DETERMINATION OF INDIVIDUAL 17-KETOSTEROIDS BY GRADIENT ELUTION CHROMATOGRAPHY

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DINGEMANSE *et al.*[1] were first to describe a method of quantitative analysis for the major individual urinary 17-ketosteroids in micro amounts by use of alumina as an adsorbent and benzene, containing increasing concentrations of ethanol, as eluent. Subsequently, a number of adsorption methods were described in which various adsorbents and eluents were employed[2-6], but, in general, the overlapping of various fractions limited the efficiency of these procedures. This difficulty was overcome to some extent by the use of partition columns by JONES AND STITCH[7], who used nitromethane on silicic acid columns as the stationary phase, and later by EDWARDS AND KELLIE[8], who employed aqueous sodium acetate solution on silicic acid for the separation of the glucuronides of urinary steroid ketones. WILSON *et al.*[9] achieved the separation of urinary corticosteroids and C_{19} steroids on a micro column of aluminum silicate with 50 % ethanol as the stationary phase by use of a "stepwise gradient" of hexane containing increasing concentrations of chloroform.

A major improvement in the analysis of individual 17-ketosteroids was accomplished by LAKSHMANAN AND LIEBERMAN[10,11], through the use of gradient elution, which eliminates much of the "tailing" inherent in adsorption methods. KELLIE AND WADE[12] have recently described a very useful application of the gradient elution principle to the DINGEMANSE[1] method, in which a relatively simple device was used for producing the desired gradient automatically.

The development of a method in this laboratory for the determination of individual adrenocorticosteroids on water-impregnated silicic acid[13,14] prompted us to investigate the possibilities of adapting this method to the fractionation of 17-ketosteroids. The gradient elution method described has the advantages of employing simple apparatus and lends itself well to completely automatic operation.

METHODS

1. *Column chromatography*

Just prior to packing a column, 7.5 ml of water, or 7.5 ml of water and 1.5 ml of methanol is added dropwise to 60 g of silicic acid while grinding it in a mortar. Since the water content of commercial silicic acid (Merck) as determined by heating several 1-g samples for 3 h and reweighing immediately, was found to be 15.8 ± 1 %, this

gives a water content of 25 %. This mixture is then suspended in water-saturated petroleum ether. The column is packed, and fractions of 400 drops each are collected, as described in the adrenal steroid method[13,14]. Gradient elution by petroleum ether, containing increasing amounts of methylene chloride, is effected by delivering the water-saturated solvents from two separatory funnels, mounted one on top of the other.

2. *Quantitative estimation*

The individual fractions, collected in test tubes, are allowed to dry and then 10 ml of 95 % ethanol is added to each tube. A 5-ml aliquot is withdrawn and set aside for subsequent identification by paper chromatography. The remaining aliquot is dried under vacuum at 40° in an automatic test tube evaporator. The dried residues in each tube are then analyzed by a modification of the M.R.C.[15] method of 17-ketosteroid determination. The modification involves the use of ascorbic acid-protected ethanolic 2.5 *N* KOH and development of the color at 0° for 3 h[16]. Calibration curves are determined for each compound* since they show an appreciable difference in response to the Zimmermann test.

3. *Paper chromatography*

Identification of the fractions is based on paper chromatography with the solvent systems methylcyclohexane/propylene glycol[17], ligroin/propylene glycol[18], and benzene–cyclohexane (1:1)/propylene glycol[19].

RESULTS AND DISCUSSIONS

Fig. 1 illustrates the fractionation obtained with the silicic acid column of 25 % water content. It was found that the separation is equally good with the commercial silicic acid to which the necessary amount of water is added and with the addition

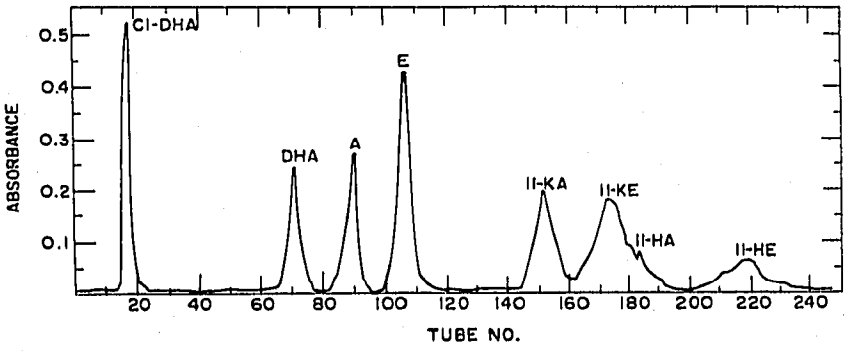

Fig. 1. Fractionation of 17-ketosteroids (100 γ of each) on a silicic acid column containing 25% water. Absorbance at 530 mμ after Zimmermann reaction.

* Standard compounds used: 3α-hydroxyandrostan-17-one (androsterone, A); 3α-hydroxyandrostane-11,17-dione (11-ketoandrosterone, 11-KA); 3α,11β-dihydroxyandrostan-17-one (11-hydroxyandrosterone, 11-HA); 3α-hydroxyetiocholan-17-one (etiocholanolone, E); 3α-hydroxyetiocholane-11,17-dione (11-ketoetiocholanolone, 11-KE); 3α,11β-dihydroxyetiocholan-17-one (11-hydroxyetiocholanolone, 11-HE); 3β-chloro-Δ^5-androsten-17-one (chlorodehydroepiandrosterone, Cl-DHA); 3β-hydroxy-Δ^5-androsten-17-one (dehydroepiandrosterone, DHA).

of water to silic acid dried as described. All 17-ketosteroids used were completely separated, except 11-KE and 11-HA. Separation of these two substances is achieved by the use of 7.5 ml of water and 1.5 ml of absolute methanol per 60 g of commercial silicic acid (Fig. 2). This shifts the position of the elution peaks and somewhat decreases their sharpness, but there is no change in resolution. Repeated chromatograms have shown only minor variations in the position of elution peaks.

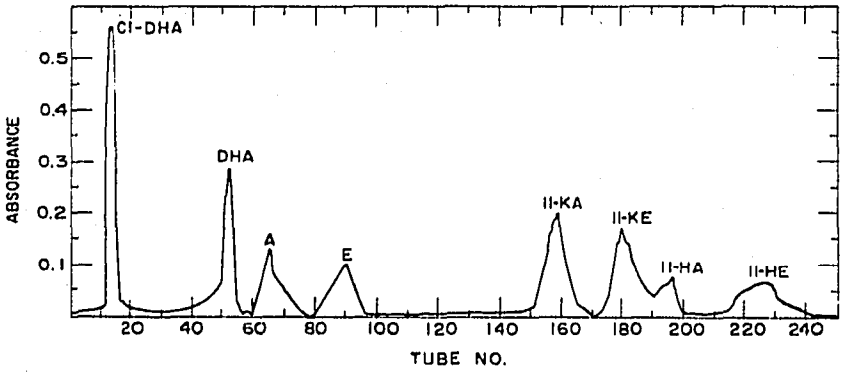

Fig. 2. Fractionation of 17-ketosteroids (100 γ of each) on columns of 60 g silicic acid plus 7.5 ml of water and 1.5 ml of methanol. Absorbance at 530 mμ after Zimmermann reaction.

Recoveries of each of the standard compounds, calculated with reference to individual calibration curves, are 95–100%, but lower for the mixed zone of 11-KE and 11-HA.

A number of experimental conditions were varied in order to test their effect on separation. In these experiments Cl-DHA and DHA were not included.

1. *Stationary phase of methanol–water (2:1)*

In preliminary experiments with 45 ml of this stationary phase per 60 g of dried silicic acid, partial separation of androsterone, etiocholanolone and their respective 11-oxygenated derivatives (11-KA, 11-HA, 11-KE, and 11-HE) was obtained, but elution occurred in the first 40 tubes. Decrease of the quantity of stationary phase to 40 ml greatly decreased the flow rate of the solvent. With 30 ml of the stationary phase, flow stopped altogether. An increase to 55 ml increased the resolution somewhat, but all the steroids were still collected in the first 40 tubes. An attempt to improve the resolution by starting the elution with pure petroleum ether and continuing the gradient elution by introducing a methylene chloride–petroleum ether mixture (7:3), instead of pure methylene chloride, was unsuccessful. Changes in the ratio of methanol:water to 4:1 and 1:1 caused the zones to coalesce and vitiated the partial resolution achieved by the 2:1 ratio.

2. *Propyl alcohol–water (2:1) and tert.-butanol–water (2:1)*

Changes in the polarity of the stationary phase by substitution of *n*-propanol and *tert.*-butanol for methanol resulted in each case in the elution of all six compounds in two peaks in the first ten tubes.

3. *Stationary phase of water*

In this series of experiments varying amounts of water were added to 60 g of the commercial silicic acid of known water content. Addition of 21 ml of water accomplished the separation of A and E in the first 40 tubes, but the remaining 4 steroids were eluted in a broad band in tubes 41 through 70. An increase in the water content to 36 ml shifted all six peaks to the first 40 tubes and gave poor resolution. Addition of 7 to 11 ml of water gave the same separation as shown in Fig. 1. However, when 6 ml of water or less was added, no resolution was obtained and the compounds were eluted in a broad zone extending over all 200 tubes analyzed. The effect of adding 1.5 ml of methanol to 7.5 ml of water has already been mentioned (Fig. 2). The quantity of methanol appears to be quite critical, and an increase to 2 ml broadens the chromatographic bands. The results obtained by use of 1.5 ml of methanol are readily reproducible.

It is apparent from the experimental results described that the relative participation of adsorption and partition in the fractionation on silicic acid columns varies with the water content of the stationary phase. This is in agreement with earlier studies of the effect of water on the adsorptive power of silicic acid[20,21]. KAY AND TRUEBLOOD[22] also indicate that water molecules may interfere with adsorption by occupying the adsorption sites on the column. They suggest that water can act as an adsorbent itself through hydrogen bonds and that methanol may be less effective in this respect because it is less apt to form hydrogen bonds. This effect may have some bearing on the improved separation of 11-KE and 11-HA obtained when a small amount of methanol is added to the water.

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SUMMARY

A method is described for the individual determination of eight 17-ketosteroids commonly found in urine. Fractionation of the compounds is achieved by use of a water-impregnated silicic acid column and elution with a concentration gradient of methylene chloride in petroleum ether. The effects of changes in the stationary phase on fractionation are presented and discussed.

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