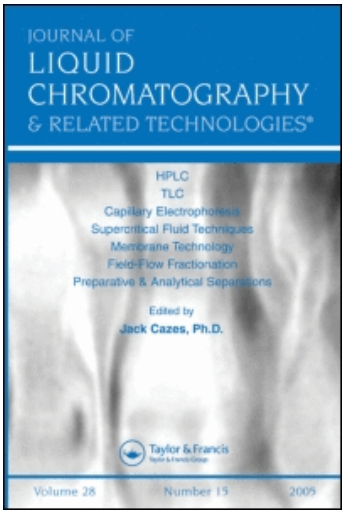

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Thin-Layer Chromatography of Bile Alcohols, Bile Acids and Conjugated Bile Acids

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THIN-LAYER CHROMATOGRAPHY OF BILE ALCOHOLS, BILE ACIDS AND CONJUGATED BILE ACIDS

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ABSTRACT

Thin-layer chromatography of bile alcohols, bile acids and bile acid conjugates has been reviewed. Particular emphasis has been placed on the separation of the glycine and taurine conjugated bile acids as a class and as individual compounds, and on the isolation of bile alcohols and C_{27} bile acids diastereoisomeric at C-25.

INTRODUCTION

Bile acids are the end products of cholesterol metabolism in the liver and are excreted by the liver as glycine and taurine conjugates. The hepatic transformation of cholesterol into bile acids proceeds via a number of bile alcohol and C_{27} bile acid intermediates. It has been observed that several liver diseases cause specific changes in the composition of bile alcohols and bile acids that are present in the bile. For a quantitative determination of the bile acid components in the bile by gas-liquid chromatography-mass spectrometry, they are first resolved by thin-layer chromatography (TLC). The TLC separation of various bile acids of biological interest has been studied in great detail (1-9). Also, the realization that the conjugation

pattern of bile acid changes in certain liver diseases has prompted attempts to separate bile acid conjugates by TLC and various solvent systems have been proposed for the complete or partial separation of various bile acid conjugates (3,7-9, 10-19). However, little is known about the TLC separation of the intermediate bile alcohols and C_{27} bile acids, particularly the compounds that have an asymmetric centre at C-25.

The purpose of the present paper is to review the TLC separation of bile alcohols, bile acids and conjugated bile acids with particular emphasis on the separation of the different isomeric compounds.

MATERIALS AND METHODS

The C_{27} bile alcohols and bile acids used for TLC in the present study were obtained as described previously (20-22), and the C_{24} bile acids and conjugated bile acids were purchased from Calbiochem, Los Angeles, CA. Glycine and taurine conjugates of ursodeoxycholic acid were synthesized according to Tsern, etal. (23). The compounds to be analyzed by TLC on silica gel G plates were dissolved in methanol and 2-4 μg was applied at room temperature to 0.25 mm thick plate (Brinkman, Westbury, N.Y.) and the bile alcohols to be analyzed on neutral alumina plates were dissolved in methanol and 5-7 μg was applied at 40°C to 0.25 mm thick plate (Analtech Inc., Newark, DE) activated before use at 110°C for 1 hour. The plate was then developed in the appropriate solvent system at ambient temperature and the solvent was allowed to rise 16-18 cm. from the starting line, followed by drying with hot air. The plate was then subsequently developed in the same or different solvent system and finally dried at 110°C. The plate

was then sprayed with 20% sulfuric acid and phosphomolybdic acid (3.5% in isopropanol, E M Labs., Westbury, N.Y.) and heated at 110°C for 2-3 minutes. The spots thus obtained had a maximum diameter of 1 cm. on a silica gel G plate and 2 cm. on a neutral alumina plate.

RESULTS AND DISCUSSION

Bile Alcohols: Thin-layer chromatographic behaviour of several bile alcohols has been summarized by Eneroth (24) and it has been reported that bile alcohols that differ from one another with respect to the number of -OH groups are very well separated, but the bile alcohols that differ with respect to the position of a side chain -OH group are not well resolved. The TLC separation of the various side chain hydroxylated bile alcohols is very important for enzymatic studies since the hepatic enzymes that are responsible for the side chain hydroxylation of bile alcohols are non-specific and may yield several products hydroxylated at C-22, C-23, C-24, C-25 or C-26. Bile alcohols with epimeric C-22, C-23 or C-24 hydroxyl group have now been successfully separated on silica gel G plates (20,25-30).

We have reported the separation of several trihydroxylated bile alcohols in a mixture (31) and now, using a combination of silica gel G and neutral alumina plates, we have completely separated the following 5β-cholestane-tetrols from one another: 5β-cholestane-3α,7α,12α,24(R)-tetrol, 5β-cholestane-3α,7α,12α,24(S)-tetrol, 5β-cholestane-3α,7α,12α,25-tetrol, (25R) 5β-cholestane-3α,7α,12α,26-tetrol and (25S) 5β-cholestane-3α,7α,12α,26-tetrol (Fig. 1). It was found that 5β-cholestane-3α,

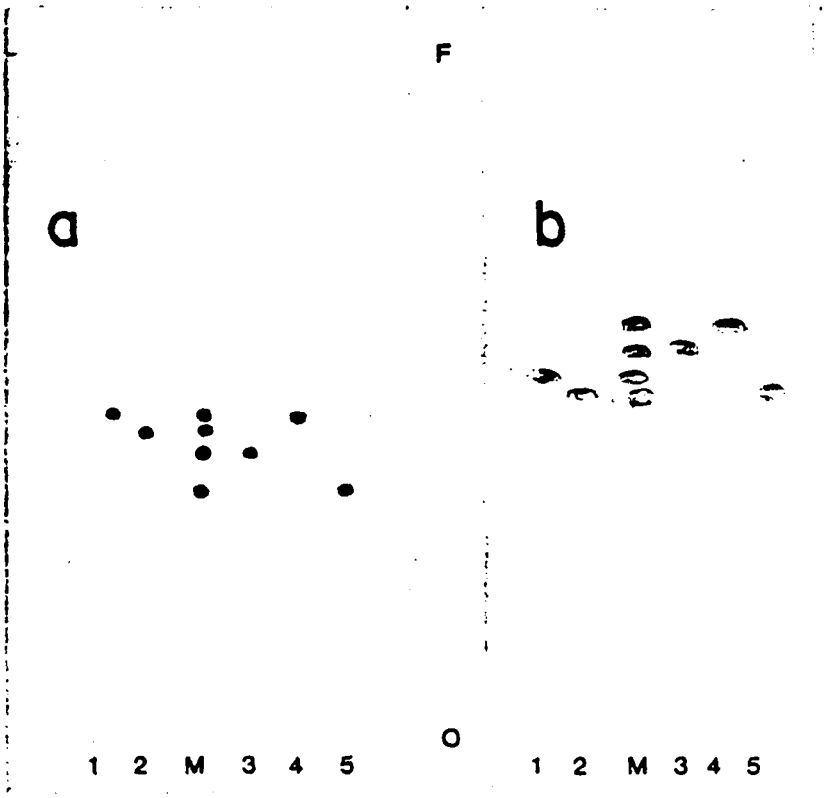

FIGURE 1

TLC Separation of 5β-Cholestane-tetrols. a, Silica Gel G Plate; Solvent System, Chloroform:Acetone:Methanol, 70:50:7, Three Developments; b, Neutral Alumina Plate; Solvent System, Benzene: Ethyl Acetate:Methanol, 90:20:14, Two Developments. 1, (25S) 5β-Cholestane-3α,7α,12α,26-tetrol; 2, (25R) 5β-Cholestane-3α,7α,12α, 26-tetrol; M, Mixture of 5β-Cholestane-tetrols; 3, 5β-Cholestane-3α,7α,12α,25-tetrol; 4, 5β-Cholestane-3α,7α,12α,24S-tetrol; 5, 5β-Cholestane-3α,7α,12α,24R-tetrol; O, Origin; F, Solvent Front.

7α,12α,24(S)-tetrol and (25S) 5β-cholestane-3α,7α,12α,26-tetrol that had identical R_f values on the silica gel G plate were well resolved on a neutral alumina plate. On the other hand, 5β-cholestane-3α,7α,12α,24(R)-tetrol and (25R) 5β-cholestane-3α,7α,12α,26-tetrol could not be resolved on the neutral alumina plate and were separable on the silica gel G plate. The R_f values of the various 5β-cholestane-triols and 5β-cholestane-tetrols on silica gel G and neutral alumina plates are shown in table I. It may be remarked here that the spots obtained on the neutral alumina plates are bigger in diameter and the resolution of compounds on these plates is sensitive to humidity.

Bile Acids: TLC of bile acids has been studied extensively. Eneroth has described the separation of several isomeric mono-, di- and trihydroxylated bile acids and their derivatives (4) and found that solvent systems that contained acetic acid produced better resolution than neutral solvent systems. Allo-bile acids have been separated from their 5β-analogs by several workers (11,32-34) and bile acids with a double bond have been separated from saturated bile acids on silver nitrate impregnated silica gel G plates (35,36). Morimoto separated 3α, 7α,12α-trihydroxy bile acids with 3-10 carbon atoms in the side chain with chloroform: ethyl acetate: acetic acid (45:45:10) as the solvent system and found that the farther the COOH group from the nucleus, the less polar was the bile acid (37). Chenodeoxycholic acid can be separated from its 7β-OH epimer, ursodeoxycholic acid (4), but its separation from the 3α,12α-dihydroxy

TABLE 1

R_f Values of Some 5β-Cholestane-triols and 5β-Cholestane-tetrols on Silica Gel G and Neutral Alumina Plates

5β-Cholestane-	R_f^a	R_f^b	R_f^c	R_f^d
3α,7α,24R-triol	0.59	0.61		
3α,7α,24S-triol	0.64	0.70		
3α,7α,25-triol	0.56	0.61		
3α,7α,26-triol(25R)	0.61	0.44		
3α,7α,26-triol(25S)	0.63	0.51		
3α,7α,12α,24R-tetrol			0.35	0.48
3α,7α,12α,24S-tetrol			0.47	0.59
3α,7α,12α,25-tetrol			0.41	0.55
3α,7α,12α,26-tetrol(25R)			0.44	0.48
3α,7α,12α,26-tetrol(25S)			0.47	0.51

[a]Silica Gel G Plate; Solvent System, Chloroform:Acetone:Methanol, 70:50:2; Two Developments.

[b]Neutral Alumina Plate; Solvent System, Chloroform:Acetone:Methanol, 70:50:7 (First Development); Benzene; Ethyl Acetate:Methanol, 90:20:7 (Second Development).

[c]Silica Gel G Plate; Solvent System, Chloroform:Acetone:Methanol, 70:50:7; Three Developments.

[d]Neutral Alumina Plate; Solvent System, Benzene:Ethyl Acetate:Methanol, 90:20:14; Two Developments.

isomer, deoxycholic acid is generally found to be difficult. These two compounds have been separated as their methyl esters (5) and recently, as free acids (15).

Although, neutral solvent systems are not widely used for the TLC of bile acids, we have found that systems containing

mixtures of chloroform, acetone and methanol are very useful for the separation of C_{27} bile acids diastereoisomeric at C-25. Thus, (25R and 25S) 3α,7α-dihydroxy-5β-cholestan-26-oic acids were resolved by allowing the plate to run twice in a solvent system of chloroform:acetone:methanol (70:30,1.5) (21) and (25R and 25S) 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acids were resolved by allowing the plate to run twice in chloroform:acetone:methanol (70,50,10) (22). Table 2 shows the R_f values of these compounds. Using this TLC method, we have been able to prove that the natural isomer of 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid in the bile of alligator is 25R (38).

Conjugated Bile Acids: Bile acids are present in the bile as conjugates with glycine and taurine and it is important to

TABLE 2

R_f Values of 25R and 25S Diastereoisomers of 3α,7α-Dihydroxy-5β-cholestan-26-oic Acid and 3α,7α,12α-Trihydroxy-5β-cholestan-26-oic Acid

Compound	R_f
(25R) 3α,7α-Dihydroxy-5β-cholestan-26-oic acid	0.56[a]
(25S) 3α,7α-Dihydroxy-5β-cholestan-26-oic acid	0.60[a]
(25R) 3α,7α,12α-Trihydroxy-5β-cholestan-26-oic acid	0.41[b]
(25S) 3α,7α,12α-Trihydroxy-5β-cholestan-26-oic acid	0.44[b]

[a]Solvent System, Chloroform:Acetone:Methanol, 70:30:1.5; Two Developments.

[b]Solvent System, Chloroform:Acetone:Methanol, 70:50:10; Two Developments.

analyze them in the form of conjugates in order to overcome problems of artefact formation and partial destruction of bile acids during hydrolysis of the conjugates. However, till very recently, solvent systems were not available for the separation of individual bile acid conjugates. It was however, found that the glycine conjugates of the bile acids were separable from the corresponding taurine conjugates and that the conjugates that differed from one another with respect to the number of -OH groups could be easily resolved (3,10-14). Chavez and Krone reported a group separation of the glycine and taurine conjugates of bile acids (15) and recently, Goswami and Frey separated the glycine and taurine conjugates of chenodeoxycholic acid and deoxycholic acid from one another (16). We developed a solvent system of chloroform:isopropanol:acetic acid:water (30:30:4:1) that could achieve a group separation of the glycine and taurine conjugates of lithocholic acid, chenodeoxycholic acid, deoxycholic acid and cholic acid and also separate the individual bile acid conjugates from one another (17). Touchstone, etal. have recently separated the glycine and taurine conjugates of cholic, chenodeoxycholic and deoxycholic acids from one another and from glycolithocholic acid on K C_{18} F reverse phase TLC plates using ethanol-0.3M calcium chloride-dimethylsulfoxide (25:25:2) as mobile phase (39). Using a solvent system of chloroform:isopropanol:acetic acid:water (60:60:3:0.75), we have now been able to separate the glycine and taurine conjugates of the three dihydroxy bile acids, ursodeoxycholic acid, chenodeoxycholic acid and deoxycholic acid from one another (Fig. 2).

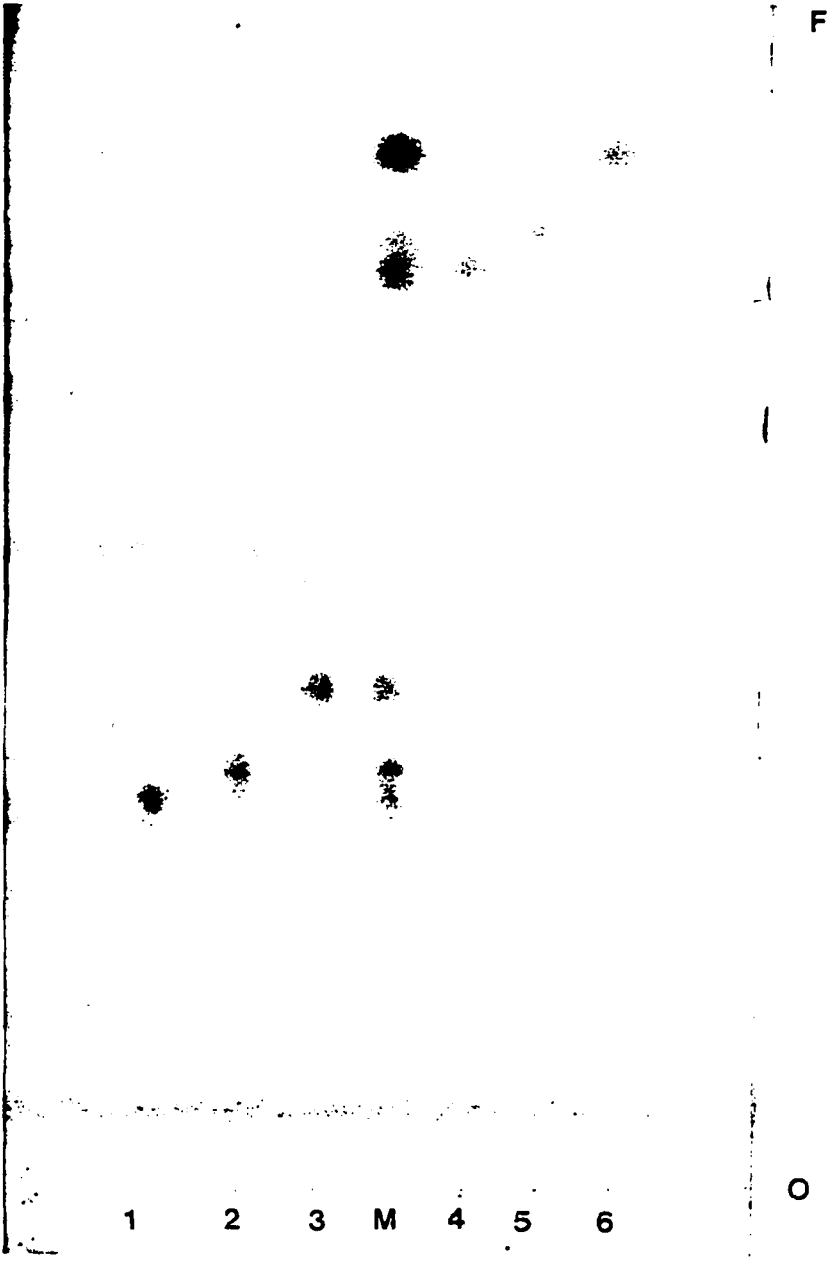

FIGURE 2

TLC Separation of Conjugated Ursodeoxycholic, Chenodeoxycholic and Deoxycholic Acids. Solvent System, Chloroform:Isopropanol: Acetic Acid:Water, 60:60:3:0.75; Two Developments. 1, Taurodeoxycholic Acid; 2, Taurochenodeoxycholic Acid; 3, Tauroursodeoxycholic Acid; M, Mixture of the Conjugated Dihydroxy Bile Acids; 4, Glycodeoxycholic Acid; 5, Glycochenodeoxycholic Acid; 6, Glycoursodeoxycholic Acid; O, Origin; F, Solvent Front.

This TLC separation has enabled us to study the conjugation pattern of ursodeoxycholic acid in the bile when it is fed to patients with gallstones.

In the course of our studies, we also found that the mobilities of the glycine conjugates of the bile acids were markedly reduced on increasing the pH of the solvent system. The effect of pH was much less marked on the mobilities of the taurine conjugated bile acids (Table 3). By adjusting the pH of the solvent system, the mobilities of the compounds could be changed to the extent that the least polar of the glycine conjugated bile acids, glycolithocholic acid moved slower than the most polar taurine conjugated bile acid, taurocholic acid (Fig. 3). Thus, although the neutral or basic solvent systems tested do not separate all the conjugated bile acids, they may be very useful to obtain a group separation of the glycine and taurine conjugated bile acids. Furthermore, the effect of the change of pH from acidic to basic in the solvent system can serve as a criterion to establish whether a given conjugated bile acid belongs to the taurine or the glycine class.

TABLE 3

Effect of Change of pH on the R_f Values of Conjugated Bile Acids

Compound	R_f value in solvent system[a]					
	A_1	N_1	B_1	A_2	N_2	B_2
Glycolithocholic acid	0.86	0.41	0.51	0.86	0.50	0.52
Glycochenodeoxycholic acid	0.72	0.34	0.46	0.65	0.40	0.45
Glycodeoxycholic acid	0.72	0.32	0.46	0.65	0.40	0.45
Glycocholic acid	0.63	0.23	0.41	0.58	0.31	0.35
Taurolithocholic acid	0.60	0.56	0.68	0.73	0.73	0.74
Taurochenodeoxycholic acid	0.52	0.49	0.64	0.63	0.67	0.69
Taurodeoxycholic acid	0.52	0.47	0.64	0.63	0.67	0.69
Taurocholic acid	0.41	0.36	0.58	0.46	0.60	0.62

[a]The Compositions of the Various Solvent Systems Used are as Follows: A_1, n-Butanol:Acetic Acid:Water, 20:4:3; N_1, n-Butanol:Water, 20:3; B_1, n-Butanol:Pyridine:Water, 20:4:3; A_2, Ethyl Acetate:Methanol:Acetic Acid:Water, 35:12:2:2, N_2, Ethyl Acetate:Methanol:Water, 35:12:2; B_2, Ethyl Acetate:Methanol:Pyridine:Water, 35:12:5:2.

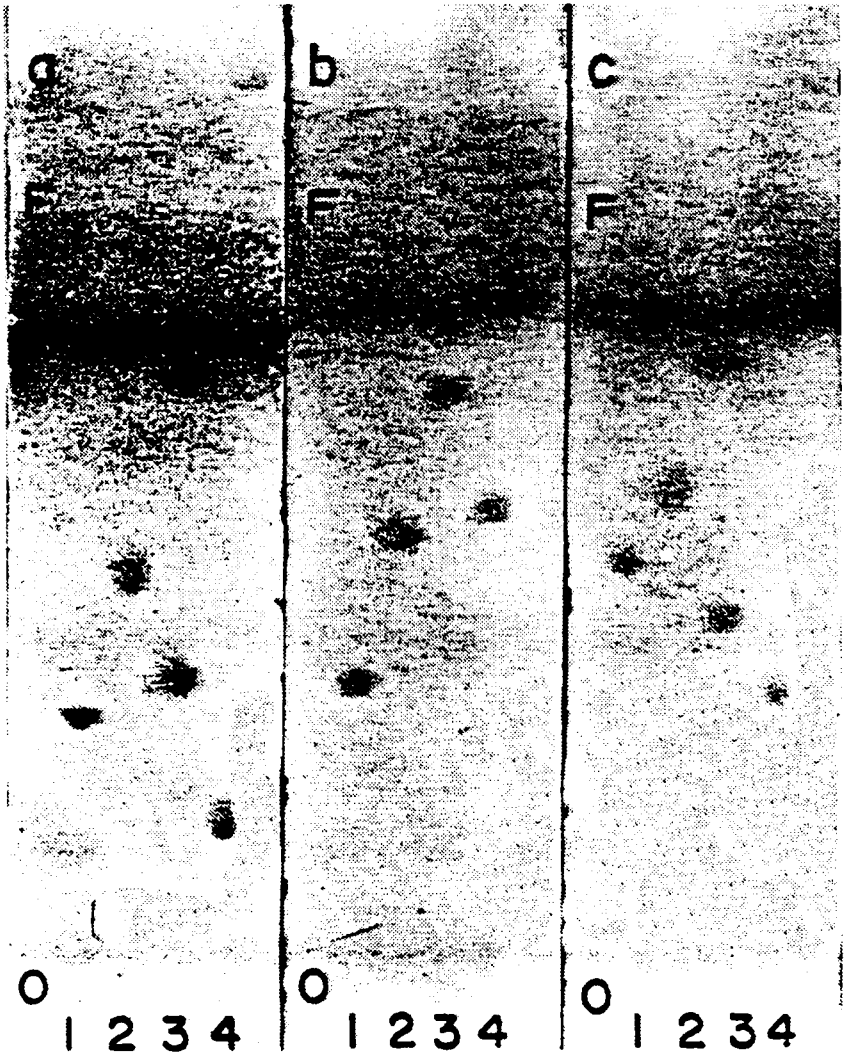

FIGURE 3

Effect of pH on R_f Values of Conjugated Bile Acids. Solvent System: a, n-Butanol:Water, 20:3; b, n-Butanol:Acetic Acid: Water, 20:4:3; c, n-Butanol:Pyridine:Water, 20:4:3. 1, Taurocholic Acid; 2, Taurolithocholic Acid; 3, Glycolithocholic Acid; 4, Glycocholic Acid; O; Origin, F, Solvent Front.

ACKNOWLEDGEMENTS

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