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REVERSED PHASE PARTITION CHROMATOGRAPHY OF SOME C_{27}-STEROIDS*

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For metabolic studies and synthetic work on a micro-scale we needed a method for the separation of different, neutral C_{27}-hydroxy- and keto-steroids. The chromatographic systems worked out by MOSBACH *et al.*[1] for the separation of the air-oxidation products of cholesterol were not entirely satisfactory for our purposes, since cholesterol and less polar compounds move with the solvent front in these systems. The reversed phase partition chromatography technique of HOWARD AND MARTIN[2] has been found very useful for the separation of such compounds as higher fatty acids (HOWARD AND MARTIN[2]) and bile acids (BERGSTRÖM, SJÖVALL AND NORMAN[3-5]), and it was to be expected that this method could be used for the separation of neutral C_{27}-steroids.

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A preliminary report was read at the meeting of the Norwegian Biochem. Soc. in Oslo, June 1956[9].

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Different solvent-systems have been tried out. Benzene, chloroform, cyclohexane, and heptane have been used as stationary phase, and aqueous ethanol, isopropanol, and pyridine as moving phase. It has been found that systems containing 50–55% aqueous isopropanol as moving phase and 20% chloroform in heptane as stationary phase provide a rather efficient means for the separation of these substances.

MATERIALS AND METHODS

Solvents

Isopropanol: *p.a.* (Baker, Chemical Co., Phillipsburg, N.J., U.S.A.). Chloroform: techn. grade, carefully washed with conc. H_2SO_4, 5% Na_2CO_3 and H_2O; then dried over K_2CO_3, distilled, and stabilized with 1% EtOH. Heptane: redistilled.

Filter-aid

Hyflo Supercel (Johns Manville and Co.) was washed with 2 *N* HCl and water, and dried at 110° (SJÖVALL[4]). To remove fat the Hyflo was washed with acetone. After drying, it was treated according to the method of HOWARD AND MARTIN[2] with dimethyldichlorosilane in a desiccator until completely hydrophobic, then washed with methanol, and dried.

Preparation of columns

The columns were prepared as described by HOWARD AND MARTIN[2]. The best proportion between stationary phase and hydrophobic Supercel was found also in our case to be 4 ml of the former to 4.5 g of the latter. For standard runs with this amount of Supercel columns of an internal diameter of 13 mm and a length of 500 mm were used. The capacity of a column with 4.5 g of Supercel is 25–30 mg of steroids, which can be recovered in yields of 95–98%. However, it is possible to vary the size of the columns and thereby the capacity by increasing proportionally the amount of Supercel and stationary phase.

Phase systems

	Moving phase		Stationary phase	
I.	Isopropanol	165 ml	Chloroform	10 ml
	Distilled water	135 ml	Heptane	40 ml
II.	Isopropanol	157.5 ml	Chloroform	10 ml
	Distilled water	142.5 ml	Heptane	40 ml
III.	Isopropanol	150 ml	Chloroform	15 ml
	Distilled water	150 ml	Heptane	60 ml

The phases were equilibrated and the chromatograms run at constant temperature (23°). The sample to be run was dissolved in a small amount of moving phase (1–2 ml for up to 20–30 mg) and put on top of the column. In case of low solubility, the mixture can be warmed and a few drops of stationary phase added. When dealing with amounts of 50 mg and more, it is also possible to dissolve it in a small volume of stationary phase (0.5–1 ml). The effluent was collected in test tubes by using an automatic fraction collector. Fractions of about 2 ml each per 10 min were taken.

Identification of the C_{27}-steroids used

7-hydroxy-cholesterol was identified by means of the Lifschütz color-reaction as described by BERGSTRÖM AND WINTERSTEINER[6] and the extinction measured at 635 mμ. Cholesterol was assayed with the Tschuageff reaction[7] and measured at 529 mμ. Cholestan-3-one and 3α,7α-dihydroxy-coprostane were treated with conc. H_2SO_4 for 2 h at room temperature and the extinction at 310 mμ read. Furthermore, excluding cholestanone, we had access to all substances tested as labelled either with tritium or ^{14}C.

RESULTS

Fig. 1 shows the result of a run of C_{27}-hydroxy- and keto-steroids with phase system I: 55% aqueous isopropanol as moving phase and chloroform–heptane 1:4 as stationary phase, 4 ml of which was supported on 4.5 g of hydrophobic Supercel. It is seen that the elution rate is determined mainly by two factors: the amount of hydroxyls

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or keto-groups, and the presence or absence of a double bond. The tri-, di- and monohydroxy-coprostanes[8] are separated from each other and from the corresponding unsaturated compounds. It should be noted that in the chromatogram shown in Fig. 1 no monohydroxy-coprostane is included, but in this system on a column with the same amount of Supercel 3α-hydroxy-coprostane moves somewhat slower than cholesterol with its peak about 10 ml after the cholesterol-peak. Of C_{27}-monoketo-

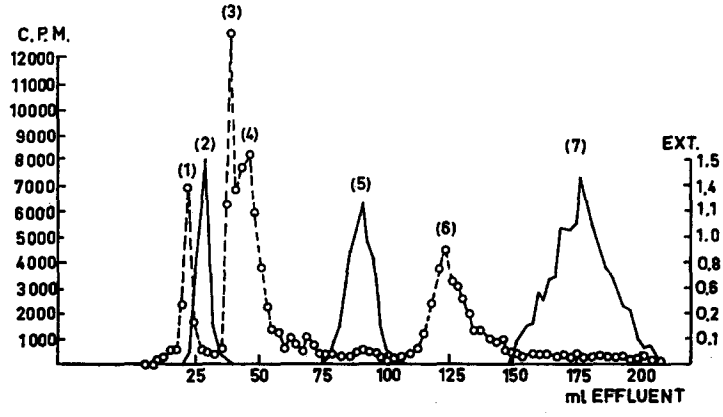

Fig. 1. Moving phase: 55% (v/v) aqueous isopropanol. Stationary phase: 20% (v/v) chloroform in heptane. 4 ml of stationary phase supported on 4.5 g of hydrophic Supercel. The compounds in the order in which they are eluted from the column: 3α-,7α-,12α-trihydroxy-coprostane (1), 7α-cholesterol (2), 3α-,7α-dihydroxy-coprostane (3), 3α-,12α-dihydroxy-coprostane (4), cholesterol (5), cholestenone (6) and cholestanone (7).

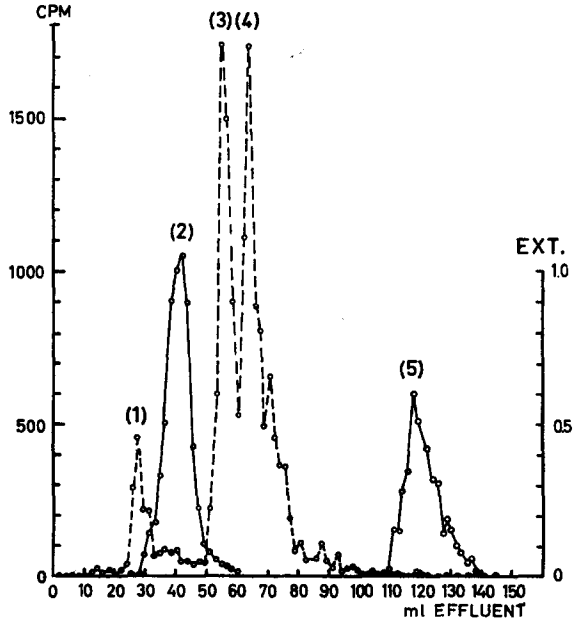

Fig. 2. Moving phase: 52.5% (v/v) aqueous isopropanol. Stationary phase: 20% (v/v) chloroform in heptane. 4 ml of stationary phase supported on 4.5 g of hydrophobic Supercel. The compounds in the order of their elution from the column: 3α-,7α-,12α-trihydroxy-coprostane (1), 7α-cholesterol (2), 3α-,7α-dihydroxy-coprostane (3), 3α-,12α-dihydroxy-coprostane (4) and cholesterol (5).

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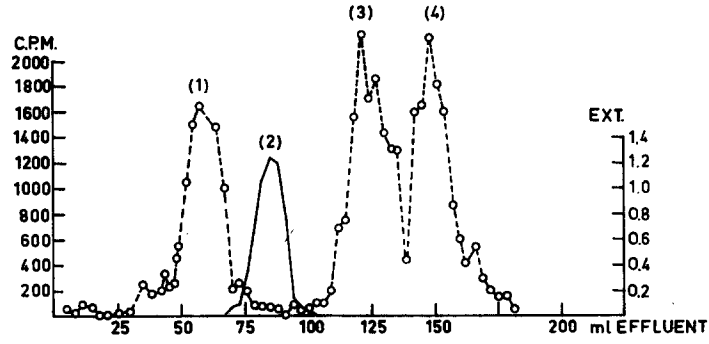

Fig. 3. Moving phase: 50% (v/v) aqueous isopropanol. Stationary phase: 20% (v/v) chloroform in heptane. 4 ml of stationary phase supported on 4.5 g of hydrophobic Supercel. The compounds in the order of their elution from the column: 3α-,7α-,12α-trihydroxy-coprostane (1), 7α-hydroxy-cholesterol (2), 3α-,7α-dihydroxy-coprostane (3) and 3α-,12α-dihydroxy-coprostane (4).

steroids cholestenone and cholestanone have been run and as seen in Fig. 1 are eluted later than cholesterol, and further, the unsaturated ketone is separated from the saturated one. Of isomers, 3α,7α- and 3α,12α-dihydroxy-coprostane have been tested, but with the phase system containing 55% aqueous isopropanol as moving phase, a resolution of these two substances is impossible. Using phase system II (52.5% aqueous isopropanol as moving phase) (Fig. 2) 3α,7α- and 3α,12α-dihydroxy-coprostane are partly separated and an almost complete resolution of these can be obtained with phase system III (50% aqueous isopropanol as moving phase) (Fig. 3). Overlapping of the two dihydroxy-coprostanes occurs but can be diminished by increasing the length of the column. 3α,7α,12α-trihydroxy-coprostane and 7α-cholesterol, which are incompletely separated with phase systems I and II, can be separated with III (Fig. 3). Using phase system II (Fig. 2) on a column with 4.5 g of Supercel cholesterol is eluted about 40 ml later than with phase system I under the same conditions and this increases the possibility of a separation from substances, which are eluted close to it with phase system I.

These systems have proved to be useful for the purification of different neutral C_{21}-steroids and also for biological work with such substances. Fig. 4 shows a chromatogram of commercial cholesterol-4-^{14}C, which in this case contained at least three different impurities.

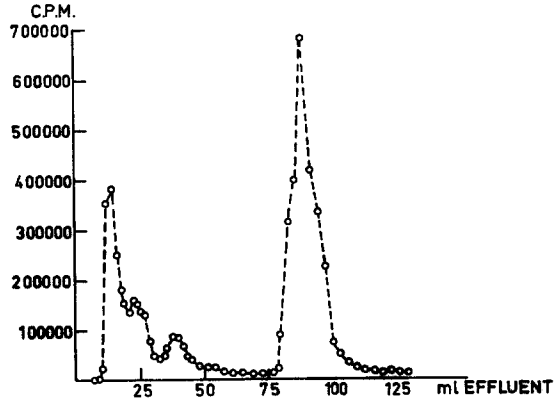

Fig. 4. Chromatography of autoxidized cholesterol-4-^{14}C. Moving phase: 55% (v/v) aqueous isopropanol. Stationary phase: 20% (v/v) chloroform in heptane. 4 ml of stationary phase supported on 4.5 g of hydrophobic Supercel.

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SUMMARY

Reversed-phase partition chromatography of some neutral C_{27}-hydroxy and keto-steroids using aqueous isopropanol as moving phase and chloroform in heptane as stationary phase is described. The method has proved useful for separating cholesterol from its autoxidation products.

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Short Communications

Chromatography of follicle-stimulating hormone (FSH) on hydroxyl apatite

Calcium phosphate has been used by several groups of investigators[1,2] to purify crude urinary gonadotropins. Recently, TISELIUS *et al.*[3] have reported that hydroxyl apatite is an excellent material for the chromatography of proteins. Using column chromatography with this substance it has been possible to prepare FSH with a high specific activity.

Swine FSH prepared by the method of STEELMAN *et al.*[4] and having an activity of 2.5 times the Armour Standard (264-151X)[5] was first purified by the use of diethylaminoethyl (DEAE)-cellulose*. A 12 × 450 mm column of DEAE-cellulose (0.5 mequiv./g) was equilibrated at pH 7.0 with 0.005 *M* sodium phosphate buffer. The FSH (750 mg), dissolved in 40 ml of the same buffer, was placed on the column and followed with 40 ml buffer. A solution consisting of 80 ml 0.025 *M* NaCl in 0.005 *M* phosphate buffer, pH 7.0, was then used to remove inactive impurities. The FSH activity was then eluted with 120 ml of a solution consisting of 0.065 *M* NaCl in 0.005 *M* phosphate, pH 7.0. The yield after dialysis and lyophilization was approximately 150 mg with an activity of 8–10 times standard.

Hydroxyl apatite was prepared according to TISELIUS *et al.*[3]; however, it was found that a bulking agent was necessary to insure an adequate flow rate. Solka Floc (α-cellulose*) washed with dilute alkali and equilibrated with the 0.01 *M* phosphate buffer, pH 6.8, was found to be satisfactory. A mixture of approximately one part of hydroxyl apatite to one part of Solka Floc did not appreciably alter the chromatographic properties of the hydroxyl apatite with respect to FSH. 100 mg FSH, purified as above, was dissolved in 15 ml 0.01 *M* phosphate buffer, pH 6.8 and placed on a column (10 × 300 mm) of hydroxyl apatite equilibrated with 0.01 *M* phosphate, pH 6.8. The sample was followed successively with 0.01 *M*, 0.04 *M* and 0.2 *M* phosphate solutions at a constant pH of 6.8 using a flow rate of 10–15 ml/h. Fig. 1 shows the elution pattern obtained by collecting

* Obtainable from Brown Paper Co.