Chromatographic separation of 17-ketosteroids

Chromatographic separation of individual 17-ketosteroids has been performed mainly by the stepwise or gradient elution method, using an alumina[1–4] or a silica gel column[5].

Although these methods provide satisfactory separation of the common 17-ketosteroids in a relatively short time (a few hours), application of the gradient or changing of the eluants during chromatography is somewhat tedious.

On the other hand it has been shown that estrogens[6] and some Δ^4-3-ketosteroids[7] can be separated by a one-step elution method, using partially esterified Amberlite IRC-50 as the adsorbent and a mixture of alcohol and water as the eluant. This method has now been applied to the separation of 17-ketosteroids and it was possible to separate fourteen common 17-ketosteroids (Table I) by using three solvent systems. The mixture of steroids was first fractionated by using a mixture of methanol, ethanol and water (3:9:8 by vol., eluant A) as the eluant. The components that were eluted in the same fraction were next fractionated by using a mixture of methanol, ethanol and water (3:15:8 by vol., eluant B) or a mixture of isopropanol and water (15:8 by vol., eluant C) as eluants. The former eluant provided a good separation of monoketo-monohydroxy compounds from diketo compounds, and the latter eluant eluted dehydro-*epi*-androsterone, 11β-hydroxyandrosterone and 11-ketoandrosterone in the order described.

TABLE I

KEY TO STEROID DESIGNATION

No. in the figures	*Chemical name**	*Trivial name*
1	3-Hydroxyestra-1,3,5(10)-trien-17-one	Estrone
2	3α,11β-Dihydroxy-5β-androstan-17-one	11β-Hydroxyetiocholanolone
3	3α-Hydroxy-5β-androstane-11,17-dione	11-Ketoetiocholanolone
4	3α,11β-Dihydroxy-5α-androstan-17-one	11β-Hydroxyandrosterone
5	3α-Hydroxy-5α-androstane-11,17-dione	11-Ketoandrosterone
6	11β-Hydroxyandrost-4-ene-3,17-dione	11β-Hydroxyandrostenedione
7	Androst-4-ene-3,11,17-trione	Adrenosterone
8	3β-Hydroxyandrost-5-en-17-one	Dehydro-*epi*-androsterone
9	3α-Hydroxy-5β-androstan-17-one	Etiocholanolone
10	3α-Hydroxy-5α-androstan-17-one	Androsterone
11	3β-Hydroxy-5α-androstan-17-one	*epi*-Androsterone
12	5β-Androstane-3,17-dione	Etiocholanedione
13	5α-Androstane-3,17-dione	Androstanedione
14	Androst-4-ene-3,17-dione	Androstenedione

* International Union of Pure and Applied Chemistry, *Nomenclature of Organic Chemistry, 1957*, Butterworths Sci. Publs., London, 1958.

Experimental

Materials. 11β-Hydroxyetiocholanolone and 11-ketoetiocholanolone were supplied by Dr. S. LIEBERMAN. Dehydro-*epi*-androsterone, androsterone, etiocholanolone, androstanedione, etiocholanedione, *epi*-androsterone and androstenedione were made available by Dr. A. KANBEGAWA and Miss M. KIMURA. 11-Ketoandrosterone was supplied by Dr. J. ENDO. Adrenosterone was prepared by chromic acid oxidation

of hydrocortisone, and 11β-hydroxyandrostenedione was prepared from hydrocortisone by sodium bismuthate oxidation. 11β-Hydroxyandrosterone was isolated from the urine of a patient with congenital adrenal hyperplasia. Estrone was a commercial product. The potassium hydroxide, dinitrobenzene and ethanol (99.5 %) used for the Zimmermann reaction were of analytical grade. Methanol, ethanol (99 %) and isopropanol were distilled before use.

Ion-exchange resin. Amberlite IRC-50 (A.G.) was pulverized and washed as described previously (200 to 300 mesh)[8]. It was partially esterified by refluxing for 40 h with aqueous acidic alcohol D, E or F (Table II). One liter of acidic alcohol was

TABLE II

COMPOSITION OF AQUEOUS ACIDIC ALCOHOL

Aqueous acidic alcohol	*Composition*
D	Methanol–ethanol–*N* HCl (1:3:2, by vol.)
E	Methanol–ethanol–2 *N* HCl (1:5:2, by vol.)
F	Isopropanol–2 *N* HCl (5:2, by vol.)

used for 70 to 100 ml of H form Amberlite IRC-50, and bumping of the mixture was prevented by adding two or three pieces of porcelain boiling stone (8 mm cube), the edges of which were removed by grinding[9].

Preparation of the chromatographic column. The partially esterified resin was transferred to a glass filter, washed with the eluant to be used for chromatography (Table III) and suspended by swirling in about two volumes of the same solvent.

TABLE III

CONDITIONS OF THE CHROMATOGRAPHIC SEPARATION

Figure	*Aqueous acidic alcohol with which Amberlite IRC-50 was boiled*	*Size of the column (cm)*	*Eluant*	*Temperature (°C)*
1	D	0.69 × 137	A	28 – 28.5
2	E	0.78 × 195	B	20 – 20.2
3	F	0.78 × 181	C	24 – 24.5

The suspension was poured into a jacketed chromatographic tube through a small funnel fitted with a ground glass joint and allowed to settle. After about 200 ml of the eluant have been passed through, the column is ready for use.

Chromatographic separation of synthetic mixtures. One ml of a solution of steroids in the eluant was applied to the column and elution was performed with the same eluant. The effluent was collected in fractions of 40 or 42 drops in test tubes, using a drop count type automatic fraction collector. Analysis of the steroids was performed by the Nathanson–Wilson modification of the Holtorff–Koch method[10] after evap-

oration of the eluant. The eluant was evaporated by placing the test tubes in a suitable rack and heating in a boiling water bath for about 1 h. The recovery of steroids with a Δ^4-3-keto-group was measured by ultraviolet absorption at 240 mμ and that of estrone at 280 mμ using a Beckman model DU quarz spectrophotometer after dilution of each fraction with aqueous ethanol (70 %) to a total volume of 3.5 ml.

Results and discussion

As shown in Figs. 1 to 3, fourteen 17-ketosteroids have been separated by using three solvent systems. They were first separated by using solvent A (Fig. 1). The fractions

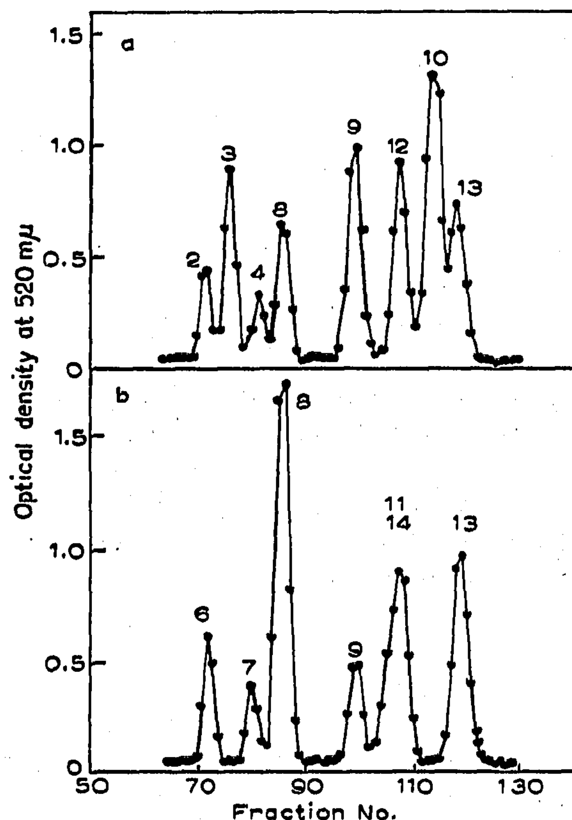

Fig. 1. Elution patterns of 17-ketosteroids. Elution patterns a and b were obtained in separate experiments performed under the same conditions. 11-Ketoandrosterone was eluted between 11β-hydroxyandrosterone and dehydro-*epi*-androsterone and estrone overlapped 11β-hydroxyetiocholanolone. The effluent was collected in fractions of 42 drops and the flow rate was 1.5 fractions per hour.

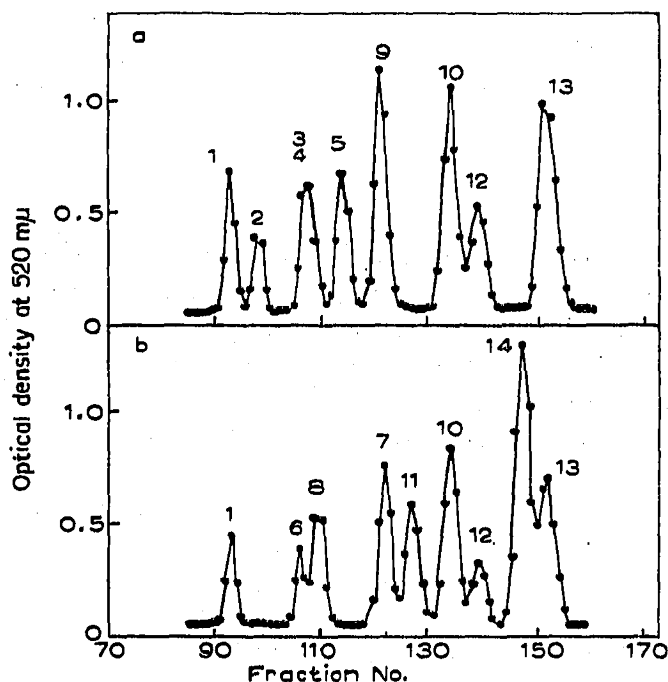

Fig. 2. Elution patterns of 17-ketosteroids. Elution patterns a and b were obtained in separate experiments performed under the same conditions. The effluent was collected in fractions of 40 drops and the flow rate was 2 fractions per hour.

containing two or more steroids were pooled and separated by using solvent B or C (Figs. 2 and 3). Good separation was obtained of the 5α- and 5β-isomers of the steroids, and since the chromatographic method described utilizes non-ionic adsorption of steroid molecules on partially esterified carboxylic acid type ion-exchange resin, the order of elution from the column is determined by both carbon number and degree of oxygenation of the molecule. The C_{18} steroid (estrone) is eluted faster than C_{19}

steroids (*e.g.* dehydro-*epi*-androsterone) if the number of oxygen atoms in the molecule is the same.

Increase of the alcohol concentration of the eluant exerted two effects on the elution volume of the steroids: (1) among steroids with the same number of oxygen atoms in the molecule, those with the greater number of hydroxyl group(s) were

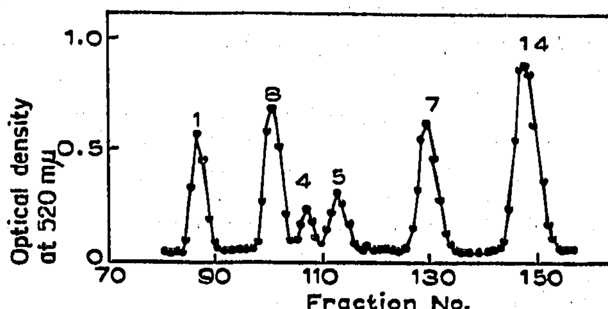

Fig. 3. Elution pattern of 17-ketosteroids. 11β-Hydroxyandrostenedione was eluted between dehydro-*epi*-androsterone and 11β-hydroxyandrosterone. The effluent was collected in fractions of 40 drops and the flow rate was 2 fractions per hour.

preferentially accelerated, and (2) less oxygenated steroids were preferentially accelerated. The elution volume was also sensitive to changes of column temperature. When the column temperature was elevated, less oxygenated steroids were preferentially accelerated and among steroids with the same number of oxygen atoms in the molecule, those with the greater number of keto-groups were preferentially accelerated.

The recovery of 17-ketosteroids with a Δ^4-3-keto-group and estrone, shown in Table IV, was satisfactory and the elution pattern was reproducible when the temperature of the column was kept constant. The optimum load was 100 to 500 μg/cm^2 for each component and as much as 5 mg/cm^2 could be added if there was an efficient separation of components.

TABLE IV

PER CENT RECOVERY OF Δ^4-3-KETOSTEROIDS AND ESTRONE FROM THE CHROMATOGRAPHIC COLUMNS

Steroids	*Eluant*		
	A	*B*	*C*
11β-Hydroxyandrostenedione	93	96	99
Adrenosterone	93	95	100
Androstenedione	91	90	100
Estrone	93	96	95

Acknowledgements

The authors wish to express their appreciation to Prof. H. KIKKAWA and Prof. K. OKANO for their encouragement and interest in this work; to Dr. S. LIEBERMAN,

who kindly provided samples of 11β-hydroxyetiocholanolone and 11-ketoetiocholanolone; to Dr. A. KANBEGAWA and Miss M. KIMURA, who kindly provided samples of dehydro-*epi*-androsterone, androsterone, *epi*-androsterone, etiocholanolone, androstanedione, etiocholanedione and androstenedione; and to Dr. J. ENDO, who kindly provided a sample of 11-ketoandrosterone.

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Received August 6th, 1962

Determination of ornithine, lysine, arginine, citrulline and histidine

Separation of the common amino acids in plant material with good resolution on a single sheet of filter paper cannot be accomplished with present procedures. MCFARREN[1] and MCFARREN AND MILLS[2] used a series of buffers and solvents, but these procedures have been found to be long and tedious. THOMPSON *et al.*[3] partially overcame this problem by separating the amino acids on columns of Dowex 50X4 resin into a basic fraction and an acidic and neutral fraction. This preliminary separation also purified the acids from extraneous materials which was necessary for high resolution. In

TABLE I

R_F VALUES OF AMINO ACIDS AND AMINES SEPARATED ON EDTA BUFFERED PAPER

Compound	R_F
Hydroxylysine	0.03
Ornithine	0.07
Lysine	0.12
Arginine	0.24
Citrulline	0.31
Histidine	0.40
Homocitrulline	0.52
Tyramine	0.65
Ethionine	0.76