Alkaline solvent systems for thin-layer chromatography of bile acids

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SUMMARY Thin-layer chromatographic separation of the common bile acids and their taurine and glycine conjugates in chloroform–methanol–ammonia is reported. An alkaline system offers advantages for the separation and nondestructive staining of bile acid conjugates.

KEY WORDS thin-layer chromatography · ammonia system · conjugated · nonconjugated · bile acids · nondestructive staining

Bile acids and their conjugates and synthetic derivatives have been separated on TLC both with neutral and acidic solvent systems (1, 2). The great success of the acidic systems in resolving most positional isomers and epimers of the natural bile acids has led to a virtual dismissal of the neutral and any basic chromatographic media (3). The presence of relatively nonvolatile organic acid in the developing system, however, is undesirable when sensitive and nondestructive staining of the separated bile acids is desired. This communication describes ammonia-containing solvent systems that give useful separations of physiologically important bile acids and their conjugates and greatly increase the sensitivity of detection of the components with iodine and 2′, 7′-dichlorofluorescein.

Bile Acids. Reference bile acids were obtained commercially and were purified before use by standard TLC techniques (3). The bile acid conjugates were synthesized in the laboratory by the method of Norman (4) and purified by crystallization and TLC. The methyl esters of free and conjugated bile acids were prepared by reaction either with diazomethane (5) or with BF_3–methanol. The latter reagent was particularly suitable for preparative work. About 5 mg of a bile acid or its conjugate was treated with 3 ml of BF_3–methanol (Applied Science Laboratories Inc., State College, Pa.) at room temperature overnight (12 hr). We recovered the esters by diluting the reaction mixture with an equal volume of water and extracting with diethyl ether (methyl esters of bile acids) or ethyl acetate (methyl esters of bile acid conjugates).

Natural mixtures of bile acids and bile acid conjugates were extracted from the bile and plasma of man and rat by standard techniques (2).

Abbreviation: TLC, thin-layer chromatography.

Experimental Conditions. A layer of Silica Gel G or H (Merck and Co.), 0.25 mm thick, is prepared by standard methods on 20 × 20 cm glass plates and with Desaga (Desaga, Heidelberg, Germany) equipment. The plates are activated at 110°C for 1 hr and used immediately after cooling to room temperature. The bile acids or their conjugates (0.5–10 μg) dissolved in either methanol or chloroform are applied as spots or bands about 2 cm from the bottom of the plate by means of a 50 μl Hamilton syringe (Hamilton Company Inc., Whittier, Calif.). After evaporation of the solvent the plates are developed in Desaga jars with the solvent systems described in the footnote to Table 1. The solvent front is allowed to rise to a height of about 14 cm, which requires 40–80 min at room temperature. At the end of the run the plates are air-dried in the fume hood for 2 min, and then either placed in another jar containing iodine crystals or sprayed with a 0.05% solution of dichlorofluorescein in 50% methanol for inspection under UV light.

Separation of Standards. Table 1 gives the R_f values for some of the alkaline solvent systems investigated. The absolute mobilities of these compounds are presented in Fig. 1 in the form of schematic chromatograms. System I separates the mono-, di-, and trihydroxy bile acid conjugates of glycine and taurine as groups. The taurine conjugates migrate faster than the glycine conjugates. Within each conjugate group, the trihydroxy derivatives move more slowly than those of the dihydroxy bile acids, and the latter again more slowly than those of the monohydroxy bile acids. The positional isomers and epimers of the conjugated dihydroxy bile acids overlap. Furthermore, any free dihydroxy bile acids overlap with taurocholic acid and any free cholic acid overlaps with glycocholic acid. While the partial overlap of the conjugates with the free bile acids is a disadvantage, it is more than offset by the ability of alkaline systems to move the taurine conjugates away from the origin.

System II allows the resolution of free bile acids into groups with three, two, one, or no hydroxyl functions per molecule. The conjugated bile acids overlap the corresponding free bile acids nearly completely and only a few useful resolutions of free and conjugated acids are obtained with this system.

System III invariably effects a complete separation of the mono-, di-, and trihydroxy bile acids and their methyl esters, such as is achieved only rarely with the common acidic systems. Under the conditions selected all the methyl esters (including those of the conjugates) are carried to the top of the plate. The system is useful for the purification of bile acid methyl esters which are to be used as standards.

Mixed bile acid methyl esters may be resolved with System IV, which retains all the free and conjugated bile acids together in one group near the origin. The

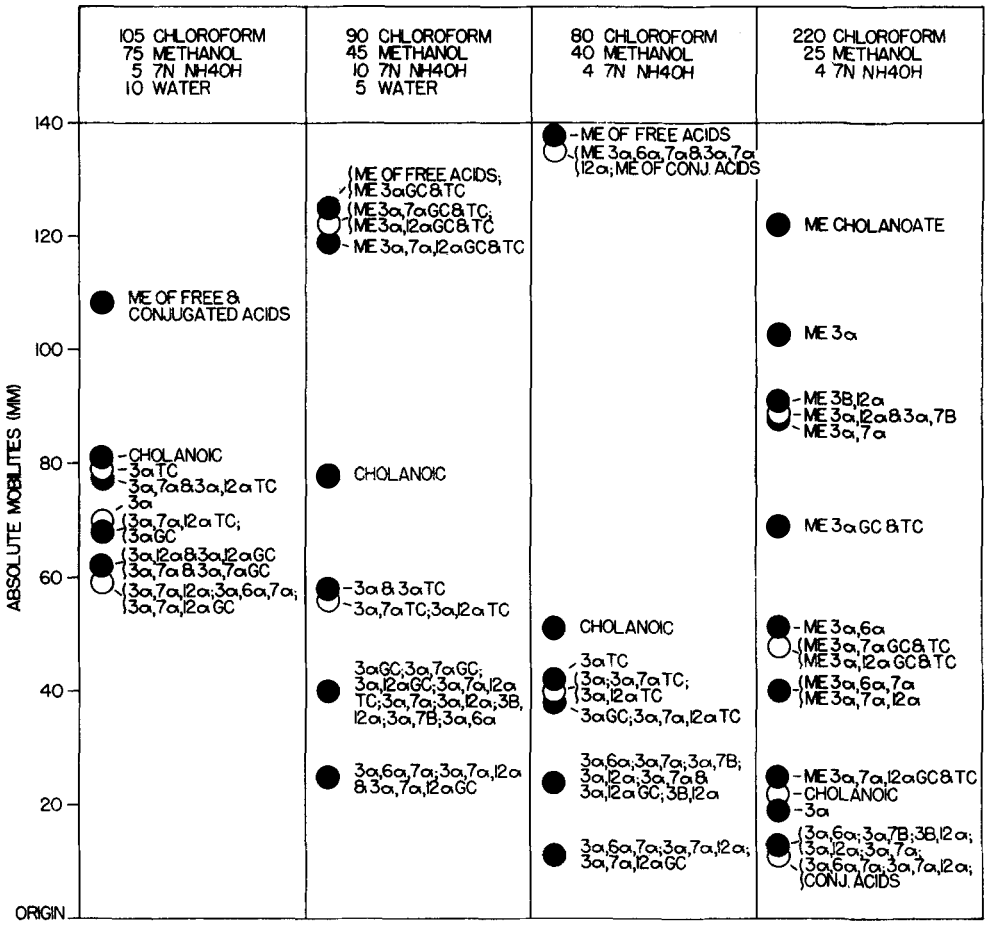

FIG. 1. Schematic chromatograms of the TLC separation of bile acids in ammonia-containing systems. Solvent ratios as given at the top of the diagrams.

Abbreviations: ME, methyl esters; GC, glycine conjugate; TC, taurine conjugate; 3α, 6α, 7α, 12α, 3β, and 7β indicate the location and configuration of the hydroxyl functions on the cholanoic acid molecule.

order to migration of the bile acid methyl esters in this alkaline system is nearly identical with that obtained in most acidic systems, except that the 7β-OH derivative (ursodeoxycholic acid) moves ahead of the 7α-OH derivative (chenodeoxycholic acid). Other bile acid derivatives, such as the acetates and even trifluoroacetates, can also be resolved in the alkaline developing systems, but no examples of resolution are given here.

The kind of separation obtained in the alkaline systems is illustrated in Fig. 2 with the methyl esters of selected bile acids and conjugates. In general, the chromatoplates of both free and methylated bile acids or conjugates are characterized by almost complete absence of tailing, which results in compact spots or bands for all components. This permits more sensitive detection and more effective recovery of the materials from the TLC plates.

Separation of Unknowns. The ammonia systems have been successful in the isolation of taurine- and glycine-conjugated bile acids from the bile and plasma of the rat (5) and man. The separation of the free bile acids or their methyl esters into well-defined classes containing uniform numbers of functional groups provides an adequate preliminary segregation of unknown bile acids for a reliable further separation and identification by gas–liquid chromatography (6).

Limit of Detection. The lower limit of detection varies with the nature of the component and the method of staining used. With the alkaline systems, iodine was the most sensitive stain and allowed the recognition of all components in a concentration of 0.1 μg/cm² or higher within a few seconds of exposure to iodine vapour. This was a nearly 20-fold increase over the sensitivity of iodine staining after development with acidic systems, where about 2 μg/cm² can be detected after a 30–45-min exposure.

When the plates have been developed in ammonia, the bile acids also become more sensitive to dichlorofluorescein; as little as 0.5 μg/cm² of taurocholate, and even less of the other derivatives of cholanoic acid, could be detected under UV light. Since the difficulty of staining

TABLE 1 RELATIVE MOBILITIES OF BILE ACIDS AND CONJUGATES IN DIFFERENT SOLVENT SYSTEMS

Compounds	System I*		System II		System III		System IV	
	COOH	Me	COOH	Me	COOH	Me	COOH	Me
Bile Acids								
Cholanoic	1.40	1.81	3.10	5.00	1.37	3.50	0.49	3.06
Lithocholic	1.16	"	2.35	"	1.00	"	0.44	2.64
Deoxycholic	1.02	"	1.60	"	0.60	"	0.31	2.22
Chenodeoxycholic	"	"	"	"	"	"	"	"
Ursodeoxycholic	"	"	"	"	"	"	"	"
Hyodeoxycholic	"	"	"	"	"	"	"	1.29
3β,12α-Dihydroxycholanoic	"	"	"	"	"	"	"	2.25
Hyocholic	1.00	"	1.00	"	0.25	3.42	0.27	1.00
Cholic	1.00	"	1.00	"	"	"	"	1.00
Conjugates								
Taurolithocholic	1.32	1.81	2.36	5.00	1.05	3.42	0.27	1.70
Taurodeoxycholic	1.28	"	2.30	4.90	1.01	"	"	1.14
Taurochenodeoxycholic	1.28	"	"	"	1.01	"	"	"
Taurocholic	1.13	"	1.60	4.80	0.98	"	"	0.64
Glycolithocholic	1.13	"	"	5.00	0.98	"	"	1.70
Glycodeoxycholic	1.02	"	1.60	4.90	0.60	"	"	1.14
Glycochenodeoxycholic	1.02	"	"	4.90	0.60	"	"	"
Glycocholic	1.00	"	1.00	4.80	0.25	"	"	0.64

The common names refer to the following cholanoic acid derivatives: lithocholic, 3α-hydroxy; deoxycholic, 3α,12α-dihydroxy; chenodeoxycholic, 3α,7α-dihydroxy; ursodeoxycholic, 3α,7β-dihydroxy; hyodeoxycholic, 3α,6α-dihydroxy; hyocholic, 3α,6α,7α-trihydroxy; cholic, 3α,7α,12α-trihydroxy. The prefixes tauro and glyco indicate taurine and glycine conjugates, respectively.

* System I: chloroform–methanol–7 N NH_4OH–water 105:75:5:10. System II: same four components, 90:45:10:5. System III: chloroform–methanol–7 N NH_4OH 80:40:4. System IV: same three components, 220:25:4. Free fatty acids and any neutral lipids run with the solvent front in all systems except in System IV, where they overlap with the methyl ester of cholanoic acid. In all systems free cholesterol overlaps with the methyl ester of lithocholic acid, or the free lithocholic acid.

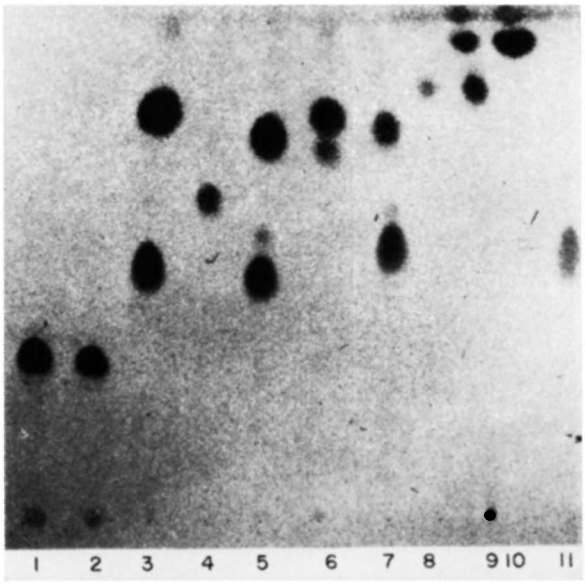

FIG. 2. TLC of the methyl esters of free and conjugated bile acids. Solvent System IV. In ascending order the acids are (preceded by the number of the lane): *1*, taurocholic; *2*, glycocholic; *3*, cholic and deoxycholic; *4*, hyodeoxycholic; *5*, cholic and chenodeoxycholic; *6*, chenodeoxycholic and ursodeoxycholic; *7*, taurodeoxycholic and deoxycholic; *8*, 3β,12α-dihydroxycholanoic; *9*, taurolithocholic and lithocholic; *10*, lithocholic; *11*, hyocholic.

with iodine and dichlorofluorescein can be traced to residual acid in the silica gel, neutral solvent systems would be expected also to permit detection of bile acids at similar low concentrations. Such systems, however, were not extensively investigated.

Adequate staining with phosphomolybdic acid (2) requires prior removal of residual ammonia by heating (about 10 min at 80°C).

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REFERENCES

1. Hoffman, A. F. 1964. *In* New Biochemical Separations. A. T. James and L. J. Morris, editors. Van Nostrand Company, Ltd., London. 262.
2. Sjövall, J. 1964. *Methods Biochem. Analy.* **12:** 97.
3. Eneroth, P. 1963. *J. Lipid Res.* **4:** 11.
4. Norman, A. 1955. *Arkiv Kemi.* **8:** 331.
5. Subbiah, M. T., A. Kuksis, and S. Mookerjea. 1967. *Proc. Can. Federation Biol. Soc.* **10:** 5.
6. Kuksis, A. 1966. *Methods Biochem. Analy.* **14:** 325.