ACTA CHEMICA SCANDINAVICA 7 (1953) 1413—1419

Separation of Conjugated Bile Acids by Partition Chromatography. Bile Acids and Steroids 6

ARNE NORMAN

Department of Physiological Chemistry, University of Lund, Lund, Sweden

A method for obtaining a quantitative separation of some conjugated bile acids was described by Ahrens and Craig [1]. They applied counter-current distribution to the extraction of bile acids from other components of bile and to the subsequent separation of mixtures of various conjugated and free bile acids into individual components. This method is, however, too time-consuming for routine analyses. More convenient for this purpose is the reversed phase partition chromatography of Howard and Martin [2], used by Bergström and Sjövall [3,4] for separation of free bile acids. Some results with this technique for the separation of conjugated acids have been reported recently by Bergström and Norman [6].

In this paper a more systematic study is described of the separation of the taurine and glycine conjugates of the common bile acids from each other and from free bile acids.

EXPERIMENTAL

The samples of conjugated bile acids used have been synthesized according to a method recently described by Bergström and Norman [5]. Cholic acid-24-^{14}C was material prepared by Bergström, Rottenberg and Voltz [7].

For details of the chromatographic procedure see Bergström and Sjövall [3] and Sjövall [4].

Preparation of columns for reversed phase partition chromatography

The columns were prepared essentially as described by Howard and Martin [2]. 4 ml of the stationary phase and 4.5 g hydrophobic kieselguhr was stirred until homogeneous. Moving phase was added to obtain a slurry that could be poured into the chromatographic tube, int. diam. 12 mm, height about 300 mm. After homogenization with a perforated plunger the kieselguhr was left to settle under weak suction. When all the material was packed, a solution of the bile acids to be analyzed in the smallest possible volume of moving phase was added.

Preparation of columns for ordinary partition chromatography

For separation of the most hydrophilic taurine conjugates ordinary partition chromatography has been used with amyl alcohol as moving phase and water as stationary phase. When the stationary phase was added to the kieselguhr, homogenized and suspended in the moving phase the material was very lumpy. Even after careful homogenization in the chromatographic tube with a plunger the kieselguhr had a marked tendency to form inhomogeneous columns. The best results were obtained when the columns were prepared in the following way:

9 g of hydrochloric acid washed, ordinary kieselguhr and enough stationary phase to make a slurry was poured into a chromatographic tube, int. diam. 17 mm and height about 400 mm. The slurry was homogenized with a perforated plunger and allowed to settle by gravity. The stationary phase is allowed to drain and at the moment when the solvent meniscus disappears in the kieselguhr the moving phase is added. The excess of the stationary phase was then removed by adding the moving phase until no stationary phase could be detected in the effluent, generally after 24 hours in a thermostat room (23°). The solution of the bile acids to be run were then introduced on to the top of the column.

The chromatographic procedure

All chromatographic work was done at a constant temperature of 23° C.

The effluent was collected in test tubes by using an automatic fraction collector, each fraction containing 1.5—2 ml (5—10 minutes). The eluted bile acids were determined by micro titration under N_2 with 0.02 *N* KOH in methanol using bromothymol blue as indicator. When ^{14}C-labelled acids were used, each fraction was brought to dryness after titration and then transferred to copper planchets for determination of the isotop content.

Phase systems for reversed phase partition chromatography

	Moving phase.*	Stationary phase.
A.	180 ml of methanol 120 ml of distilled water	45 ml of chloroform 5 ml of heptane
C.	150 ml of methanol 150 ml of distilled water	15 ml of *iso*-octanol 15 ml of chloroform
D.	300 ml of distilled water	100 ml of *n*-butanol
F.	165 ml of methanol 135 ml of distilled water	45 ml of chloroform 5 ml of heptane
G.	255 ml of methanol 45 ml of distilled water	50 ml of heptane

* For the preparation of a certain phase system the volymes of solvents indicated were mixed in a separatory funnel and equilibrated when the mixture had reached the temperature of the room (23°).

Phase system for ordinary partition chromatography

E. Moving phase: 510 ml of *iso*-amyl alcohol and 90 ml of chloroform. Stationary phase: 200 ml of distilled water.

RESULTS

A. Separation of the tauroconjugates from the glycine conjugated and free bile acids

The separation of the taurine-conjugated acids from other bile acids offers no difficulties since the taurine-conjugates are more hydrophilic than all other bile acids. The solvent system used for this purpose has recently been described by Bergström and Norman [6].

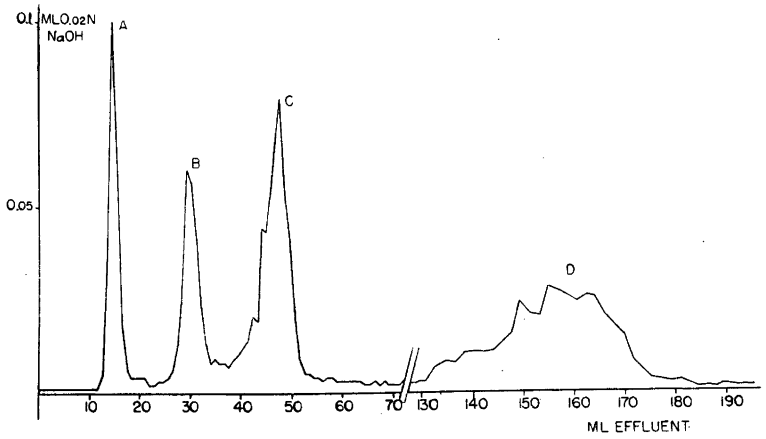

Fig. 1. Separation of taurocholic acid (A. 4.1 mg), taurocholanic acid (B. 4.4 mg), glycocholic acid (C. 6.2 mg) and cholic acid (D. 5.9 mg). Moving phase: 46 % methanol. Stationary phase: 50 % chloroform and 50 % iso-octanol. Cf. Table 1.

Table 1. Rate of elution of bile acids in relation to the composition of the moving phase. Stationary phase: isooctanol / chloroform 1 : 1.

Moving phase:	50% MeOH	47.5 % MeOH	46 % MeOH	40% MeOH	30% MeOH	25%MeOH
Cholic acid	80—98—112	110—140—170	130—160—175			
Glycocholic acid	32—37—42	38—44—50	40—48—54			
Taurocholic acid	12—14—16	12—14—16	12—14—16	12—14—16	14—17—20	14—17—20
Taurodesoxycholic acid	12—14—16	12—14—16	12—14—16			
Taurolithocholic acid	16—22—28				20—25—30	30—38—44
Taurocholanic acid	22—27—34	24—29—36	26—30—36	30—35—40	48—56—70	60—70—90

Column: 4.5 g hydrophobic Supercel with 4 ml stationary phase. The phases have been prepared by mixing 15 ml chloroform, 15 ml *iso*octanol and 300 ml aqueous methanol of the composition indicated (v/v). The figures give the beginning, peak and end of each bile acid band as the total volume (ml) of effluent at these points.

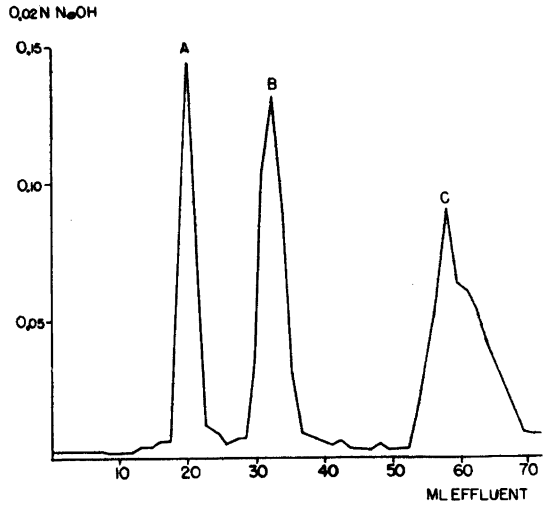

Fig. 2. Separation of taurocholic acid (A. 4.5 mg), taurodesoxycholic acid (B. 5.6 mg) and taurolithocholic acid (C. 6.5 mg). Phases: Type D, see p. 1414.

The influence of the taurine group is so pronounced that even the taurine conjugate of unsubstituted cholanic acid can be eluted from column before glycocholic acid using phase system C. This is best illustrated in a chromatography with 46 % methanol as moving phase and chloroform-*iso*octanol 1 : 1 as stationary phase (Fig. 1), where taurocholic, taurocholanic and glycocholic acid are clearly separated from each other. It is of interest to point out the great influence on the rate of elution of cholic acid caused by lowering the methanol concentration in the moving phase from 50 to 46 %. By comparison, the elution of taurolithocholic acid is not delayed more than 20 ml when the methanol concentration in the moving phase is lowered from 50 to 25 %, cf. Table 1.

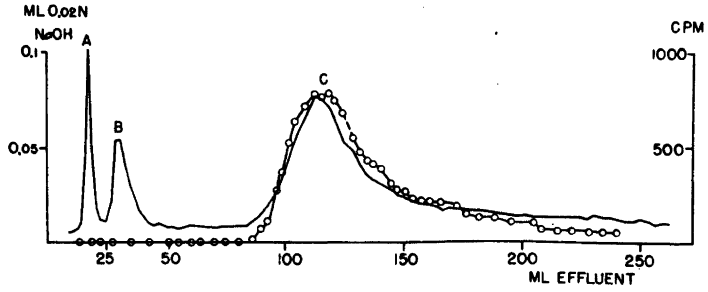

Fig. 3. Separation of taurolithocholic acid (A. 2.5 mg), taurodesoxycholic acid (B. 3.5 mg) and taurocholic acid (C. 15 mg) containing a sample of taurocholic acid-24-^{14}C. Phases: E see p. 1414. The solid lines in the figure connect the titration values and the open circles connected by a broken line show the c.p.m. of each sample.

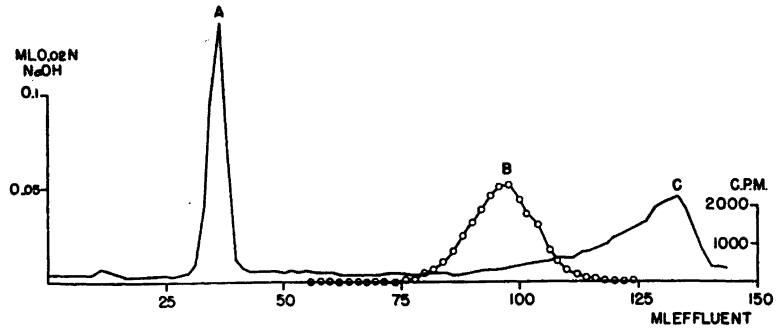

Fig. 4. Separation of glycocholic acid (A. 6.5 mg), cholic acid-24-^{14}C (B. tracerdose) and glycodesoxycholic acid (C. 7.1 mg). Phases: Type C. *See p. 1414.*

B. Separation of the tauroconjugates

For separation of the tauroconjugates from each other it is necessary to use an aqueous system because of the strong hydrophilic properties of these compounds. The best results were obtained with *n*-butanol as stationary phase and water as moving phase. Fig. 2 shows a separation of the taurocompounds of cholic, desoxycholic and lithocholic acid. The more hydrophobic taurocompounds *viz.* taurolithocholic and taurocholanic acid can be separated using 25 % MeOH as moving phase and chloroform-*iso*octanol 1 : 1 as stationary phase, *cf.* Table 1.

As taurocholic acid is the most commonly occurring compound in bile it was found necessary to have another system for separating this compound from other substances leaving the column with, or just after, the front in phase system C. Various systems with acetic acid/water as stationary phase and chloroform, amyl alcohol, and heptane as moving phase have been tried but very bad tailing of the eluted bile acids was observed. Using water as stationary phase and amyl alcohol as moving phase tailing of the eluted taurocholic acid (the peak at 100 ml) was minimised. The elution can be delayed by addition of chloroform to the moving phase. The peak appears at 110 ml with 15 % chloroform and at 220 ml with 20 % chloroform in the moving phase. Fig. 3 shows a separation of the taurine conjugates of cholic, desoxycholic and lithocholic acid by ordinary partition chromatography.

3. Separation of a glycine conjugated bile acid from free bile acids

The influence of conjugation with glycine is not so pronounced as the taurine group, so that a separation of the glycine conjugated bile acids as a group from the free acids is not possible. Conjugation with glycine of a bile acid changes its partition behaviour roughly corresponding to the introduction of a hydroxylgroup. Phase system C offers a possibility for separating the glycoconjugates and free forms of cholic and desoxycholic acid (Fig. 4). Gly-

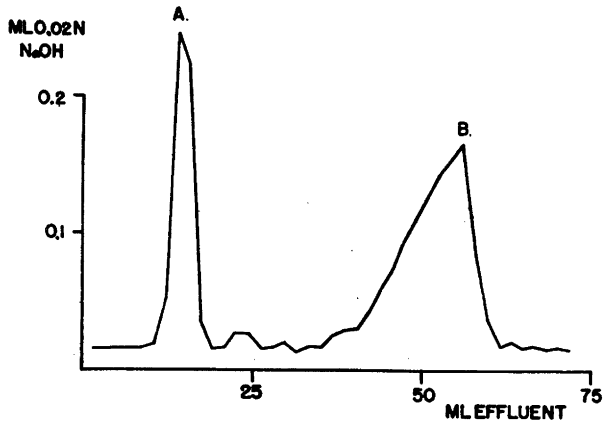

Fig. 5. Separation of glycocholanic acid (A. 4.0 mg) and cholanic acid (B. 5.5 mg). Phases: Type G. See p. 1414.

cocholic acid is clearly separated from cholic acid. Between cholic acid and glycodesoxycholic acid there is, however, some overlapping, whereas free desoxycholic aad chenodesoxycholic acid stay in the column. There exists a difference in the rate of elution of glycochenodesoxycholic and glycodesoxycholic acid in this phase system (*cf.* Table 2). This is, however, too small for a complete separation of these acids.

By using a system described by Bergström and Sjövall (phase system A) one can separate the glycine conjugate and the free form of lithocholic acid, *cf.* Table 2. Separation of glycocholanic acid from cholanic acid requires a more hydrophobic system (Fig. 5).

Table 2. Appearance of free and glycine conjugated bile acids with different phase systems.

Acid	Phase system C	Phase system G	Phase system A
Cholic acid	80—98—112	10—13—18	10—13—18
Desoxycholic acid }	> 250	48—60—66	24—30—36
Chenodesoxycholic acid }			
Lithocholic acid	> 250	> 250	70—78—93
Glycocholic acid	32—37—42	14—16—20	10—13—18
Glycodesoxycholic acid	100—132—148	18—24—28	10—13—18
Glycochenodesoxycholic acid	95—120—132	18—24—28	10—13—18
Glycolithocholic acid	> 250	60—80—90	32—40—45
Glycocholanic acid	> 250	> 250	> 250

Column: 4.5 g hydrophobic Supercel with 4 ml stationary phase.
The figures give the beginning, peak and end of each bile acid band as the total volume (ml) of effluent at these points.

SUMMARY

Partition chromatography on kieselguhr columns has been used for the separation of the taurine and glycine conjugates of cholic, desoxycholic, lithocholic and cholanic acid. Solvent systems are presented for (1) separation of the taurine conjugates from other bile acids, (2) individual separation of the taurine conjugates and (3) separation of the glycine conjugate from the corresponding free acids.

This work has been supported by a grant from the Medical Faculty, University of Lund, and by *"Knut och Alice Wallenbergs Stiftelse"*.

REFERENCES

1. Ahrens, E. H., and Craig, L. C. *J. Biol. Chem.* **195** (1952) 763.
2. Howard, G. A., and Martin, A. J. P. *Biochem. J. (London)* **46** (1950) 532.
3. Bergström, S., and Sjövall, J. *Acta Chem. Scand.* **5** (1951) 1267.
4. Sjövall, J. *Acta Physiol. Scand.* **29** (1953) 232.
5. Bergström, S., and Norman, A. *Acta Chem. Scand.* **7** (1953) 1126.
6. Bergström, S., and Norman, A. *Proc. Soc. Exptl. Biol. Med.* **83** (1953) 71.
7. Bergström, S., Rottenberg, M., and Voltz, J. *Acta Chem. Scand.* **7** (1953) 481.

Received September 29, 1953.