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CHROMATOGRAPHIC SEPARATION OF 17-KETOSTEROIDS AND 17-HYDROXYCORTICOSTEROIDS ON SEPHADEX LH-20

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SUMMARY

The chromatographic behaviour of 17-ketosteroids and 17-hydroxycorticosteroids normally present in human urine was studied on a column of Sephadex LH-20 equilibrated with 99% 1-butanol; 99% 1-butanol was also used as eluent. Most of the steroids were eluted in order of their increasing polarity and 17-ketosteroids were separated into two groups, namely, $C_{19}O_2$-17-ketosteroid and $C_{19}O_3$-17-ketosteroid. Tetrahydrocortisone and tetrahydrocortisol were completely separated from each other, but cortisone and cortisol were eluted between the former. This method was applied to the analysis of urinary steroids, and the efficiency of separation and the purity of steroids eluted in four main peaks was studied by other chromatographic systems.

INTRODUCTION

Sephadex LH-20 has been used for chromatographic separation of conjugated steroids[1] and 17-hydroxycorticosteroids[2]. In these studies, a less polar mobile phase was used and the steroids were eluted in order of their increasing polarity.

On the other hand it has been shown that polycyclic hydrocarbons are eluted from a Sephadex LH-20 column in order of their increasing complexity, isopropanol being used as eluent[3]. This prompted us to investigate the chromatographic behaviour of various steroids on a column of Sephadex LH-20 using alcohols as the mobile phase, and a good separation of 17-ketosteroids and 17-hydroxycorticosteroids was obtained when 99% 1-butanol was used as eluent. The steroids were eluted in the order of their increasing polarity.

EXPERIMENTAL

Materials

Tetrahydrocortisol and tetrahydrocortisone were supplied by Dr. T. F. GALLAGHER. 11β-Hydroxyetiocholanolone and 11-ketoetiocholanolone were supplied by Dr. S. LIEBERMAN, 11-ketoandrosterone was supplied by Dr. W. KLYNE and 11β-hydroxyandrosterone by Dr. K. MATSUMOTO. Other steroids used in this work were available commercially. The potassium hydroxide, dinitrobenzene, ethanol (99.5%),

sulfuric acid, 1-butanol and phenylhydrazine sulfate used were of analytical grade. Methanol, ethanol (99%), benzene, *n*-hexane and cyclohexane were distilled before use. A 10% (w/v) aqueous solution of Hyamine 1622 was purchased from Sankyo Co., Ltd.

Preparation of chromatographic column

Sephadex LH-20 (lot 222) was classified by the sedimentation method[4] in 1-butanol–water (99:1) and the smaller particles were collected. They were washed with 99% 1-butanol and poured into a chromatographic tube and allowed to settle at room temperature. After the particles had settled, the temperature of the column was gradually elevated to 33°.

Chromatographic separation of a synthetic mixture

A mixture of standard samples of steroids and N-2,4-dinitrophenylethylamine (DNP-ethylamine) was dissolved in 99% 1-butanol, and 0.6 ml of the solution was placed on the column. After the solution had drained into the column, elution was started with 99% 1-butanol. The eluate was collected using a time-flow type automatic fraction collector. The test tubes were changed every 30 min. Each fraction (0.8 ml) was divided into two equal parts. One was analyzed by the Zimmermann reaction and the other was analyzed for 17-hydroxycorticosteroids. Each divided fraction was made up to 0.5 ml by adding 99% 1-butanol, and 0.75 ml of 0.1% (w/v) solution of phenylhydrazine sulfate in diluted sulfuric acid (sulfuric acid–water, 3:2) was added with mixing[5]. The mixture was allowed to stand at room temperature for

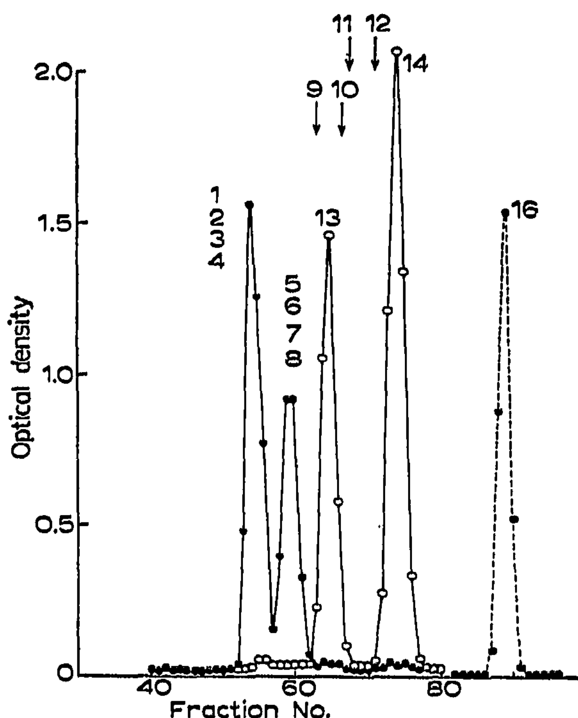

Fig. 1. Elution of standard steroids. The size of the column was 0.66 × 188 cm. Vertical arrows show the elution position of the steroids as obtained in separate experiments performed under the same conditions. For numbers, see Table I; 16 represents DNP-ethylamine. In Figs. 1 to 4, optical density at 520 mμ, (●—●); at 410 mμ, (○—○); and at 350 mμ, (●---●).

15 h and the optical density was measured at 410 mμ. The Zimmermann reaction was performed according to EPSTEIN's method after evaporation of the eluent[6]. The eluent was removed as follows: Two and a half volumes of 60% ethanol were mixed with each divided fraction and mixtures were placed in a rack and heated in a boiling water bath for about 30 min. The recovery of steroids having a Δ_4-3-keto group was estimated from their U.V. absorption at 240 mμ.

TABLE I

STEROID NOMENCLATURE

No. in figures	*Chemical name*	*Trivial name*
1	3α-Hydroxy-5α-androstan-17-one	Androsterone
2	3α-Hydroxy-5β-androstan-17-one	Etiocholanolone
3	3β-Hydroxy-5α-androstan-17-one	Epiandrosterone
4	3β-Hydroxyandrost-5-en-17-one	Dehydroepiandrosterone
5	3α,11β-Dihydroxy-5β-androstan-17-one	11β-Hydroxyetiocholanolone
6	3α-Hydroxy-5β-androstane-11,17-dione	11-Ketoetiocholanolone
7	3α-11β-Dihydroxy-5α-androstan-17-one	11β-Hydroxyandrosterone
8	3α-Hydroxy-5α-androstane-11,17-dione	11-Ketoandrosterone
9	17α,21-Dihydroxypregn-4-ene-3,20-dione	11-Deoxycortisol
10	11β,21-Dihydroxypregn-4-ene-3,20-dione	Corticosterone
11	17α,21-Dihydroxypregn-4-ene-3,11,20-trione	Cortisone
12	11β,17α,21-Trihydroxypregn-4-ene-3,20-dione	Cortisol
13	3α,17α,21-Trihydroxy-5β-pregnane-11,20-dione	Tetrahydrocortisone
14	3α,11β,17α,21-Tetrahydroxy-5β-pregnan-20-one	Tetrahydrocortisol
15	3α,11β,17α,21-Tetrahydroxy-5α-pregnan-20-one	Allotetrahydrocortisol

TABLE II

RECOVERY OF Δ_4-3-KETOSTEROIDS FROM THE CHROMATOGRAPHIC COLUMN

Steroid	*Added* (μg)	*Recovered* (μg)	*Recovery* (%)
Testosterone	260	264	102
Cortisone	218	210	96
Cortisol	294	288	98

Analysis of steroids in human urine

Urine (1/5 of a 24 h sample from a 42 year old normal male subject) was hydrolyzed with β-glucuronidase and the neutral fraction was extracted and purified as described previously, without removing the less polar lipids, by partition between aqueous methanol and *n*-hexane[7]. The purified neutral fraction was dissolved in 0.6 ml of 99% 1-butanol containing a small amount of DNP-ethylamine. The solution was applied to a column of Sephadex LH-20 and eluted with the 99% 1-butanol. Effluent was collected as described above, and each fraction was divided into three equal parts. 17-Ketosteroids and 17-hydroxycorticosteroids were assayed as described above and the fractions containing $C_{19}O_2$-17-ketosteroid, $C_{19}O_3$-17-ketosteroid, tetrahydrocortisone, or tetrahydrocortisol were determined. These fractions were pooled separately and separated on columns of esterified Amberlite IRC-50[7].

RESULTS AND DISCUSSION

As shown in Fig. 1, the separation of $C_{19}O_2$-17-ketosteroid and $C_{19}O_3$-17-ketosteroid was satisfactory. Tetrahydrocortisone and tetrahydrocortisol were separated from each other completely, whereas cortisone was eluted more slowly and partially overlapped with tetrahydrocortisone while cortisol partially overlapped with tetrahydrocortisol. 11-Deoxycortisol was eluted more rapidly, but the elution of corticosterone was slower than that of tetrahydrocortisone. Testosterone was eluted with $C_{19}O_2$-17-ketosteroid. Thus, with the exception of tetrahydrocortisone, the order of elution of the steroids studied coincided with the order of their polarity. The recoveries of testosterone, cortisone and cortisol are shown in Table II. The capacity of the column is high and mg amounts of steroid could be separated with the size of column

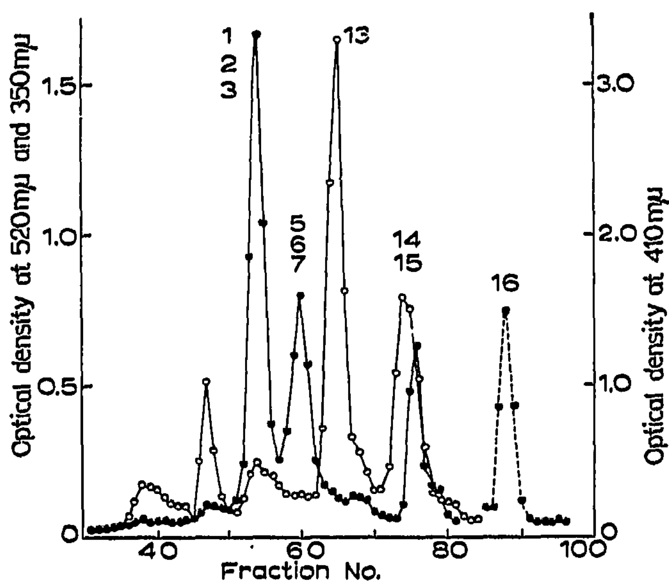

Fig. 2. Elution of the neutral fraction of β-glucuronidase hydrolysate of human urine performed under the same conditions as in Fig. 1. For numbers, see Table I and Fig. 1.

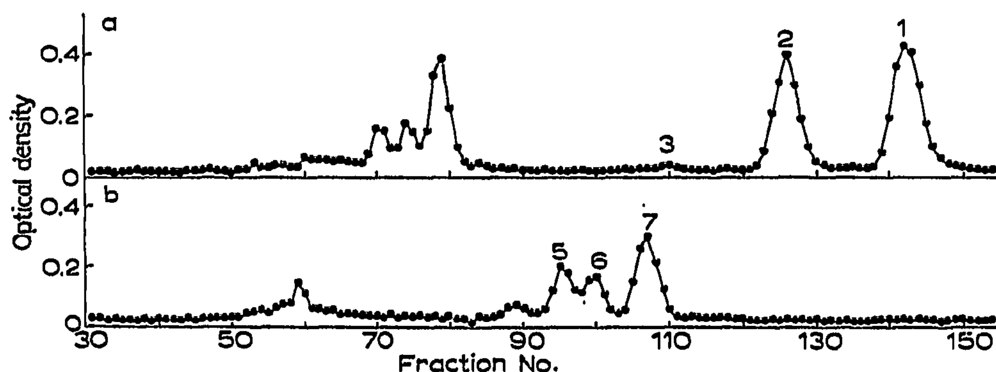

Fig. 3. Elution of the $C_{19}O_2$-17-ketosteroid fraction (a = fractions 52 to 56 of Fig. 2) and the $C_{19}O_3$-17-ketosteroid fraction (b = fractions 57 to 62 of Fig. 2) from a column of partially esterified Amberlite IRC-50. The size of the column was 0.6 × 121 cm and the eluent was a mixture of methanol, ethanol (99%) and water (3:9:8). The temperature of the column was 33°, the eluate was collected in fractions of 21 drops at a flow rate of 3 fractions per hour. For numbers, see Table I. 11-Ketoandrosterone could not be detected by another method[7].

used in this experiment. The elution pattern was quite reproducible when the temperature of the column was kept constant, and the column could be used repeatedly. The elution pattern of the neutral fraction of a β-glucuronidase hydrolyzate of urine of a normal male subject is shown in Fig. 2. Although separation of $C_{19}O_2$-17-ketosteroid and $C_{19}O_3$-17-ketosteroid was complete, each peak contained considerable amounts of impurities, as shown in Fig. 3, which amounted to 30 to 40% of the total color value of each fraction. The tetrahydrocortisone and tetrahydrocortisol fractions were almost

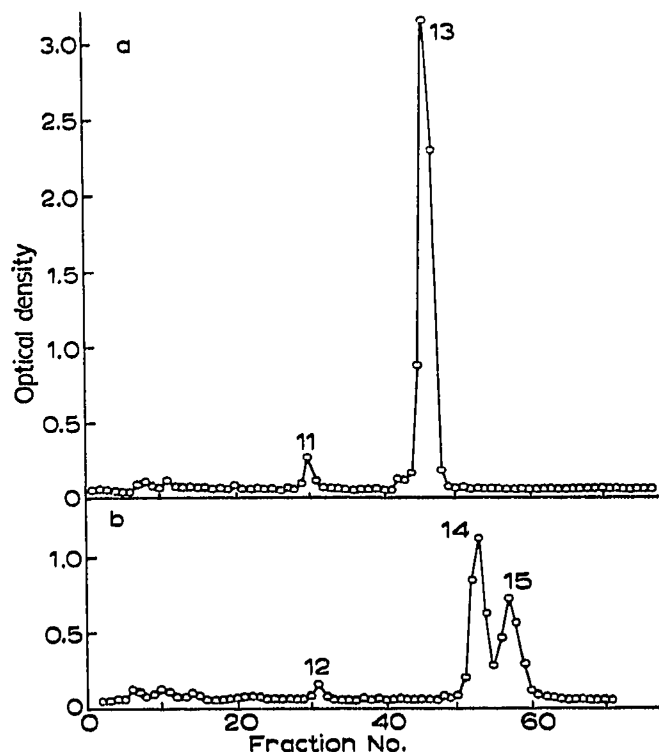

Fig. 4. Elution of the tetrahydrocortisone fraction (a = fractions 63 to 69 of Fig. 2) and the tetrahydrocortisol fraction (b = fractions 70 to 79 of Fig. 2) from a column of esterified Amberlite IRC-50. The size of the column was 0.5 × 56 cm and the eluent was a mixture of ethanol (99.5 %), benzene, *n*-hexane and water (50:350:80:3.3). The temperature of the column was 22°, the eluate was collected in fractions of 21 drops at a flow rate of one fraction per hour. For numbers, see Table I.

pure, as shown in Fig. 4. Fig. 2 shows that most of the impurities giving a positive Porter-Silber reaction were eluted more rapidly than tetrahydrocortisone. Elution of less polar lipids was faster than that of $C_{19}O_2$-17-ketosteroid and fractions 35 to 49 became turbid when mixed with the Porter-Silber reagent.

From these results it is concluded that the method described above gives a satisfactory value for total 17-hydroxycorticosteroid, but only an approximate value for the $C_{19}O_2$-17-ketosteroid and $C_{19}O_3$-17-ketosteroid present in human urine.

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