A RAPID METHOD FOR THE ESTIMATION OF TOTAL 11-DEOXY-17-OXOSTEROIDS IN URINE

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The main components of the 11-deoxy-17-oxosteroid fraction of human urine are 3β-hydroxy-androst-5-ene-17-one (dehydroepiandrosterone, DHA), 3α-hydroxy-5α-androstan-17-one (androsterone) and 3α-hydroxy-5β-androstan-17-one (aetiocholanolone). Separate estimation of these compounds by paper chromatography or gradient elution from alumina is time-consuming and for certain purposes measurement as a group may serve equally well. This paper describes a rapid method for such an estimation based on modifications of the methods of SAVARD[1] and KELLIE AND WADE[2].

MATERIALS AND APPARATUS

Chemicals

All solvents were of analytical reagent grade, except for "heptane" ("fraction from petroleum" B.D.H.) and were redistilled shortly before use. Ethanol (Burroughs Ltd., A. R. Grade) was kept in the dark over *m*-phenylenediamine for 7 days and then redistilled twice. Neutral alumina (Woelm) was deactivated with 6 % water (w/v). β-Glucuronidase (Powder B) was prepared by the method of DODGSON AND SPENCER[3] from limpets (*Patella vulgata*) and assayed by the method of DODGSON, LEWIS AND SPENCER[4]. The activity found was approx. 900,000 units/g. *m*-Dinitrobenzene (B.D.H. "purified for 17-KS determinations") was further purified by the method of CALLOW, CALLOW AND EMMENS[5]. Ethanolic KOH (2.5 *N*) was prepared by the method of WILSON AND CARTER[6] and stored under N_2 at —20°. Sodium acetate–acetic acid buffer solution (2.5 *M*, pH 4.0) was kept as stock solution and diluted to 0.5 *M* before use.

Chromatographic apparatus

The chromatographic apparatus is shown in Figs. 1 and 2. It is constructed of aluminium alloy and consists of a circular platform (A) supported on three legs (B), two of which have adjustable feet for levelling purposes. Around the top of the platform and 0.2 cm in from the outer edge is channelled a gully (C) 0.5 cm wide and 0.2 cm in depth. This acts as a reservoir and ensures an even supply of eluant to the chromatography papers. Circumscribing the legs and 1½ in. above the base is fitted a circular ring (D) for supporting 10 tapered graduated 10 ml test tubes. On top of the platform and of similar diameter is a detachable ring (E), in the centre of which fits a 4 in. diameter Petri dish (F). The ring is fixed to the platform by means of knurled screws (G).

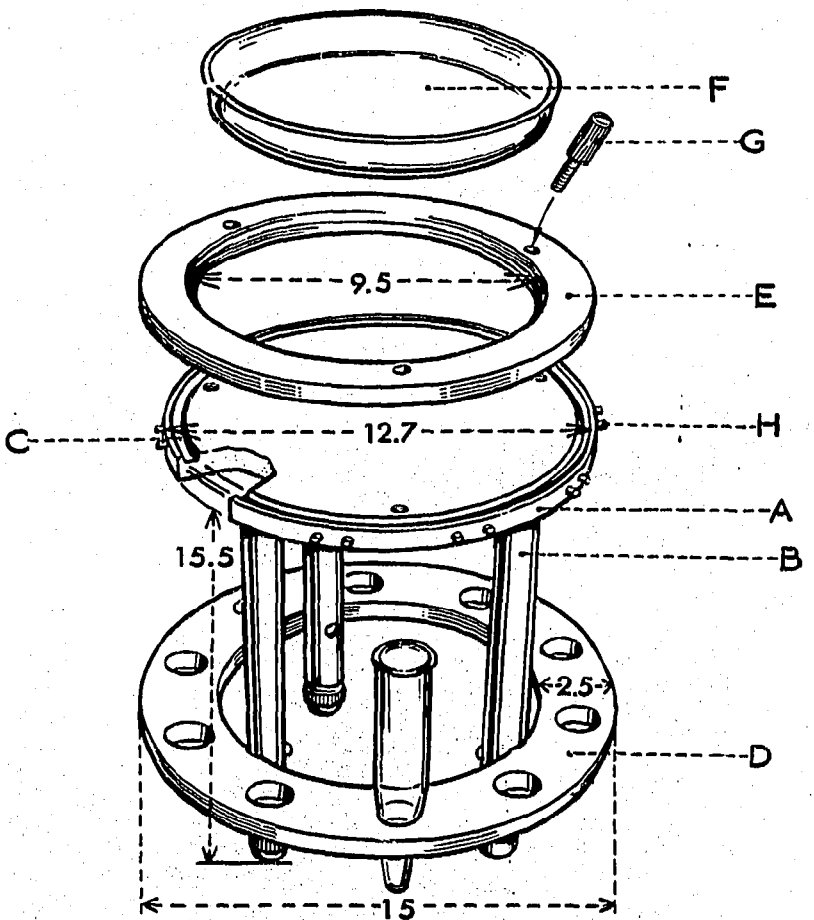

Fig. 1. Exploded diagram of chromatographic apparatus. The important measurements (in cm) are given in the figure or in the text. All other dimensions are not critical. The alphabetical key is explained in the text.

A circular disc of Whatman glass fibre paper is cut to 12 cm diameter and is fitted between the detachable ring (E) and the platform (A), and beneath the Petri dish. Two strips of glass fibre paper (2.5 cm wide) run under the Petri dish and cross at right-angles at the centre. The ends of these strips are looped over the edges of the Petri dish so that they clear the sides (a circular metal supporting frame protruding 2–3 mm above the rim of the dish is helpful but not essential). The ends of the strips are weighed down and held in place by a square of glass in the centre of the dish (see Fig. 2). If the wet paper strips which feed solvent to the circular disc of glass fibre paper touch the edge of the dish when it is filled with developing solvent there is excessive capillary flow.

Chromatography

Strips of Whatman No. 4 paper are shaped as shown in Fig. 3. The strips are dipped into a freshly prepared solution of 30 % (v/v) propylene glycol in methanol. They are removed with forceps, shaken to remove excess liquid and hung up by a corner for 10 min at room temperature to allow the methanol to evaporate. Before application of the steroid extracts, the papers are sandwiched between four strips of glass plate leaving exposed an area 0.5 cm either side of the start line.

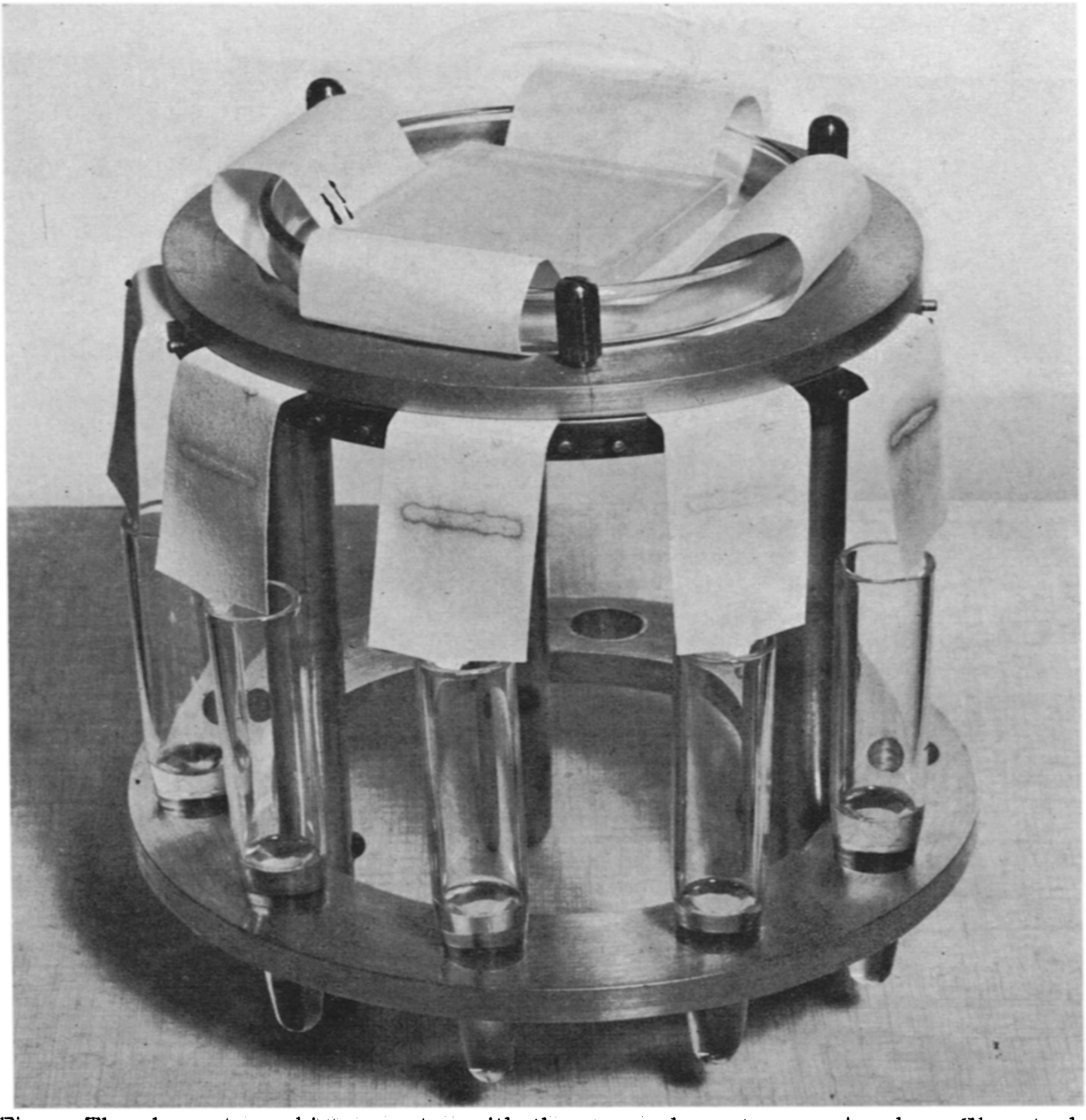

Fig. 2. The chromatographic apparatus with the paper chromatograms in place. Glass tank not shown.

Urine extracts (see subsequent section for preparation) in tapered tubes are dissolved in approx. 50 μl of benzene–ethanol (1:1) mixture and applied along the start line using a pipette with a capillary end. At least 2 mm clear space must be left at each end of the start line or the steroids will streak down the edges of the chromatogram during the run. Three further applications each of approx. 20 μl of solvent are necessary to transfer the extract quantitatively to the paper strip. Drying between each application is carried out with a stream of cold air.

The strips are then left for 10 min to allow a backflow of propylene glycol displaced by the application of the benzene–ethanol mixture. This backflow narrows the band of extract at the start line.

The strips are then bent at right angles approximately half-way between the top and the start line. The top flap so formed is inserted with forceps between the detachable ring (E) and the platform of the elution rack (A), making sure that each

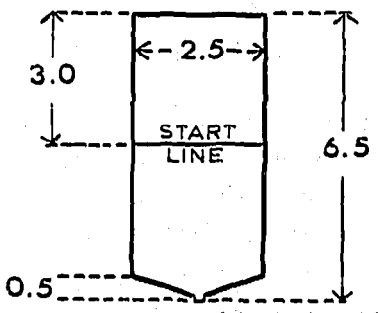

Fig. 3. Diagram of paper chromatographic strip. All measurements are in cm.

paper is spaced between the protruding pins (H) on the side of the platform. The chromatograms must protrude about 2 mm from the side of the platform or capillary action will cause excessive flow of solvent. The small tongue at the end of the chromatogram (Fig. 3) should rest just inside the rim of the collection tube. The knurled screws are tightened and the whole rack is placed in a circular glass jar (approx. 8 in. high, 8 in. diameter), the atmosphere of which has been equilibrated with heptane by placing about 50 ml of this solvent in the tank at least 30 min previously.

Heptane (35–40 ml) is then poured into the Petri dish, a lid placed on the jar, and the chromatogram is over-run until 3.5–4.0 ml of eluate has collected in the graduated test tube. Initial equilibration of the chromatogram is unnecessary. At a temperature of 20–25° the solvent drips from the chromatogram at a rate of 1–1.2 ml/h. It may occasionally be necessary to recharge the Petri dish with heptane during the run.

THE METHOD IN DETAIL

(1) *Extraction of conjugated 17-oxosteroids*

24 h urine specimens are made up to 2 l with water if below this volume and two 50 ml aliquots taken. To each is added ammonium sulphate (25 g) which is stirred until dissolved. The urine is then extracted with ether–ethanol (3:1) mixture (3 × 25 ml). The extracts are pooled and filtered through a fluted 15 cm Whatman No. 4 paper into a 250 ml round-bottomed flask. Using a rotary evaporator the extract is taken to near dryness under partial vacuum at the water pump at a temperature less than 45°. Ethanol (5 ml) is added and the full vacuum applied. The dry extract is dissolved in ethanol (4 ml) and transferred to a tapered centrifuge tube graduated at 10 ml. Three further washes of ethanol (2, 2 and 1 ml) are transferred from the flask to the centrifuge tube which is then made up to the 10 ml mark with ethanol. After mixing, the tube is centrifuged for 3 min at 1400 g and an 8 ml aliquot of supernatant (equivalent to 40 ml original urine) is pipetted into a 50 ml tube and blown to dryness under N_2 at 45° (*cf.* EDWARDS, KELLIE AND WADE[7]).

(2) Enzymic hydrolysis and solvolysis: estimation of total 17-oxosteroids

β-Glucuronidase powder (approx. 25 mg, depending on the activity) is homogenized with acetate buffer (10 ml, 0.5 *M*, pH 4.0) and then centrifuged at 1400 *g* for 5 min. The supernatant (which contains the bulk of the enzyme activity) is decanted into a measuring cylinder and made up with buffer solution to give a final concentration of approx. 2000 units/ml. This solution (5 ml) is added to the dry conjugate extract and stood overnight at 40°. NaCl (4 g) is then added, followed by water (15 ml). The solution is then taken to pH 1 (glass electrode) with 4 *N* H_2SO_4 (approx. 0. 25 ml) and then extracted with ethyl acetate (2 × 20 ml). These extracts are pooled and incubated at 50° for 4 h (*cf.* BURSTEIN AND LIEBERMAN[8]).

The volume of the ethyl acetate, which now contains the steroids freed by β-glucuronidase hydrolysis and by solvolysis, is reduced to approx. 5 ml under N_2 at 45°. Benzene (35 ml) is added, the mixture transferred to a separating funnel and washed (3 × 5 ml) with a freshly prepared solution of sodium dithionite (5 % w/v, in *N* NaOH) until any red pigment present (indirubin) is decolourized. The organic phase is then washed with water (2 × 2.5 ml) and taken to dryness (rotary evaporator). The extract is transferred with ethanol (1 × 2 ml, 3 × 1 ml) to a test tube, taken to dryness under N_2 and redissolved in 1.0 ml ethanol. A single aliquot (0.2 ml) is removed and the total 17-oxosteroids estimated by the method of CALLOW *et al.*[5] except that after 1 h at 25°, the reaction mixture is diluted with only 5 ml of 95 % ethanol. The colours are read at 440, 520 and 600 mμ and a correction applied (ALLEN[9]).

(3) Separation and estimation of the 11-deoxy-17-oxosteroid fraction

From the results of the estimation of the total 17-oxosteroids, the content in the remaining 0.8 ml of ethanol solution can be calculated. From this a further aliquot containing 30–60 μg of 17-oxosteroid is taken into a small tapered tube, blown to dryness with N_2 and transferred to the paper chromatogram and run as previously described. The eluate from the chromatogram which contains the 11-deoxy-17-oxosteroid fraction is taken to dryness under N_2 at 50° and the Zimmerman reaction carried out as previously described.

(4) Separation and estimation of the 11-oxygenated 17-oxosteroid fraction

The method can also be used for the estimation of the 11-oxygenated 17-oxosteroids by subtracting the results of the determination of the 11-deoxy fraction from those obtained for the total 17-oxosteroids. Alternatively, the paper chromatograms may be transferred to a second chromatographic tank in which the mobile phase is toluene–methylene chloride (1:1). The 11-oxygenated fraction is eluted from the paper when 3.0 ml of eluant have been collected in the test tube. This method does not remove stationary phase from the paper. The Zimmerman reaction is then carried out as for the 11-deoxy fraction except that half quantities of reagents are used and the final reaction mixture is diluted with 2.5 ml of 95 % ethanol.

In a subsequent section, independent evidence is given for the validity of results obtained with the method for the 11-deoxy fraction. Since similar evidence is at present not as complete for the 11-oxygenated fraction, the method for this group of compounds will not be discussed further.

(5) *General comments on the method*

(*a*) *Extraction of conjugates and hydrolysis.* The method of EDWARDS *et al.*[7] must be used if enzymic hydrolysis is to be complete in 18 h. Furthermore, the extraction of the conjugates results in a substantial saving in the amount of enzyme used. Centrifugation of the solution of enzyme in acetate buffer removes a considerable portion of inactive protein and reduces the chances of subsequent formation of emulsions. The free steroids released by hydrolysis with limpet enzyme are extracted together with unhydrolysed sulphates into ethyl acetate and the latter are subsequently cleaved by solvolysis (BURSTEIN AND LIEBERMAN[8]).

Interference by urinary pigments is reduced considerably by shaking the extract obtained after hydrolysis with alkaline dithionite, and the bulk of any remaining pigments remain on the start line of the chromatogram. Occasionally a red pigment is found which is not reducible with dithionite. This runs with the solvent front and can be eluted with the first two drops of heptane and discarded without loss of 17-oxosteroids. Evaporation of most of the ethyl acetate extract and addition of benzene results in a more efficient removal of pigments by alkaline dithionite.

(*b*) *Chromatography.* The behaviour of various components of the 17-oxosteroid fraction during paper chromatography and their elution from the strips by overrunning is shown in Fig. 4.

These results show that there is a considerable safety margin between the complete elution of DHA (the most polar of the 11-deoxy-17-oxosteroid fraction) and the start of the elution of 11-keto-androsterone, the least polar of the 11-oxygenated

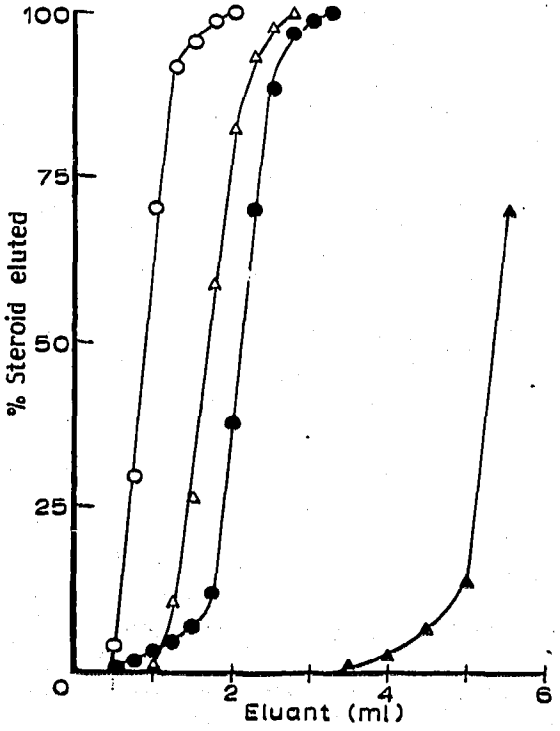

Fig. 4. The elution curves of 17-oxosteroids from paper chromatograms. Pure standards were run on paper chromatograms (see text) and analyses carried out for each steroid for each 0.25 ml of eluant. For 11-keto-androsterone the analyses were done for each 0.5 ml of eluant. —○—○—, androsterone; —△—△—, actiocholanolone; —●—●—, DHA; —▲—▲—, 11-keto-androsterone.

17-oxosteroids normally encountered in urine. In some urine specimens from pregnant women androst-4-ene-3,11,17-trione is present and this compound runs between DHA and 11-keto-androsterone, partly eluting into the 11-deoxy fraction.

RESULTS

(1) *Evaluation of the method*

The definition of the terms used in this section and the details of the calculations have been described by BROWN, BULBROOK AND GREENWOOD[10]. The accuracy of the method, measured by recovery experiments, is given in Table I. These experiments show that pure steroids added at various stages of the method, either free or unconjugated, can be recovered in amounts of 75 % or over of the original starting material.

TABLE I

RECOVERY OF PURE STEROIDS ADDED AT VARIOUS STAGES IN THE METHOD

Steroid added	*Amount (μg) of steroid added to:*			*No. of estimations*	*Mean recovery (%)**	*Range (%)*
	Paper	*Hydrolysed urine extract*	*Urine*			
DHA Androsterone Aetiocholanolone	25–40	—	—	20	98.2 (±2.72)	93–103
DHA Androsterone Aetiocholanolone	—	10	—	18	95.3 (±6.89)	84–112
DHA Androsterone Aetiocholanolone	—	—	40	12	85.7 (±7.46)	75–99
$Na \cdot DHA \cdot SO_4 \cdot 2H_2O$**	—	—	50	11	78.8 (±16.25)	62–107
Paper blanks	—	—	—	5	0.005***	—
11-OH-Aetiocholanolone	30	—	—	4	97.3	91–102
11-OH-Aetiocholanolone	—	30	—	4	84.8	81–99
11-OH-Aetiocholanolone	—	—	40	4	73.0	71–74

* Figures in brackets represent standard deviations.
** Obtained from Steraloids Ltd.
*** Corrected optical density.

The precision of the method was calculated from differences between duplicate estimations in a series of 51 assays on different urine specimens. At a mean level of 6.68 mg of 11-deoxy-17-oxosteroids/24 h the estimate of the standard deviation(s) is 0.43 mg/24 h. From this it can be calculated that the lower limit of sensitivity of the method is about 0.8 mg/24 h (if duplicate determinations are performed).

(2) *Comparison of results with those obtained by gradient elution from alumina*

In 36 instances, aliquots of urine were taken after the hydrolytic procedures already described had been carried out. The amounts of DHA androsterone, and aetiocholanolone in one aliquot were then determined by the method of KELLIE AND WADE[2] and the amounts of total 11-deoxy-17-oxosteroid in a second aliquot were determined

by the paper chromatographic method described. The results of the comparison are shown in Fig. 5. In a further 36 instances, the comparisons were made by processing aliquots of the original urine completely separately by either method (also shown in Fig. 5). These comparisons show clearly that the titre of total 11-deoxy-17-oxosteroids

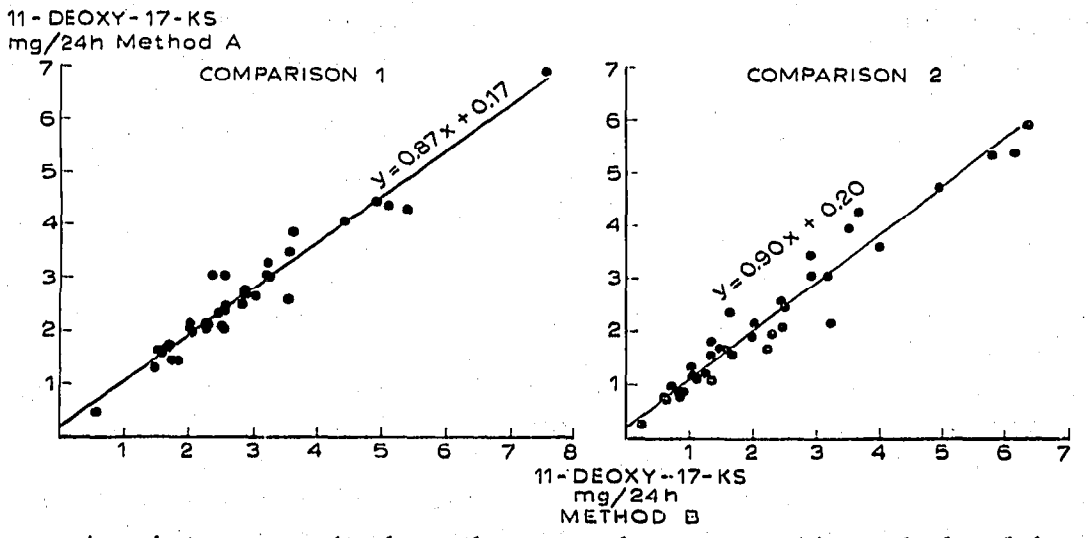

Fig. 5. Comparison between results from the paper chromatographic method and from gradient elution from alumina. Method A refers to the paper chromatographic method. The results of determinations of DHA, androsterone and aetiocholanolone by gradient elution (method B), expressed in terms of DHA standard were summed for this comparison. Comparison 1: hydrolytic procedure common to both methods. Comparison 2: separate processing throughout (see text). The lines are the fitted regression curves of the paper chromatography result (y) on the gradient elution results (x).

obtained by the paper chromatographic method can be almost entirely accounted for by summing the amounts of DHA, androsterone and aetiocholanolone determined by gradient elution from alumina. The correlation coefficients (r) are 0.978 and 0.980 respectively.

DISCUSSION

Various methods have been described for the separation and determination of individual 17-oxosteroids in urine (SAVARD[1], KELLIE AND WADE[2], BUSH AND WILLOUGHBY[11], BROOKS[12]). All are time-consuming and require a high degree of skill for constant reliability. In some clinical investigations the amounts of the individual 11-deoxy-17-oxosteroids may not be required and all that is needed is a measurement of the total 11-deoxy fraction. The paper chromatographic method described here is suitable for this purpose. It is especially suitable for handling large numbers of determinations and a trained worker can conveniently assay 12 urine specimens in duplicate in one week. It is also useful as a preliminary purification step for subsequent separation of the 17-oxosteroids by paper chromatography and may be useful in this context for gas chromatography.

The use of 6.5 cm strips obviates the need for paper blank corrections which may be considerable if 3 MM paper of conventional size is used. Recoveries of 17-oxosteroids from the short strips have been consistently higher in our hands than from 3 MM paper. Also use of the latter sometimes results in incomplete resolution of the

less polar from the more polar 17-oxosteroids (see BUSH AND WILLOUGHBY[11], BUSH[13], JAMES[14]).

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SUMMARY

(1) A paper chromatographic method is described for the rapid separation and estimation of the 11-deoxy-17-oxosteroids in urine. The method may also be useful for the measurement of the 11-oxygenated compounds.

(2) The method is based on extraction of conjugates from urine, enzymic hydrolysis and solvolysis followed by paper chromatography in a specially designed apparatus on 6.5 cm strips of paper impregnated with propylene glycol.

(3) The results are comparable with those obtained by summing the individual components of the 11-deoxy-17-oxosteroid fraction separated and estimated by gradient elution from alumina.

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