pregnane.

Preparation of urine extracts

Twenty-four hour specimens were collected. Specimens of less than 2·0l. were made up to that volume with distilled water. They were stored at $-18°$.

Unconjugated fraction. The urine was extracted with chloroform (3×1vol.) and the extracts washed with water ($2\times 0{\cdot}1$vol.). The combined extracts were evaporated to dryness in a rotary evaporator at 40°. The extracted urine, combined with the washings, was used for the determination of conjugated steroids. In a few instances ethyl acetate was used for extraction.

Glucuronide fraction. Urine (or urine freed from unconjugated steroids) was made 0·1M with respect to sodium acetate and adjusted to pH5·0. β-Glucuronidase (Ketodase; Warner–Chilcott Laboratories, New York, U.S.A.) was added to give a concentration of 1000 Fishman units/ml. of final solution. After 48hr. at 37° the mixture was extracted with ethylene dichloride or with ethyl acetate as described above.

Glucuronide-plus-sulphate fraction. Urine (or urine freed from unconjugated steroids) was treated with the digestive juice of *Helix pomatia* (L'Industrie Biologique Française) under the conditions given above for treatment with β-glucuronidase.

Sulphate fraction. Urine (or urine freed from unconjugated steroids) was made 0·3M with respect to pyridinium sulphate, extracted with chloroform (0·6vol.) and the extract evaporated to dryness. The dry residue in dioxan (5ml.) was kept for 6hr. at 37°. Chloroform (25ml.) was added and the mixture washed with water (2ml.), 2N-NaOH (5ml.) and water (5ml.). The washed organic phase was evaporated to dryness.

Standard analytical procedure

Reduction. Urine (5ml.) was treated with KBH_4 (50mg.) overnight at room temperature.

Glycol cleavage. (1) With sodium bismuthate. After completion of the reduction with KBH_4, acetic acid (5ml.) and, a few minutes later, sodium bismuthate (2·5g.) were added to the urine. The mixture was shaken for 1hr. and then filtered through a sintered-glass funnel. The surplus reagent was washed with aq. 50% (v/v) acetic acid (2ml.). A small quantity (on the tip of a spatula) of sodium metabisulphite (disulphite) and water (10ml.) were added to the combined filtrates and the mixture was extracted with ethylene dichloride (3×10ml.). The extract was washed with water (6ml.), 2N-NaOH (6ml.) and again with water (4ml.); it was then dried over anhydrous Na_2SO_4 and evaporated to dryness. Part of this material (2×0·05) was taken for the micro-assay of Zimmermann chromogens according to the method of Corker, Norymberski & Thow (1962).

(2) With periodate. After completion of the reduction with KBH_4, acetic acid (0·1ml.) and, a few minutes later, freshly prepared aq. 10% (w/v) sodium metaperiodate solution (2ml.) were added to the urine. The mixture was immediately adjusted to pH6·5–7·0 with N-NaOH (0·2–0·4ml.) and then shaken for 2hr. at room temperature. It was next adjusted to pH9·0 with 5N-NaOH (0·2ml.), kept for 15min. at 37° and finally extracted with ethylene dichloride (2×10ml.). The extract was washed with water (2ml.), 5% (w/v) sodium dithionite solution in 2·5N-NaOH (2ml.), again with water (2ml.), and then evaporated to dryness. As under (1) above the material was assayed by the Zimmermann reaction.

Oxidation with periodate is the method of choice whenever analysis of fraction KA (see below) is intended.

Girard separation. Method 1. The products of the glycol cleavage were treated with the Girard T reagent (7·5mg.) in acetic acid (0·15ml.) overnight at room temperature. The mixture was chilled in ice, diluted with 3·0ml. of a cold buffer (pH7·0) prepared from citric acid (0·1M) and Na_2HPO_4 (0·2M) and extracted with cold ether (3×4·0ml.). The extract was washed with water (1ml.), N-NaOH (1·5ml.), again with water (1ml.) and then evaporated to dryness (non-ketonic fraction A). The aqueous phase and the first washing were combined and acidified with conc. HCl (1·0ml.). After 2hr. at room temperature the mixture was extracted with ethylene dichloride (10ml.), the extract washed with water (2ml.), 2N-NaOH (2ml.) and again with water (2×2ml.), and evaporated to dryness (ketonic fraction K).

Method 2. This method differs from method 1 in that the reaction mixture was diluted with 4N-K_2CO_3 (3ml.) in place of the buffer and the extracted aqueous phase acidified with more of the conc. HCl (1·5ml.). All other operations were as before. Method 2 is recommended since it is more efficient in separating 3,20-diones.

Oxidation with tert.*-butyl chromate.* Fraction K (see above) in pyridine (0·1ml.) was treated with the chromate reagent (0·1ml.) overnight at room temperature. After the addition of a freshly prepared saturated ethanolic solution of citric acid (0·2ml.) the mixture was shaken for 15min., diluted with N-H_2SO_4 (1ml.) and extracted with ethylene dichloride (3·0ml.). The extract was washed with water (1·0ml.), aqueous 10% (w/v) sodium metabisulphite (1·0ml.), N-NaOH (1·0ml.) and again with water (1·0ml.), and then evaporated to dryness (fraction KK).

Fraction A (see above) was treated in the same manner. The oxidation products were submitted to the Girard separation as described giving a non-ketonic fraction (which was discarded) and a ketonic fraction (fraction KA).

Samples of fractions KK and KA (2×0·05 of each) were taken for the micro-assay of Zimmermann chromogens. Another portion of each fraction (usually containing approx. 5μg. of Zimmermann chromogens) was admixed with 3α,20α-diacetoxy-5β-pregnane (usually 2·8μg.), and the mixture deposited on a stainless-steel gauze and submitted to gas–liquid chromatography as described above.

Variants of the method. Most of the quantitative results were obtained by the method outlined. Larger samples of urine (25ml. or more) were analysed when fractions KK and KA were submitted to paper chromatography; the amounts of reagents and solvents were then suitably adjusted but the method was essentially the same. Extracts of urines were made up with water to a suitable steroid concentration and analysed by the same method. When initial treatment with KBH_4 was omitted all other operations remained unchanged.

RESULTS

Gas–liquid chromatography

Gas–liquid chromatography of steroids was carried out essentially according to the methods of VandenHeuvel, Sweeley & Horning (1960) and Brooks & Hanaineh (1963). The results were in good agreement with those reported by the cited authors. Injection of samples deposited on stainless-steel gauzes (cf. Chamberlain, Knights & Thomas, 1963) proved convenient and made it possible to develop a technique for accurate sampling (see the Materials and Methods section). Table 1 lists the retention times of steroidal di- and tri-ketones likely to be produced by the proposed analytical procedure.

A linear relationship between the ratio of the peak heights of two compounds and their molar ratio was established for a reference compound (3α,20α-diacetoxy-5β-pregnane) and each of the following: 5β-androstane-3,17-dione, 5α-androstane-3,17-dione, 5β-androstane-3,11,17-trione, 5α-androstane-3,11,17-trione and 5β-pregnane-3,20-dione (see Fig. 1). By using these relationships, each compound (0·5–1·5μg.) could be determined with

Table 1. *Retention times (t_R) and paper-chromatographic mobilities (R_F) of some steroidal diones and triones*

Gas–liquid chromatography was carried out on SE-30 columns at 220°. Paper chromatography was carried out at 22° with the systems E_4 (Eberlein & Bongiovanni, 1955) and B_3 (Bush, 1952). Experimental details are given in the text. The retention time of the internal standard (diacetoxypregnane) was 25·8 min.

Compound	t_R (min.)	Separation factor	R_F	
			E_4	B_3
5β-Androstane-3,17-dione	7·7		0·46	0·78
		1·10		
5α-Androstane-3,17-dione	8·5		0·44	0·76
		1·08		
5β-Androstane-3,11,17-trione	9·2		0·19	0·52
		1·08		
Androst-4-ene-3,17-dione	9·9		0·32	0·60
		1·05		
5α-Androstane-3,11,17-trione	10·4		0·16	0·49
		1·14		
Androst-4-ene-3,11,17-trione	11·9		0·11	0·34
		1·02		
5β-Pregnane-3,20-dione	12·1		0·58	0·84
		1·11		
Androst-4-ene-3,6,17-dione	13·4		0·10	0·41
		1·01		
5α-Pregnane-3,20-dione	13·5		0·55	0·82
		1·16		
5β-Pregnane-3,11,20-trione	15·7		0·28	0·61
		1·03		
Pregn-4-ene-3,20-dione	16·2		0·47	0·78
		1·10		
5α-Pregnane-3,11,20-trione	17·8		0·20	0·47
		1·15		
Pregn-4-ene-3,11,20-trione	20·4		–	–
		1·12		
Pregn-4-ene-3,6,20-trione	22·8		0·21	0·56
		3·34		
Cholest-4-ene-3,6-dione	76·0		0·78	0·85

an accuracy of 5%. The accuracy of measurement of the same compounds present in a mixture depended on their relative quantities. The gas–liquid chromatograms of two such mixtures are shown in Fig. 2 and their quantitative evaluation is given in Table 2. The quantitative estimate was accurate even when two components were not completely resolved, provided that they were present in similar amounts. A minor component not completely separated from a major one was, however, seriously overestimated. A peak with the retention time of 21·2 min. was seen in all chromatograms (peak *X* in Fig. 2); its origin remains unknown. Impurities with retention times of less than 6 min. (see Fig. 2*b*) were frequently found even when pure compounds were chromatographed. The frequency of their occurrence was greatly diminished by briefly flaming the gauzes before use. Even then unaccountable peaks in the 0–6 min. region were occasionally observed.

Qualitative analysis of urines

Analyses reported in the present paper were performed either according to the basic scheme outlined in the introductory section or according to a modified scheme in which the initial reduction with borohydride is left out. In all instances sodium bismuthate was used for the cleavage of glycols. The end fractions (KK and KA) were resolved on paper in the system E_4 (Eberlein & Bongiovanni, 1955), and the chromatograms viewed in ultraviolet light (approx. 250 mμ) and then treated with the Zimmermann reagent. Assignations of structure are tentative and in most instances incomplete. They are based on chromatographic mobilities and response to the tests applied. No attempt was made to distinguish between 5-epimers: thus a chromatographic fraction referred to as androstane-3,17-dione may be the 5α- or the 5β-epimer or a mixture of the two. The quantities of the chromatographic

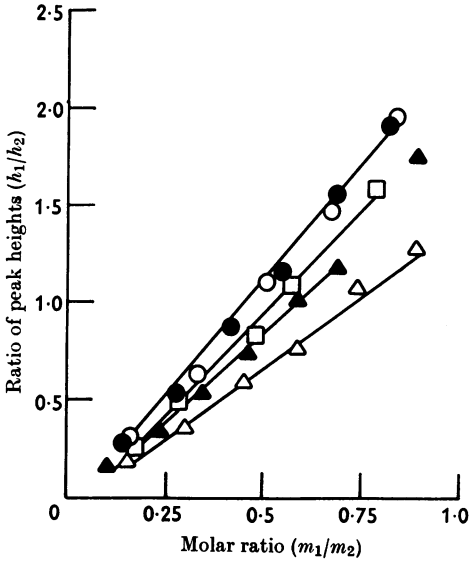


Fig. 1. Calibration curves for the gas–liquid-chromatographic determination of the major components of fractions KK and KA. Experimental details are given in the text. h_1, Peak height of compound measured; h_2, peak height of pregnanediol diacetate; m_1, moles of compound measured; m_2, moles of diacetoxypregnane. ○, 5α-Androstane-3,17-dione; ●, 5β-androstane-3,17-dione; △, 5α-androstane-3,11,17-trione; ▲, 5β-androstane-3,11,17-trione; □, 5β-pregnane-3,20-dione.

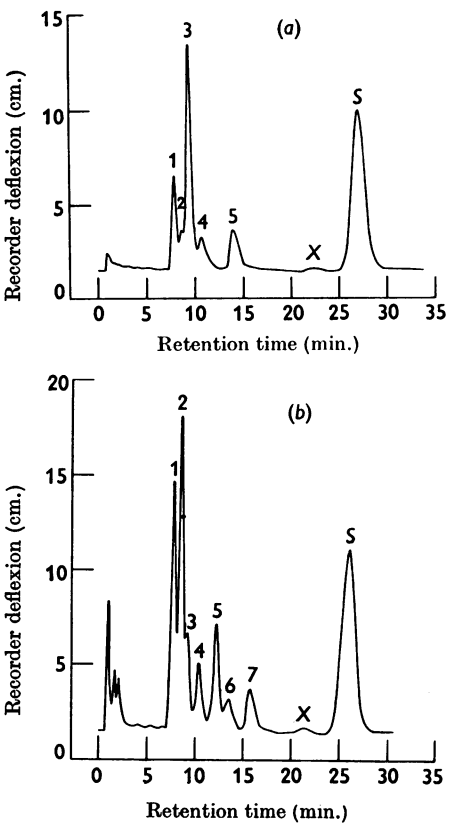


Fig. 2. Gas–liquid chromatography of mixtures of pure steroids. Experimental details are given in the text. *S*, Internal standard (3α,20α-diacetoxy5-β-pregnane; 3·06μg.); *X*, unidentified impurity (see the text). (*a*) 1, 5β-Androstane-3,17-dione (0·61μg.); 2, 5α-androstane-3,17-dione (0·23μg.); 3, 5β-androstane-3,11,17-trione (1·61μg.); 4, 5α-androstane-3,11,17-trione (0·31μg.); 5, androst-4-ene-3,6,17-trione (0·96μg.). (*b*) 1, 5β-Androstane-3,17-dione (1·22μg.); 2, 5α-androstane-3,17-dione (1·41μg.); 3, 5β-androstane-3,11,17-trione (0·55μg.); 4, 5α-androstane-3,11,17-trione (0·57μg.); 5, 5β-pregnane-3,20-dione (0·76μg.); 6, 5α-pregnane-3,20-dione (0·28μg.); 7, 5β-pregnane-3,11,20-trione (0·60μg.).

fractions detected are roughly indicated by: (+++), (++), (+) and (±), the last symbol denoting very weak spots. Allowance should be made for the fact that the response to the Zimmermann test varies with the compound tested and that, in our hands, the sensitivity of the test was variable.

Basic scheme. Results are recorded in Table 3. Analysis of urine 1 (from a healthy non-pregnant woman) gave androstane-3,11,17-trione in fraction KK (+++) and in fraction KA (++), and androstane-3,17-dione in fraction KK (+) and in fraction KA (+++). This result accounts for the most abundant neutral steroids normally encountered in the human urine, the tetrahydro and hexahydro derivatives of cortisol giving rise to fraction-KK 3,11,17-triones, 5β-pregnane-3α,17α,20α-triol to fraction-KK 5β-3,17-dione, 11-oxygenated 17-oxo steroids to fraction-KA 3,11,17-triones, and saturated 11-deoxy-17-oxo steroids to fraction-KA 3,17-diones. An exception is the 17-deoxy corticosteroids, which, though excreted by man in substantial quantities (Exley, Ingall, Norymberski & Woods, 1961), escaped detection through their conversion into carboxylic acids. Fraction KK of urine 1 contained an unknown component (++) with R_F 0·24. A substance of similar mobility (R_F 0·28) was present in fraction KA; in the system B_3 (Bush, 1952) it had R_F 0·13. Since the R_F values of keto steroids are higher in the B_3 system than in the E_4 system (see Table 1) it seems unlikely that the unknown substance is a steroid. It was not detected in any other urine. In fraction KK of urine 2 (from another healthy non-pregnant woman) androst-4-ene-3,11,-17-trione (+) was detected, presumably derived from urinary cortisol and its metabolites with unchanged ring A. Saturated 3,11,17-triones and 3,17-diones were present in the same relative

Table 2. *Measurement of components in two mixtures of different composition*

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

Compound	Separation factor	Mixture 1 Content (μg.)	Mixture 1 Determined mean ± S.D. (μg.)	Mixture 2 Content (μg.)	Mixture 2 Determined mean ± S.D. (μg.)
5β-3,17-Dione		0·61	0·63 ± 0·01	1·22	1·26 ± 0·04
	1·10				
5α-3,17-Dione		0·23	0·35 ± 0·00	1·41	1·55 ± 0·06
	1·09				
5β-3,11,17-Trione		1·61	1·71 ± 0·07	0·55	0·72 ± 0·02
	1·14				
5α-3,11,17-Trione		0·31	0·38 ± 0·01	0·57	0·63 ± 0·02
	1·16				
5β-3,20-Dione		—	—	0·76	0·73 ± 0·01

Table 3. *Paper chromatography of fractions KK and KA in the system E_4*

Urines 1 and 2 were from non-pregnant women, urine 3 from a pregnant woman. Experimental details are given in the text.

Urine	Initial treatment	Fraction	Size of sample*	Δ⁴-3,11,17-Trione	3,11,17-Triones	3,17-Diones	3,20-Diones	Other components
1	None	KK	0·005	−	+++	+	−	R_F 0·24 (++)
		KA	0·01	−	++	+++	−	R_F 0·28 (++)
2	Ketodase	KK	0·01	+	+++	+	−	None
		KA	0·01	−	−	−	−	None
2	None	KK	0·01	+	+++	+	−	None
		KA	0·01	−	−	−	−	None
3	*Helix pomatia*	KK†	0·01	+	+++	++	−	None
		KA†	0·01	−	++	+	+++	None
3	None	KK	0·01	+	+++	++	−	None
		KA	0·02	−	++	+	+++	None
3	Ethyl acetate extract	KK	0·05	+++	+++	−	−	None
		KA	0·10	−	−	+++	+++	Cholest-4-ene-3,6-dione (+++)

* As fraction of a 24 hr. specimen.

† The fraction was resolved also by gas–liquid chromatography (see the text).

amounts as in the corresponding fraction of urine 1. No ketones were detected in the KA fraction of urine 2: a demonstration of an unusually low content of urinary 17-oxo steroids. Analysis of steroids liberated by β-glucuronidase and extracted from the same urine gave results identical with those obtained by the direct analysis of the urine.

Analysis of a pregnancy urine (urine 3) revealed significant and expected changes in the composition of both end fractions. Fraction KK contained more androstane-3,17-dione (increased urinary pregnanetriol and tetrahydro-11-deoxycortisol), and fraction KA contained very large amounts of pregnane-3,20-dione (increased urinary pregnanediol and pregnanolone). Direct analysis of this urine gave the same result as analysis of an extract obtained after treatment with the mixed enzymes of *Helix pomatia*. Gas–liquid chromatography of fraction KK gave (in order of emergence from the column and with assignations of structure based on retention times) a large peak of 5β-androstane-3,17-dione with a shoulder at its base indicating a small quantity of the 5α-epimer, a large peak of 5β-androstane-3,11,17-trione, a small peak of 5α-androstane-3,11,17-trione, a small peak of either 5β-pregnane-3,20-dione or androst-4-ene-3,11,17-trione and a small peak of either 5α-pregnane-3,20-dione or androst-4-ene-3,6,17-trione (see Table 1). No other components were detected in this fraction. The last two peaks recorded were of the same magnitude. However, if the last peak was 5α-pregnane-3,20-dione derived from the corresponding

diol(s) 'spilled over' into the ketonic fraction K, then it should have been accompanied by a much larger amount of its 5β-epimer. It seems altogether more likely that the two peaks represent androst-4-ene-3,11,17-trione and androst-4-ene-3,6,17-trione respectively, which were not resolved by paper chromatography. Fraction KA gave a peak of 5β-androstane-3,17-dione, a peak of similar height of the 5α-epimer, a small peak of 5α-androstane-3,11,17-trione, a large peak of an unknown compound, a very large peak of 5β-pregnane-3,20-dione, a substantial peak of the 5α-epimer and a small peak of pregn-4-ene-3,6,20-trione. No other components were detected in this fraction. Except for the large unidentified peak of fraction KA, there is good agreement between the results of paper and gas–liquid chromatography, which strongly supports the structures assigned to the major components of the two end fractions.

Paper chromatography of fraction KK obtained from an extract of unconjugated steroids of the same pregnancy urine (urine 3) revealed the presence of approximately equal amounts of androst-4-ene-3,11,17-trione and of the corresponding saturated trione. Fraction KA contained large amounts of androstane-3,17-dione, pregnane-3,20-dione and cholest-4-ene-3,6-dione. This finding suggests that the urine contained substantial amounts of unconjugated disubstituted derivatives of androstane and pregnane as well as cholesterol.

Modified scheme. When initial reduction with borohydride is omitted from the basic analytical procedure, fraction KK will be expected to contain keto steroids derived not only from 17-hydroxy corticosteroids but also from all other steroids with one or more reactive carbonyl groups, and fraction KA to contain only compounds derived from steroids lacking both vicinal oxygen functions and reactive carbonyl groups. The modified procedure was applied to the analysis of four urines, three of which had already been analysed according to the basic scheme. The results recorded in Table 4 offer a few points of special interest. Detection of androstane-3,11,17-trione in fraction KA of two pregnancy urines (urines 3 and 4) and of androstane-3,17-dione in one of them demonstrates the presence of 17-hydroxyandrostanes in these urines. This is in contrast with the absence of ketones from the same fraction of urines from two non-pregnant women (urines 1 and 2), though in urine 2 the negative finding is not significant in view of the exceptionally low concentration of all 17-oxygenated androstanes (see Table 3). The large amount of pregnane-3,20-dione in fraction KK of both pregnancy urines is presumably due to urinary pregnanolone.

Comparison of these results (Table 4) with those recorded in Table 3 shows that omission of the reductive step gives rise to increased amounts of androst-4-ene-3,11,17-trione and to the detection of androst-4-ene-3,17-dione, as well as of another ultraviolet-absorbing substance with R_F 0·05. The apparent discrepancy is readily understood when one considers that urinary 4-en-3-ones are reduced by borohydride to corresponding allylic alcohols, which are likely to undergo dehydration at three

Table 4. *Paper chromatography in the system E_4 of fractions KK and KA derived from analyses in which treatment with borohydride was omitted*

Urines 1 and 2 were from non-pregnant women, urines 3 and 4 were from pregnant women. Experimental details are given in the text.

Urine	Initial treatment	Fraction	Size of sample*	Δ⁴-3,11,17-Trione	3,11,17-Triones	3,17-Diones	3,20-Diones	Other components
1	None	KK	0·005	−	+++	+++	−	R_F 0·24 (++)
		KA	0·025	−	−	−	−	None
2	Ketodase	KK	0·005	++	+++	+	−	None†
		KA	0·01	−	−	−	−	None
2	None	KK	0·005	++	+++	++	−	None
		KA	0·01	−	−	−	−	None
3	*Helix pomatia*	KA	0·0025	++	+++	+++	++	R_F 0·05 (±) and Δ⁴-3,17-dione (±)
		KA	0·033	−	+	±	+++	None
3	None	KK	0·0033	++	+++	+++	++	R_F 0·05 (±)
		KA	0·01	−	+	+	+++	Δ⁴-3,17-Dione (±)
4	*Helix pomatia*	KK	0·01	+	+++	+++	++	R_F 0·05 (±) and Δ⁴-3,17-dione (±)
		KA	0·01	−	+	−	+++	None

* As fraction of a 24 hr. specimen.

† Androst-4-ene-3,6,17-trione, pregn-4-ene-3,6,20-trione and another 4-ene-3,6-dione were found in a larger sample of this fraction (see the text).

subsequent stages of the assay: during treatment with bismuthate in acetic acid, during treatment with the Girard T reagent and during cleavage of the Girard derivatives with mineral acid. In fact, after treatment of pure androst-4-ene-3β,17β-diol with the Girard reagent only 60% of the unchanged material could be accounted for. Preliminary experiments suggest that even greater losses occur on exposure to mineral acid. It follows that the basic procedure accounts only for a small fraction of urinary 4-en-3-ones. Detection of trace amounts of androst-4-ene-3,17-dione in fraction KA of urine 3 suggests the presence of a androst-4-ene-3,17-diol in that urine. The unsaturated dione was not detected in a larger sample of fraction KA of the same urine after its treatment with the enzymes of *Helix pomatia*. Although no explanation is needed for the failure to detect a component present in amounts close to its limit of detection, the negative finding could be attributed to dehydration of the allylic alcohol during incubation (48hr. at 37° and pH 5) or possibly to its oxidation, which would also explain the detection of the unsaturated dione in the KK fraction.

A large sample (corresponding to 0·05 of a 24hr. specimen) of the KK fraction of enzyme-treated urine 3 was partitioned between ether and N-sodium hydroxide to separate any 4-ene-3,6-diones from other components (Menini & Norymberski, 1962*b*). Paper chromatography in the system E_4 of the alkali-soluble (enolic) fraction gave three ultraviolet-absorbing spots that were separately eluted and rechromatographed in the system B_3. Two of them had R_F values identical with those of andost-4-ene-3,6,17-trione and pregn-4-ene-3,6,20-trione respectively. The R_F values of the third substance were close to those calculated for androst-4-ene-3,6,11,17-tetrone. All three substances gave with cold alkali yellow strongly-fluorescing spots characteristic of the 4-ene-3,6-dione system (Menini & Norymberski, 1962*b*). The presumptive 3,6,11,17-tetraone may have derived from 6β-hydroxycortisol of pregnancy urine. Formation of the two other compounds containing the conjugated enedione fragment was already indicated by gas–liquid chromatography of the products obtained from the same urine by analysis according to the basic scheme. Pregn-4-ene-3,6,20-trione was then found in the KA fraction and therefore presumably derived either from a pregn-4-ene oxygenated at positions 3, 6 and 20 or from a pregn-5-ene oxygenated at positions 3 and 20. Androst-4-ene-3,6,17-trione was then found in the KK fraction and therefore presumably derived either from a pregn-4-ene oxygenated at positions 3, 6, 17 and 20 (and possibly 21) or from a pregn-5-ene oxygenated at positions 3, 17 and 20 (and possibly 21). The question remained whether dehydroepiandrosterone (a common constituent of urine) contributed to the formation of androst-4-ene-3,6,17-trione found in the KK fraction of the modified scheme. It should have been present in the KA fraction of the basic scheme but may have been obscured by androst-4-ene-3,11,17-trione in the paper chromatogram and by 5α-pregnane-3,20-dione in the gas–liquid chromatogram (see Table 1). Since dehydroepiandrosterone is excreted largely in the form of its hydrogen sulphate, the compound was sought in a steroid sulphate concentrate prepared from the same urine (0·1 of a 24hr. specimen) and then treated with dioxan (McKenna & Norymberski, 1960). The products of solvolysis contained 285μg. of Zimmermann chromogens. Fraction KA prepared from this material according to the basic scheme contained only 88μg. of Zimmermann chromogens. The loss of 17-oxo steroids is attributed to the reaction of dehydroepiandrosterone with sodium bismuthate (Plantin, Diczfalusy & Birke, 1956; Jørgensen, 1957). Paper chromatography of fraction KA gave only trace amounts of androst-4-ene-3,6,17-trione (the product expected from dehydroepiandrosterone) accompanied by relatively large amounts of androstane-3,17-dione and pregnane-3,20-dione. The detection of the latter compound suggests that the urine contained a sulphate of pregnanediol or of prenanolone, or both.

Quantitative analysis of urines

Urines were treated directly with borohydride, then with sodium bismuthate (or with periodate; see below) and the extracted material was submitted to the other operations of the present analytical scheme. Thus, for reasons stated in the preceding section, loss of information on urinary 5-en-3-ols and 4-en-3-ones is expected. The end fractions were resolved by gas–liquid chromatography and their components determined as described above.

Analysis with the use of sodium bismuthate for the cleavage of glycols. Urines from five normal subjects (three male and two female) were analysed. In each case gas–liquid chromatography of the end fractions gave only five major components: 5β-androstane-3,17-dione, 5α-androstane-3,17-dione, 5β-androstane-3,11,17-trione, 5α-androstane-3,11,17-trione and 5β-pregnane-3,20-dione. Paper chromatography confirmed the presence of the 17-oxo steroids but failed to detect the 3,20-dione or any other component. Tetrahydrocortisone added to urine was accounted for by the increase of the Zimmermann chromogens in the KK fraction (71%) and by the increase of the gas–liquid-chromatographic peak of 5β-androstane-3,11,17-trione (77%). Recovery of androsterone was appreciably lower, and those of 11-oxoaetiocholanolone and of 5β-pregnane-3α,20α-diol were very

Table 5. *Recovery of steroids added to urine when sodium bismuthate was used as glycol-cleaving agent*

Experimental details are given in the text.

Compound	No. of experiments	Amount added (μg./ml.)	Percentage recovery (mean ± S.D.) ZM*	GLC†
Tetrahydrocortisone	5	2–10	71 ± 2	77 ± 2
Androsterone	3	6–10	59 ± 3	67 ± 1
11-Oxoaetiocholanolone	1	6	35	—
5β-Pregnane-3α,20α-diol	2	1, 3	—	46, 54

* Calculated from the increase of Zimmermann chromogens in the appropriate fraction (KK or KA).
† Calculated from the gas–liquid-chromatographic peak of the expected product.

Table 6. *Recovery of steroids added to urine when periodate was used as glycol-cleaving agent*

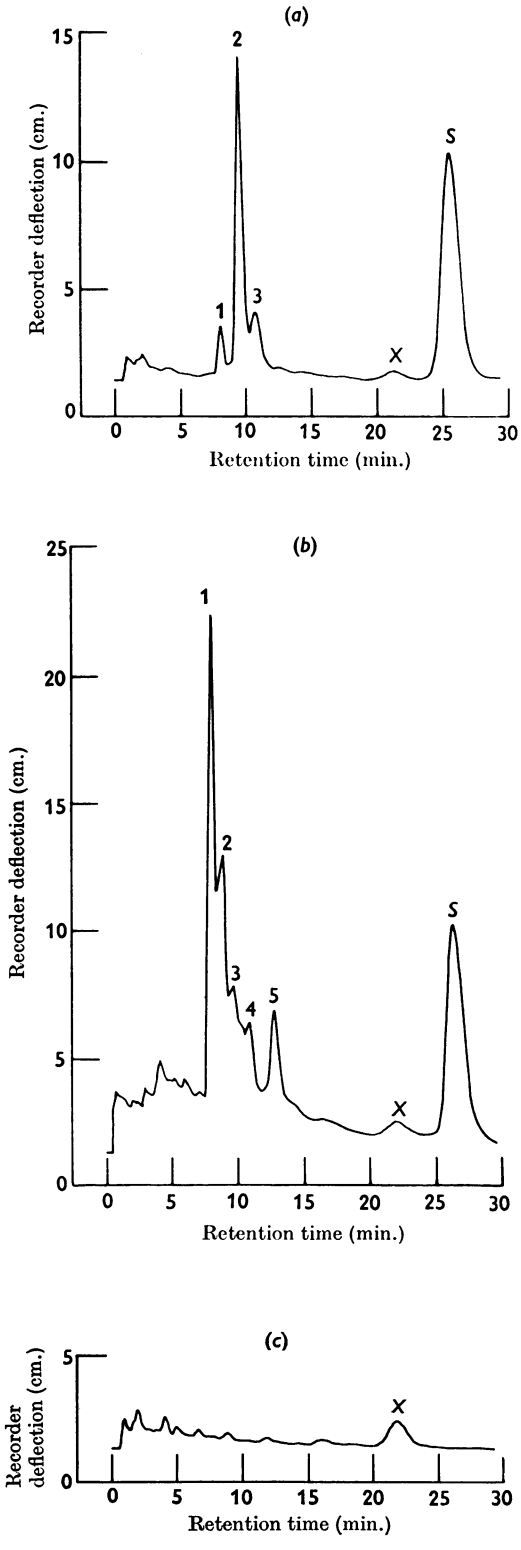
Experimental details are given in the text.

Compound	Girard method*	No. of experiments	Amount added (μg./ml.)	Percentage recovery (mean ± S.D.) ZM†	GLC‡
Tetrahydrocortisone	1	4	5–10	89 ± 7	72 ± 1
Androsterone	1	5	6–16	93 ± 7	87 ± 2
11-Oxoaetiocholanolone	1	5	6–10	79 ± 3	72 ± 4
5β-Pregnane-3α,20α-diol	1	7	10–18	—	41 ± 2
	2	2	—	—	69, 88

* The non-ketonic fraction was extracted from a neutral solution (method 1) or from a strongly alkaline solution (method 2).
† Calculated from the increase of Zimmermann chromogens in the appropriate fraction (KK or KA).
‡ Calculated from the gas–liquid-chromatographic peak of the expected product.

low (see Table 5). Each model compound gave rise to the increase of only the expected gas–liquid-chromatographic peak in the appropriate end fraction. The low recovery of 11-oxoaetiocholanolone was due to the loss of its reduction product (5β-androstane-3α,11β,17β-triol) during treatment with sodium bismuthate. This was shown by treating the pure compound with borohydride and then with a large excess of sodium bismuthate: the extracted material was oxidized with *tert.*-butyl chromate and the formed 5β-androstane-3,11,17-trione determined. It accounted for only 17% of the starting material. Reduction of the surplus reagent with sodium metabisulphite and extraction of the homogeneous solution failed to improve the recovery. A possible explanation of this finding is that 5β-androstane-3α,11β,17β-triol formed a water-soluble bismuth complex.

Analysis with the use of periodate for the cleavage of glycols. Oxidations with periodate were carried out essentially according to the method of Few (1961). All other operations were as before. Tetrahydrocortisone, androsterone and 11-oxoaetiocholanolone added to urine were accounted for satisfactorily by their respective reaction products (see Table 6). In contrast, the recovery of 5β-pregnane-3α,20α-diol was poor (41%). This could be attributed to the conditions used for the Girard separation: namely, the extraction of the non-ketonic fraction from a neutral solution. Though the Girard derivatives of 17-ketones are stable in neutral solution and therefore quantitatively separated (Brooks & Norymberski, 1953), those of saturated 3-ketones and 20-ketones are apparently partly hydrolysed since (as now found) only 60–70% of 5β-pregnane-3,20-dione was recovered in the ketonic fraction. However, the recovery of the dione was quantitative when the reaction mixture was made strongly alkaline before extraction of the non-ketonic fraction. Under the latter conditions, 5β-pregnane-3α,20α-diol added to urine was satisfactorily accounted for by the measurement of the corresponding dione formed during the assay (see Table 6). Unfortunately, the recorded values



for 5β-pregnane-3,20-dione (Tables 7, 8 and 9) were obtained before the Girard separation was modified and, in consequence, they give a gross underestimate of the compound's urinary precursors.

Analyses were performed on urines from seven healthy women, five healthy men and four subjects with adrenal dysfunction. Gas–liquid chromatography revealed the presence of the following major constituents of the end fractions. Fraction KK: 5β-androstane-3,17-dione, 5β-androstane-3,11,17-trione and 5α-androstane-3,11,17-trione; fraction KA: the three constituents of fraction KK and, in addition, 5α-androstane-3,17-dione and 5β-pregnane-3,20-dione (see Fig. 3). Constituents with shorter retention times (0–6min.) varied in number and quantity. They may comprise compounds of urinary origin and accidental impurities but not steroids other than monoketones, e.g. 5α-androst-16-en-3-one derived from urinary 5α-androst-16-en-3α-ol. As in the chromatography of pure compounds, variable amounts of an impurity with the retention time of 21·2min. were found regularly. Other components were seen usually in only minute amounts. To assess their significance process blanks were prepared for the two end fractions by submitting water to all the operations of the analytical procedure. Their chromatograms (that for fraction KK is shown in Fig. 3c) showed only few very small peaks in the chromatographic region investigated.

Table 7 gives the results of quantitative analysis of urines from healthy women. The presence of trace components is indicated but, since the size of the sample chromatographed varied by up to a factor 10, not too much significance should be read into the fact that the end fractions from some urines contained more trace components than others. In one case, the same urine was analysed with periodate and sodium bismuthate respectively. The two methods gave a similar estimate of the components of the KK fraction: even the largest difference (24% of the mean, in the estimate of 5β-androstane-3,11,17-trione) is not considered

Fig. 3. Analysis of a normal female urine (urine 5b, Table 7). Experimental details are given in the test. *S*, Internal standard (3α,20α-diacetoxy-5β-pregnane; 3·06μg.); *X* unidentified impurity (see the text). (*a*) KK fraction corresponding to 0·0006 of a 24hr. specimen. Tentative assignation of structure: 1, 5β-androstane-3,17-dione; 2, 5β-androstane-3,11,17-trione; 3, 5α-androstane-3,11,17-trione. (*b*) KA fraction corresponding to 0·002 of a 24hr. specimen. Tentative assignation of structure: 1, 5β-androstane-3,17-dione; 2, 5α-androstane-3,17-dione; 3, 5β-androstane-3,11,17-trione; 4, 5α-androstane-3,11,17-trione; 5, 5β-pregnane-3,20-dione. (*c*) KK fraction of a process blank.

Table 7. *Analysis of urines of healthy women*

Experimental details are given in the text.

Urine....	5a*†	5b*	6*	7*	8a*	8b	9	10	11	Mean‡	Range
Fraction KK											
5β-3,17-Dione [mg./24hr. (% of total)]	0·73 (18)	0·67 (14)	0·54 (12)	1·01 (17)	0·66 (6)	0·76 (8)	0·93 (14)	0·30 (11)	0·75 (12)	0·70 (12)	0·30–1·01 (6–18)
5β-3,11,17-Trione [mg./24hr. (% of total)]	2·48 (60)	3·15 (68)	3·44 (73)	3·94 (65)	7·80 (76)	7·05 (72)	4·85 (76)	2·13 (77)	4·65 (75)	4·22 (73)	2·13–7·42 (65–77)
5α-3,11,17-Trione [mg./24hr. (% of total)]	0·91 (22)	0·83 (18)	0·68 (15)	1·07 (18)	1·82 (18)	2·00 (20)	0·64 (10)	0·33 (12)	0·80 (13)	0·89 (15)	0·33–1·91 (10–19)
Total (mg./24hr.)	4·1	4·6	4·7	6·0	10·3	9·8	6·4	2·8	6·2	5·8	2·8–10·0
Total ZM found (mg./24hr.)§	5·8	6·1	6·2	7·2	12·9	14·1	10·1	3·6	8·7	7·9	3·6–13·5
Total ZM calc. [mg./24hr. (% of total found)]‖	4·0 (78)	5·7 (93)	5·8 (94)	7·3 (101)	12·9 (100)	12·4 (88)	7·9 (78)	3·5 (97)	7·7 (89)	7·2 (91)	3·5–12·6 (78–101)
Other components:											
t_R 6·6min.	−	−	−	−	−	−	+	+	+		
t_R 11·9min.	−	−	+	+	+	+	+	+	+		
t_R 13·5min.	−	−	−	−	−	−	−	+	−		
t_R 15·7min.	−	−	−	+	−	−	−	+	+		
Fraction KA											
5β-3,17-Dione [mg./24hr. (% of total)]	0·75 (33)	1·54 (36)	1·40 (36)	1·47 (27)	0·96 (32)	1·00 (32)	3·89 (62)	1·41 (35)	1·77 (37)	1·78 (38)	0·98–3·89 (27–62)
5α-3,17-Dione [mg./24hr. (% of total)]	0·49 (22)	0·97 (22)	0·71 (18)	1·44 (27)	0·48 (16)	0·48 (16)	0·94 (15)	0·79 (20)	0·74 (15)	0·87 (18)	0·48–1·44 (15–27)
5β-3,11,17-Trione (mg./24hr. (% of total)]	0·43 (19)	0·61 (14)	0·59 (15)	0·75 (14)	0·56 (19)	0·58 (19)	0·58 (9)	0·92 (23)	1·09 (23)	0·73 (17)	0·57–1·09 (9–23)
5α-3,11,17-Trione (mg./24hr. (% of total)]	0·26 (12)	0·60 (14)	0·40 (10)	0·62 (12)	0·44 (14)	0·42 (14)	0·53 (8)	0·66 (16)	0·70 (15)	0·56 (13)	0·40–0·70 (8–16)
5β-3,20-Dione [mg./24hr. (% of total)]	0·31 (14)	0·60 (14)	0·80 (21)	1·06 (20)	0·56 (19)	0·60 (19)	0·36 (6)	0·24 (6)	0·50 (10)	0·59 (14)	0·24–1·06 (6–21)
Total (mg./24hr.)	2·2	4·3	3·9	5·9	3·0	3·1	6·3	4·0	4·8	4·5	3·0–6·3
Total ZM found (mg./24hr.)§	2·5	4·4	4·4	6·1	3·9	3·9	7·2	4·4	5·5	5·1	3·9–7·2
Total ZM calc. [mg./24hr. (% of total found)]‖	2·0 (80)	3·8 (86)	3·1 (71)	4·4 (73)	2·6 (67)	2·6 (67)	5·5 (76)	4·0 (91)	4·5 (82)	4·0 (78)	2·6–5·5 (67–91)
Other components:											
t_R 9·6min.	−	+	−	−	+	+	−	−	−		
t_R 11·5min.	−	−	−	−	+	+	−	−	−		
t_R 13·5min.	+	+	+	+	+	+	+	+	+		
t_R 15·7min.	+	+	−	+	+	−	+	+	+		

* Urine freed from unconjugated steroids by extraction with chloroform.
† Sample analysed with the use of sodium bismuthate as glycol-cleaving agent; periodate was used in all other instances.
‡ Results for sample 5a were excluded; those for samples 8a and 8b were pooled.
§ Zimmermann chromogens (ZM) in terms of dehydroepiandrosterone.
‖ Calculated from the amount of each compound determined by gas–liquid chromatography and from its colour equivalent in the Zimmermann reaction.

Table 8. *Analysis of urines of healthy men*

Experimental details are given in the text.

Urine.....	12	13*	14*	15	16	Mean	Range
Fraction KK							
5β-3,17-Dione [mg./24hr. (% of total)]	2·13 (18)	1·34 (11)	1·31 (14)	0·96 (15)	2·14 (91)	1·58 (15)	0·96–2·14 (11–19)
5β-3,11,17-Trione [mg./24hr. (% of total)]	7·06 (60)	7·91 (62)	6·65 (70)	4·55 (70)	7·51 (69)	6·74 (69)	4·55–7·91 (60–70)
5α-3,11,17-Trione [mg./24hr. (% of total)]	2·62 (22)	3·53 (27)	1·47 (16)	0·97 (15)	1·25 (12)	1·97 (20)	0·97–3·53 (12–27)
Total (mg./24hr.)	11·8	12·8	9·4	6·5	10·9	10·3	6·5–12·8
Total ZM found (mg./24hr.)†	18·8	15·8	13·4	11·9	16·8	15·3	11·9–16·8
Total ZM calc. (mg./24hr. (% of total found)]‡	14·3 (78)	15·9 (101)	11·6 (87)	8·0 (67)	13·2 (79)	12·6 (82)	8·0–15·9 (67–101)
Other components:							
t_R 6·6min.	+	−	−	+	+		
t_R 11·9min.	+	+	−	+	+		
t_R 13·5min.	+	+	−	+	+		
Fraction KA							
5β-3,17-Dione [mg./24hr. (% of total)]	3·60 (32)	4·29 (47)	2·38 (39)	2·18 (52)	4·83 (52)	3·46 (43)	2·18–4·83 (32–52)
5α-3,17-Dione [mg./24hr. (% of total)]	4·48 (40)	2·29 (25)	1·58 (26)	1·11 (25)	2·02 (22)	2·29 (29)	1·11–4·48 (22–40)
5β-3,11,17-Trione [mg./24hr. (% of total)]	1·23 (11)	1·04 (11)	0·95 (16)	0·45 (10)	0·75 (8)	0·88 (10)	0·45–1·23 (8–16)
5α-3,11,17-Trione (mg./24hr. (% of total)]	1·17 (11)	0·91 (10)	0·93 (15)	0·49 (11)	1·04 (11)	0·91 (12)	0·49–1·17 (10–15)
5β-3,20-Dione [mg./24hr. (% of total)]	0·64 (6)	0·63 (7)	0·25 (4)	0·19 (4)	0·56 (6)	0·45 (6)	0·19–0·64 (4–7)
Total (mg./24hr.)	11·1	9·2	6·1	4·4	9·2	8·0	4·4–11·1
Total ZM found (mg./24hr.)†	12·2	10·0	8·5	5·5	9·0	9·4	5·5–12·2
Total ZM calc. [mg./24hr. (% of total found)]‡	10·3 (84)	8·2 (82)	5·9 (70)	4·1 (75)	8·2 (91)	7·3 (80)	4·1–10·3 (70–91)
Other components:							
t_R 13·5min.	+	−	+	+	+		
t_R 15·7min.	−	−	+	−	+		

* Urine freed from unconjugated steroids by extraction with chloroform.

† Zimmermann chromogens (ZM) in terms of dehydroepiandrosterone.

‡ Calculated from the amount of each compound determined by gas–liquid chromatography and from its colour equivalent in the Zimmermann reaction.

significant for a single comparison (see below). In contrast, consistently and significantly higher values were obtained for components of fraction KA when periodate was used. When another urine was analysed before and after removal of unconjugated steroids, the results were not significantly different. The major components accounted, on the average, for 91% of the Zimmermann chromogens determined in fraction KK and for 78% of those in fraction KA. Similar values were found for other subjects (Tables 8 and 9). Components of the end fractions were found in quantities varying within wide ranges, but their relative contributions to each fraction were on the whole much more constant. Thus the major component of fraction KK (5β-androstane-3,11,17-trione) was found in the range 2·1–7·4mg./24hr. as compared with the range of its contribution to the total fraction of 65–77%. The corresponding values for the major component of fraction KA (5β-androstane-3,17-dione) are 1·0–3·9mg./24hr. and 27–62% respectively. These values are, however, weighted by one unusual result (urine 9); when this is ignored, the probably more representative values of 1·0–1·8mg./24hr. and 27–38% respectively are obtained. Another unusual result was the finding of appreciable amounts of three components of fraction KK from urine 11 with retention times of 6·6min., 11·9min. (androst-4-ene-3,11,17-trione?) and 15·7min. respectively. A minor constituent with the retention time of 5α-pregnane-3,20-dione was found regularly in the KA fraction. Analysis of urines from male subjects (Table 8) did not reveal any special features in the composition of the end fractions.

Table 9 records the results for pathological urines. In one case of adrenal hyperplasia (urine 18) the composition of the end fractions was normal. The two other cases of the same condition were characterized by abnormally large amounts of KK-fraction components and one of them by large amounts of KA-fraction components as well. In both cases the 5β-/5α-androstane-3,11,20-trione ratio in the KA

Table 9. *Analysis of pathological urines*

Experimental details are given in the text.

Urine.....	17	18	19	20
Condition.....	Adrenal hyperplasia	Adrenal hyperplasia	Adrenal hyperplasia	Virilizing adrenal tumour
Fraction KK				
5β-3,17-Dione [mg./24hr. (% of total)]	16·0 (14)	0·75 (7)	2·3 (7)	15·1 (39)
5β-3,11,17-Trione [mg./24hr. (% of total)]	80·7 (72)	8·65 (76)	28·6 (81)	18·3 (47)
5α-3,11,17-Trione [mg./24hr. (% of total)]	15·3 (14)	1·90 (17)	4·3 (12)	5·3 (14)
Total (mg./24hr.)	112·0	11·3	35·3	38·7
Total ZM found (mg./24hr.)*	173·0	18·1	38·7	48·3
Total ZM calc. [mg./24hr. (% of total found)]†	138·2 (80)	14·4 (80)	44·6 (114)	42·9 (89)
Other components:				
t_R 6·5min.	−	+	−	−
t_R 11·8min.	+	+	+	+
t_R 13·5min.	−	−	−	+
t_R 15·7min.	−	+	+	+
Fraction KA				
5β-3,17-Dione [mg./24hr. (% of total)]	12·1 (25)	1·26 (42)	2·3 (27)	39·2 (34)
5α-3,17-Dione [mg./24hr. (% of total)]	4·4 (9)	0·65 (22)	1·0 (12)	21·3 (18)
5β-3,11,17-Trione [mg./24hr. (% of total)]	20·6 (43)	0·47 (16)	3·0 (36)	15·6 (14)
5α-3,11,17-Trione [mg./24hr. (% of total)]	7·6 (16)	0·39 (13)	1·5 (18)	41·0 (35)
5β-3,20-Dione [mg./24hr. (% of total)]	3·5 (7)	0·20 (7)	0·6 (7)	9·8 (9)
Total (mg./24hr.)	48·2	3·0	8·4	126·9
Total ZM found (mg./24hr.)*	51·2	4·0	9·4	146·0
Total ZM calc. [mg./24hr. (% of total found)]†	50·8 (99)	2·8 (70)	8·7 (93)	124·9 (85)
Other components:				
t_R 9·6min.	−	−	−	+
t_R 13·5min.	+	+	+	+
t_R 15·7min.	+	+	+	−

* Zimmermann chromogens (ZM) in terms of dehydroepiandrosterone.

† Calculated from the amount of each compound determined by gas–liquid chromatography and from its colour equivalent in the Zimmermann reaction.

fraction was unusually high, suggesting that the secretion of cortisol increased out of proportion to that of 11β-hydroxyandrost-4-ene-3,17-dione (cf. Dorfman, 1954). The same ratio was abnormally low in a case of virilizing adrenal tumour, and hence preferential increase in the secretion of 11β-hydroxyandrost-4-ene-3,17-dione may be inferred. Another notable result in this case was the large contribution of 5β-androstane-3,17-dione to fraction KK, suggesting exhaustion of the available 11β-hydroxylase.

Duplicate urine analyses were performed in 11 cases. The coefficients of variation between duplicate estimates of the components of fraction KK were: 11% for 5β-androstane-3,17-dione, 8% for 5β-androstane-3,11,17-trione and 7% for 5α-androstane-3,11,17-trione. The five major components of the KA fraction were determined in duplicate only in four cases: out of altogether 20 measurements, the difference between duplicates was more than 20% of the mean in four instances.

No attempt was made to develop the quantitative method into a routine procedure aimed at maximal working efficiency. We estimate that, given good management, one person could analyse 12–16 samples within a working week.

DISCUSSION

The basic analytical scheme proposed in the introductory section and some of its variants were investigated in a qualitative manner, and eventually a quantitative method was developed that differs from the proposed scheme in the use of periodate in place of bismuthate for the cleavage of glycols. The quantitative method terminates with the gas–chromatographic separation and determination of steroidal diones and triones in two fractions (KA and KK) derived from the chemical transformation of urinary steroids. The components of fraction KA do not provide information about the nature of the oxygen functions (keto, α-hydroxy and β-hydroxy groups) of their urinary precursors but, since carbonyl and secondary hydroxyl groups are interchangeable in steroid metabolism, no information is lost about their presumptive secretory precursors. Thus 5β-pregnane-3,20-dione determined in fraction KA must be derived from urinary 3,20-disubstituted 5β-pregnanes, and therefore gives information about the secretion of progesterone at least as well as the customary determination of urinary pregnanediol. In fact, it may be safely assumed that the 5β-pregnane-3,20-dione is derived largely from urinary pregnanediol with a small contribution from pregnanolone. The same type of argument applies to the other major components of fraction KA. They are listed in Table 10 together with their most likely urinary precursors. More detailed information about the urinary precursors of this fraction may be obtained by the reported modification of the basic scheme (omission of the initial reduction with borohydride) which permits

Table 10. *Comparison of present results with some data from the literature*

1, Fotherby & Love (1960); 2, Romanoff, Rodriguez, Seelye & Pincus (1958); 3, Romanoff, Parent, Rodriguez & Pincus (1959); 4, Romanoff, Morris, Welch, Rodriguez & Pincus (1961); 5, Layne, Meyer, Vaishwanar & Pincus (1962); 6, Cost & Vegter (1962); 7, Kellie & Wade (1957); 8, Baulieu, Weinmann & Jayle (1957).

	Present results mg./2(4hr.)			Reported average excretion (mg./24hr.)		
	Men	Women	Major urinary parent compound(s)	Men	Women	Reference
Fraction KK						
5β-3,17-Dione	1·9*	0·8*	Pregnanetriol	1·4	0·9	1
5β-3,11,17-Trione	8·1*	5·0*	Tetrahydrocortisone, tetrahydrocortisol cortols and cortolones	6·4–10·1†	4·8–8·4†	2, 3, 4, 5, 6
5α-3,11,17-Trione	2·4*	1·1*	Allotetrahydrocortisol	0·5–1·4	0·3–1·3	2, 3, 4, 5, 6
Fraction KA						
5β-3,17-Dione	3·5	1·8	Aetiocholanolone‡	3·8, 4·8	2·2, 4·5	7, 8
5α-3,17-Dione	2·3	0·9	Androsterone‡	2·1, 2·9	1·1, 2·8	7, 8
5β-3,11,17-Trione	0·9	0·7	11β-Hydroxy- and 11-oxo-aetiocholanolone‡	1·0	1·3	7
5α-3,11,17-Trione	0·9	0·6	11β-Hydroxy- and 11-oxo-androsterone‡	0·5	0·5	7

* In terms of its urinary parent compound.

† Values calculated from the reported excretion of tetrahydrocortisone and tetrahydrocortisol and from the average contribution of cortols and cortolones (Fukushima *et al.* 1960).

‡ From corresponding glucosiduronic acid only.

differentiation between compounds that contain a reactive carbonyl group and those that do not.

Correlation of components of fraction KK with their urinary and secretory precursors is complicated by the loss of information about the side chain. Strictly speaking, a 17-one detected in this fraction could be derived from any urinary steroid oxygenated at positions 17 and 20, regardless of the length and other substitution of the side chain. However, in the analysis of human urine, it seems unlikely that 5α- and 5β-androstane-3,11,17-trione are derived from precursors other than the saturated metabolites of cortisol with an intact carbon skeleton (Fukushima, Bradlow, Hellman, Zumoff & Gallagher, 1960). A quantitatively significant contribution from the metabolites of 21-deoxycortisol need be considered only in cases of congenital adrenal hyperplasia (Finkelstein, 1962). Similarly, 5β-androstane-3,17-dione of this fraction is most likely derived from urinary metabolites of 17α-hydroxyprogesterone, notably from pregnane-3α,17α,20α-triol. A significant contribution from metabolites of 11-deoxycortisol would occur when the activity of the 11β-hydroxylase were seriously impaired, a situation that would result in disproportionately low values for the 3,11,17-triones. An unequivocal differentiation between pregnanes with oxygen functions at 17 and 20 and those with oxygen functions at 17, 20 and 21 is possible when urinary steroids are reduced with borohydride, the products resolved into three fractions containing respectively triols, tetraols and pentaols (Exley & Norymberski, 1964) and the fractions analysed by the present method (D. Exley, E. Menini & J. K. Norymberski, unpublished work).

In its present form, the quantitative method (direct treatment of urine with borohydride and then with periodate) provides essentially the same information as the separate determination of the glucosiduronic acids of aetiocholanolone, of androsterone, of their 11-oxygenated derivatives, of pregnanediol, of pregnanetriol, of 11-oxygenated 17-hydroxy corticosteroids of the 5β-series and of those of the 5α-series. To account for steroid sulphates (and hence for dehydroepiandrosterone and other 5-en-3β-ols) it is necessary either to hydrolyse the sulphates before analysis or to analyse a sulphate concentrate separately. Both possibilities were investigated with the use of sodium bismuthate as the glycol-cleaving agent: the results were unsatisfactory because of interaction between reagent and the homoallylic system of dehydroepiandrosterone. Periodate affected 5-en-3β-ols to a smaller extent than bismuthate (cf. Few, 1961), and the non-specific action of either reagent could be suppressed under suitable conditions (C. S. Corker & J. K. Norymberski, unpublished work). The quantitative determination of 5-en-3β-ols by the present method therefore seems practicable. Qualitative experiments also indicated that the basic analytical scheme is unsuited to the quantitative determination of steroidal 4-en-3-ones, but that the modified procedure (no initial reduction) might well suit the purpose. Of the known major neutral urinary steroids only 17-deoxy corticosteroids escape detection by the present method, owing to their conversion, via C_{20} aldehydes, into carboxylic acids. Preliminary experiments (D. Exley & J. K. Norymberski, unpublished work) suggest the possibility of separating the formed aldehydes from other components of the analytical sample and therefore of accounting for 17-deoxy corticosteroids as well.

Components of fractions KA and KK were characterized by their retention times on an SE-30 column. Supporting evidence was obtained in many instances by their paper chromatography and in one instance by their gas–liquid chromatography on a QF-1 column (cf. Brooks & Hanaineh, 1963). The absence of major gas–liquid-chromatographic fractions other than those expected from the known major urinary steroids (except one such fraction in the case of pregnancy urine) inspires confidence in the method's specificity. Since a non-discriminating detector was used and since very polar and heat-labile substances were suitably transformed, it is most unlikely that any major steroidal component of the end fractions escaped detection. The most serious shortcoming of the present method is the failure to resolve completely all chromatographic fractions, and hence the possibility of overestimation of the less abundant of the major components and of masking of minor components. The use of longer columns is indicated. Other changes in experimental design (use of temperature programming, of automatic attenuation and of micro-detectors) might well improve the method's accuracy and sensitivity and allow the quantitative determination of minor components.

In Table 10, the present results are compared with the reported normal excretion of the most likely major urinary precursors of the components of fractions KK and KA. On the whole, the agreement is as good as can be expected from a comparison of mean values determined for small groups of subjects. Poorest agreement is found between 5α-androstane-3,11,17-trione of fraction KK and urinary allotetrahydrocortisol. This might be due to the overestimation of the 5α-trione since it was not completely separated from its more abundant 5β-epimer. Determination of 5β-pregnane-3,20-dione in fraction KA is excluded from this comparison because the compound was underestimated in most experiments (see p. 9).

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