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Note

Column and thin-layer chromatography of cholic, deoxycholic and chenodeoxycholic bile acids and their sodium salts

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Column and thin-layer chromatography (TLC) are convenient techniques for separating bile acids and their derivatives. The qualitative separation of certain free and conjugated bile acids was investigated by Hofmann[1] using TLC. A group separation of bile acids and salts by column chromatography has been examined by several authors[2–4]. Hamilton[5] reported the separation of bile acids by applying two-dimensional thin-layer and glass–paper chromatography. Sundaram *et al.*[6] improved the separation of chenodeoxycholic acid from deoxycholic acid and several publications have appeared[7–9] which deal with the conjugated and keto derivatives of bile acids. Thus, although the chromatographic analysis of bile acids and their derivatives has been well studied there are apparently no data on the qualitative and quantitative separation of alkali bile salts, taking into account impurities. In the present study we describe TLC and column chromatography methods to do this which use a new developing solvent system.

MATERIAL AND METHODS

Bile salts were sodium deoxycholate, sodium cholate (Calbiochem, San Diego, CA, U.S.A.), and sodium chenodeoxycholate (Weddell, London, Great Britain). Deoxycholic acid and chenodeoxycholic acid were obtained from Sigma (St. Louis, MO, U.S.A.) and cholic acid was from Fluka (Buchs, Switzerland), puriss grade. Silica gel sheets (TLC aluminium sheets (20 × 20 cm) pre-coated with silica gel 60; layer thickness 0.2 mm) were from E. Merck (Darmstadt, G.F.R.), and were cut into plates 15 cm × 5 cm. All solvents were laboratory grade. Ceric ammonium sulphate reagent was prepared according to Sundaram *et al.*[6].

Bile salts and bile acids were dissolved in methanol and were applied with a Hamilton microlitre syringe as a thin line at 1.5 cm from the bottom of the plate. Plates were developed at room temperature in a pre-saturated chamber, lined with filter paper. The solvent was allowed to rise 13–14 cm from the starting line. The plates were removed, dried at 110°C, and sprayed with ceric ammonium sulphate reagent. Plates were dried then, heated, for 15 min at 120–130°C to develop the colour. In a study examining the qualitative separation of sodium cholate from sodium deoxycholate, nine different solvent systems at different mixing ratios were tested (Table I).

TABLE II

SOLVENT SYSTEMS TESTED FOR THE SEPARATION OF SODIUM CHOLATE FROM SODIUM DEOXYCHOLATE

Solvent system	*Ratios of mixing*	*Solvent composition*	*Comments*	*Ref.*
S-I	5	Methanol	No separation	10
	25	Acetone		
	70	Chloroform		
S-II	1, 8	Acetone	No separation	10
	1, 92	Benzene		
S-III	4	Acetone	Poor separation	
	1	Benzene		
	2	Glacial acetic acid		
S-IV	4.5	Methanol	No separation	
	0.5	Benzene		
	3	Glacial acetic acid		
S-V	10, 2, 16, 2	Glacial acetic acid	Better separation	10
	4, 1, 4, 1	Diisopropyl ether	compared with solvent	
	9, 1, 8, 2	Isooctane	systems S-III	
S-VI	4, 20, 4	Isoamyl acetate	Better separation	10
	3, 15, 3	Diisopropyl ether		
	1, 5, 1	Carbon tetrachloride		
	1, 5, 1	Benzene		
	2, 5, 4	*n*-Propanol		
	1, 2.5, 3	Glacial acetic acid		
S-VII	10*	Isooctane	Better separation	
	7	Ethyl acetate	than solvent systems	
	3	Glacial acetic acid	S-III, S-V, S-VI	
	4	*n*-Butanol		
S-VIII	3, 5	light petroleum (b.p. 40–60°C)	No separation	
	4, 10	Methanol		
	1, 2	Glacial acetic acid		
S-IX	21**	light petroleum (b.p. 40–60°C)	Optimum separation	
	8	*n*-Butanol		
	13.5	Glacial acetic acid		
	6	Ethyl acetate		
	7	Benzene		

* Developed by testing 12 different ratios.
** Developed by testing 100 different ratios.

For the column separation of bile salts, a column (25 cm × 4 cm I.D.) with glass wool at the bottom was prepared. A slurry of Kieselgel 60 (0.04–0.06 mm 230–400 mesh, ASTM, Merck) was poured into the column, containing 300 ml solvent system and was allowed to settle. The prepared column was washed with eluting solvent system. One gram samples of bile salts were applied and 50-ml portions of eluents were collected at a flow-rate of 1.2 ml/min.

RESULTS AND DISCUSSION

Thin-layer chromatography

Table I summarizes the results of nine solvent systems for the separation of sodium cholate from sodium deoxycholate. The solvent systems S-I and S-II gave no separation because of the low polarity of the mixture, whereas separation improved slightly when the polarity increased (S-III). Replacement of a solvent component by methanol did not increase separation (S-IV and S-VIII). However, with further increase in polarity, separation again improved (S-V, S-VI and S-VII). By further manipulation of the solvent composition, sodium cholate, sodium deoxycholate and sodium chenodeoxycholate were separated from each other and from impurities with solvent system, S-IX (light petroleum (b.p. 40–60°C)–benzene–glacial acetic acid–*n*-butanol–ethyl acetate, 21:8:13.5:6:7).

Commercial bile acids and their derivatives generally contain impurities (Table II). The quantities of impurities of bile acids and their sodium salts were investigated by TLC and were compared with the purity of sodium cholate which was accepted as a standard. Thus, Barry and Gray[11] reported that sodium cholate and sodium deoxycholate contained a slight amount of deoxycholate and chenodeoxycholate respectively. Fig. 1 illustrates the separation of impurities from bile acids and their sodium salts using solvent system S-IX, (Table I). The R_F values in Table III confirm that we can analyse qualitatively the compounds on a microscale with the solvent system.

Bile acids

Several TLC methods have been reported to separate bile acids[1,5,6,12]. In the

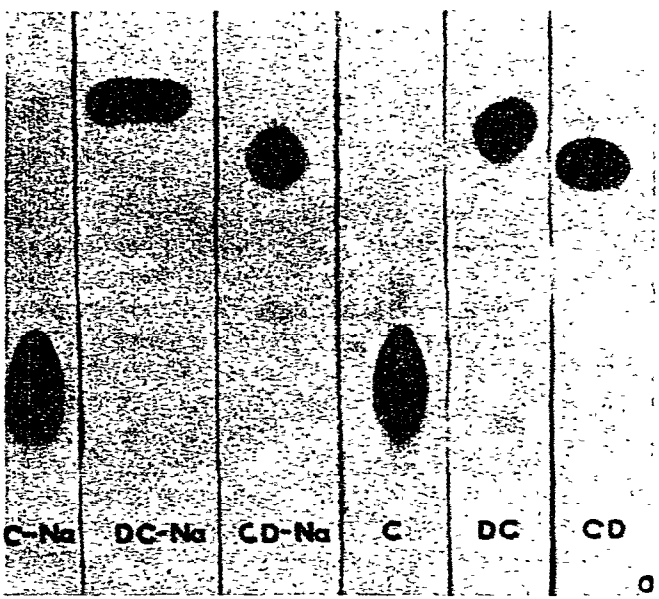

Fig. 1. Separation of bile salts and bile acids from impurities. C-Na = sodium cholate; DC-Na = sodium deoxycholate; CD-Na = sodium chenodeoxycholate; C = cholic acid; DC = deoxycholic acid; CD = chenodeoxycholic acid; O = origin.

TABLE II

GRADE AND PURITY OF COMMERCIAL BILE ACIDS AND THEIR SODIUM SALTS, AND THE ESTIMATED PERCENTAGE OF IMPURITIES D 'DUCED BY TLC

Ia = Impurity claimed by manufacturer; Ib = estimated impurity by TLC.

Bile acids	*Manufacturer*	*Ia (%)*	*Ib (%)*	*Sodium salts*	*Manufacturer*	*Ia (%)*	*Ib (%)*
Cholic	Fluka (Puriss, TLC)	<1	1–1.5	Cholate	Calbiochem (analytical grade, TLC)	3	3 (=Ia)
Deoxycholic	Sigma (grade II)	–	2–3	Deoxycholate	Calbiochem (analytical grade, TLC)	Trace spots	1
Chenodeoxycholic	Sigma	–	1–1.5	Chenodeoxycholate	Weddel (batch: MW 159)	–	2–3

TABLE III

R_F VALUES FOR THE BILE ACIDS, THEIR SODIUM SALTS AND IMPURITIES; (300 μg APPLIED)

I = Impurity.

Bile acids	$R_F \pm$ *S.D.* ($n = 6$)	*Sodium salts*	$R_F \pm$ *S.D.* ($n = 6$)
Cholic	0.38 ± 0.02	Cholate	0.51 ± 0.02
I_1	0.50	I	0.77
I_2	0.76		
Deoxycholic	0.73 ± 0.02	Deoxycholate	0.79 ± 0.02
I_1	0.30	I	0.42
I_2	0.87		
Chenodeoxycholic	0.70 ± 0.02	Chenodeoxycholate	0.70 ± 0.02
I	0.83	I	0.47

present study mixtures of the bile acids in the ratio 1:1:1 were run and the R_F values obtained were correlated with those given in the literature. Fig. 2 shows the results for mixtures of bile acids applied in total amounts of 50, 100, 200, 250, 300, and 400 μg. Table IV indicates that the separation of bile acids at 400 μg was as good as that obtained at 50 μg. Since the average R_F values of bile acids are essentially constant up to 400 μg, means of average R_F values (R_{F_m}) are also shown in Table IV together with standard deviations. The differences in R_{F_m} values for the separation of cholic acid from deoxycholic acid and chenodeoxycholic acid are slightly higher (0.45 and 0.39) than literature values (0.43 and 0.37)[1] and (0.42 and 0.34)[6]. The difference obtained for the separation of chenodeoxycholic acid from deoxycholic acid (0.06) was lower than 0.08 given by Sundaram *et al.*[6]. To check the efficiency of the solvent

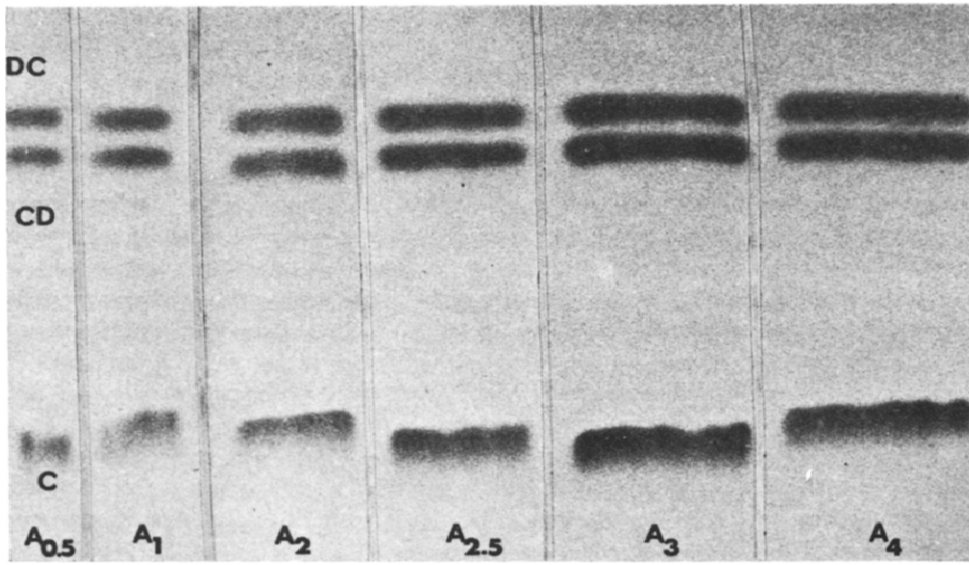

Fig. 2. Separation of bile acids from their 1:1:1 mixtures; C = cholic acid; CD = chenodeoxycholic acid; DC = deoxycholic acid. Applied quantity: $A_{0.5}$, 50 μg; A_1, 100 μg; A_2, 200 μg; $A_{2.5}$, 250 μg; A_3, 300 μg; A_4, 400 μg.

TABLE IV

R_F VALUES FOR THE BILE ACIDS OF VARIOUS APPLIED QUANTITIES

Bile acids	$R_F \pm S.D.$ $(n = 6)$						
	50 μg	*100 μg*	*200 μg*	*250 μg*	*300 μg*	*400 μg*	R_{Fm}
Deoxycholic	0.76 ± 0.03	0.76 ± 0.02	0.74 ± 0.02	0.76 ± 0.02	0.77 ± 0.02	0.77 ± 0.02	0.76 ± 0.01
Chenodeoxycholic	0.69 ± 0.02	0.70 ± 0.02	0.68 ± 0.02	0.70 ± 0.02	0.71 ± 0.01	0.71 ± 0.02	0.70 ± 0.01
Cholic	0.30 ± 0.02	0.30 ± 0.02	0.30 ± 0.03	0.31 ± 0.03	0.31 ± 0.02	0.31 ± 0.02	0.31 ± 0.01

TABLE V

R_F VALUES FOR THE BILE SALTS OF VARIOUS APPLIED QUANTITIES

Sodium salts	$R_F \pm S.D.$ $(n = 6)$						
	50 μg	*100 μg*	*200 μg*	*250 μg*	*300 μg*	*400 μg*	R_{Fm}
Deoxycholate	0.77 ± 0.03	0.75 ± 0.01	0.76 ± 0.03	0.76 ± 0.02	0.76 ± 0.02	0.76 ± 0.02	0.76 ± 0.01
Chenodeoxycholate	0.70 ± 0.02	0.68 ± 0.02	0.69 ± 0.02	0.69 ± 0.03	0.69 ± 0.01	0.69 ± 0.02	0.69 ± 0.01
Cholate	0.29 ± 0.02	0.30 ± 0.02	0.30 ± 0.01	0.31 ± 0.02	0.31 ± 0.02	0.31 ± 0.03	0.30 ± 0.01

system of these authors, mixtures of 250 μg of each acid (cholic, deoxycholic and chenodeoxycholic) were run with their solvent system (isooctane–ethyl acetate–*n*-butanol–acetic acid, 10:5:1.5:1.5). The average R_F values of six replicates for deoxycholic acid, chenodeoxycholic acid and cholic acid were 0.35 ± 0.03, 0.31 ± 0.03 and 0.12 ± 0.01 respectively. The difference between the R_F values for deoxycholic acid and chenodeoxycholic acid (0.04) is lower than 0.08 as given by Sundaram *et al.*[6], under identical conditions. The same mixture of bile acids was run with our solvent system. The average R_F values of six replicates for corresponding bile acids were 0.73 ± 0.01, 0.67 ± 0.02 and 0.29 ± 0.01. The results show that the differences between the R_F values for deoxycholic acid, chenodeoxycholic acid and cholic acid were greater for our solvent system and reproducibility was better compared with the system of Sundaram *et al.*[6].

Bile salts

Mixtures of sodium chenodeoxycholate, sodium cholate, and sodium deoxycholate (1:1:1) in amounts of 50, 100, 200, 250, 300 and 400 μg were run. Table V and Fig. 3 indicate that the bile salts separate as well from each other as do the bile acids. Again separation was good up to 400 μg, a higher limit than for the methyl esters reported in the literature[6]. The R_F values change little with applied quantity of mixture. The difference between R_{F_m} values for sodium cholate and the other salts (0.46 and 0.39 for sodium deoxycholate and chenodeoxycholate respectively) were greater than bile acid differences reported by Sundaram *et al.*[6]. However, the difference between sodium chenodeoxycholate and deoxycholate values was lower (0.07) than the value (0.08) given[6] for the corresponding bile acids.

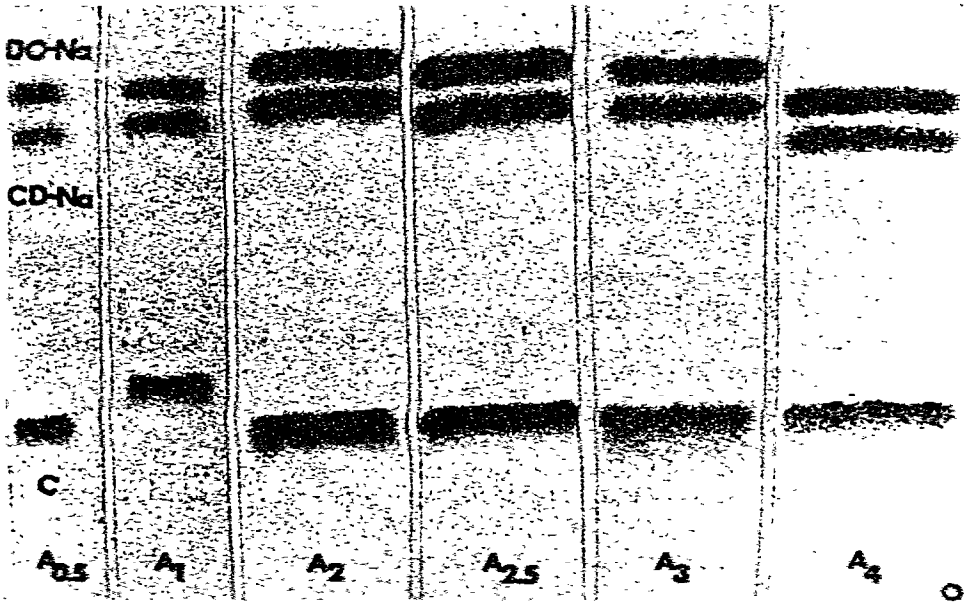

Fig. 3. Separation of bile salts from each other; C = sodium cholate; CD-Na = sodium chenodeoxycholate; DC-Na = sodium deoxycholate. Applied quantity: $A_{0.5}$, 50 μg; A_1, 100 μg; A_2, 200 μg; $A_{2.5}$, 250 μg; A_3, 300 μg; A_4, 400 μg; O = origin.

Column chromatography

The separation of bile salts from impurities by TLC using our solvent system appears to offer the possibility for preparative applications. Thus, purification of bile salts was studied by column chromatography using the same solvent system. Samples were applied to the column and the collected eluents were checked by TLC. Those portions which showed impurities were rejected. The remaining eluents were combined, dried and applied in amounts of 300 μg in methanol on TLC. Fig. 4 shows no detectable impurities of bile salts on TLC; the recovery was 70%. The R_F values for six replicates for the purified bile salts were sodium cholate (0.48 ± 0.01), sodium deoxycholate (0.83 ± 0.01) and sodium chenodeoxycholate (0.71 ± 0.01). Because of the rapid and efficient separation, column chromatography with our solvent system is a convenient technique for preparing pure samples of the bile salts.

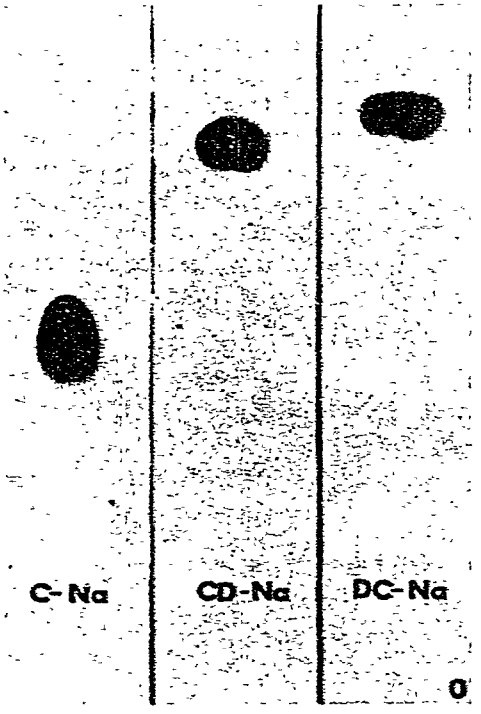

Fig. 4. TLC of purified bile salts; C-Na = sodium cholate; CD-Na = sodium chenodeoxycholate; DC-Na = sodium deoxycholate; O = origin.

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