# notes on methodology

## Separation of methylated free bile acids from their taurine and methyl glycine conjugates by thin-layer chromatography

Merry J. G. Bolt

*Department of Medicine, Division of Gastroenterology, The University of Chicago, Chicago, IL 60637*

**Summary** Class separation of methylated free bile acids from bile acids conjugated with taurine and methylglycine was accomplished using a solvent system of 2,2,4-trimethylpentane–absolute ethanol 10:1 (v/v). By developing a silica thin-layer plate two times with solvent in a Brinkmann sandwich tank, the difficult resolution between methyl cholate and methyl glycolithocholate was achieved. Evidence is presented that this separation system may be useful as a preparative step in the analysis of bile acids by gas–liquid chromatography or high pressure liquid chromatography.—**Bolt, M. J. G.** Separation of methylated free bile acids from their taurine and methyl glycine conjugates by thin-layer chromatography. *J. Lipid Res.* 1987. **28:** 1013–1015.

**Supplementary key words** bile acid separation • free bile acid methyl esters • glycine-conjugated bile acid methyl esters

Thin-layer chromatography (TLC) has been used extensively for the separation of bile acids, as reviewed recently in this journal (1). A number of TLC solvent systems have been reported that separate conjugated from free bile acids (2–4) or that resolve the methyl esters of free bile acids from each other (5–11), but no TLC method has previously been published which demonstrates class separation of free bile acid methyl esters from glycoconjugated bile acid methyl esters. This separation may be desirable at certain sequences in the extraction, purification, and analysis of bile acids from serum, urine, or bile. For example, Cantafora et al. (12) report that quantitative solvolysis and methylation can be achieved simultaneously using the 2,2-dimethoxypropane (DMP)–HCl method. Water present in the system reacts with DMP to form acetone and methanol, resulting in the anhydrous conditions that favor methylation. This method (with slight variations) has been used by others, to quantitatively solvolyze bile acid sulfates (13–15) or to quantitatively methylate bile acids (16–18). Several investigators also state that no artifact formation is observed when the methanol–DMP–HCl method is used (14, 18). Since methylation of the C-24 carboxyl group is necessary prior to gas–liquid chromatography (GLC) of free or glycine-conjugated bile acids (19), and for mass spectrometry (MS), the ability to separate bile acid groups as their methyl ester at this stage could expedite sample preparation.

The present study reports a TLC system for the class separation of methylated free bile acids from bile acids conjugated with taurine and methyl glycine.

## METHODS

Bile acid standards of greater than 98% purity were purchased as acids or salts from Steraloids, Inc., Wilton, NH; Supelco, Inc., Bellefonte, PA; Serva (via Accurate Chemical and Scientific Corp., Hicksville, NY); and Calbiochem, San Diego, CA. The methyl esters of free bile acids or their glycine conjugates were prepared by a modification of the 2,2-dimethoxypropane method (16). Bile acids (1–8 mg) were dissolved in 1 ml of MeOH, 1 ml of 2,2-dimethoxypropane (Aldrich Chemical Co., Milwaukee, WI), and 1 drop of concentrated HCl. The reactions were capped, mixed, and allowed to stand at room temperature for 3 hr. Uniplate TLC glass plates, precoated with 250 μm silica gel H, were purchased from Analtech, Newark, DE. Plates were developed in a standard rectangular glass tank with a heavy glass cover for preliminary experiments, or in a sandwich tank (Brinkmann Instruments, Inc., Westbury, NY) for the present system. Bile acid standards were dissolved in MeOH, and a 5-μl volume containing 7.5–15 μg was applied to the plate as a spot. After solvent development at room temperature, the plates were dried, gently heated, and sprayed with 10% (w/v) phosphomolybdic acid in absolute ETOH to visualize standards. Serum bile acids were extracted from 4.0 ml of pooled fasting sera from normal individuals by liquid–solid extraction (20). After solvolysis/methylation as described above, the sample was concentrated and applied to the TLC plate as a 3.5-cm band.

## RESULTS

An attempt was made to separate methyl esters of free bile acids from methyl glycolithocholate by some of the chromatographic systems reported to resolve free bile acid methyl esters (5–8). Data from **Table 1** show that methylglycolithocholate migrates farther than methylcholate in two of the four systems tested (solvents A and B) irrespective of

Abbreviations: TLC, thin-layer chromatography; GLC, gas-liquid chromatography; DMP, 2,2-dimethoxypropane; IPA, isopropyl alcohol; MS, mass spectrometry.

TABLE 1. $R_f$ values for the methyl esters of free and glycoconjugated bile acids in various solvent systems

| Bile Acid | Solvent System A Hanson et al. (5) | B Salen et al. (6) | C Norman et al. (7) | D Spears et al. (8) | E Present |
|---|---|---|---|---|---|
| | | | *$R_f$ 1.0 = 15 cm* | | |
| Me lithocholate | | | | .74 | .27 |
| Me deoxycholate | | | | .65 | .25 |
| Me chenodeoxycholate | | | | .62 | .25 |
| Me cholate | .65 | .29 | .45 | .48 | .20 |
| Me glycolithocholate | .90 | .97 | .45 | .48 | .14 |
| Me glycochenodeoxycholate | .60 | .42 | .23 | .24 | .09 |
| Me glycodeoxycholate | | | | .24 | .09 |
| Me glycocholate | | | | .09 | .04 |
| Taurocholic acid | | | | .00 | .00 |

Composition of the solvent systems is as follows: A, benzene–isopropyl alcohol–acetic acid 30:10:1 (v/v); B, chloroform–acetone–methanol 70:25:5 (v/v); C, 2,2,4-trimethylpentane–isopropyl alcohol–acetic acid 30:10:1 (v/v); D, 2,2,4-trimethylpentane–isopropyl alcohol–acetic acid 30:10:0.25 (v/v); E, 2,2,4-trimethylpentane–ethyl alcohol 50:5 (v/v). Thin-layer plates were developed once in solvents A–D by ascending chromatography using a standard rectangular glass tank with a heavy cover. For solvent E, plates were predeveloped in MeOH, dried overnight at 100°C, then developed two times in a Brinkmann sandwich tank.

differing solvent compositions and pH. $R_f$ values were equal for cholic acid methyl ester and methylated glycolithocholate in solvent systems C and D, independent of pH. From these preliminary findings, isopropyl alcohol (IPA) appeared to be the best solvent to modify concentration. It was included in the formulation of solvent E (IPA–ethyl alcohol 50:5, v/v), which successfully separates cholic acid methyl ester and all free methylated bile acids from methylated glycolithocholate and all methylglycine conjugates (Table 1, **Fig. 1**). Taurocholic acid, which cannot be methylated, remained at the origin. For optimal visualization, it was found necessary to predevelop the plates with MeOH; then for best resolution to activate the layers at 100°C overnight and to develop them two times in a sandwich tank, allowing 45 min per run and 1 hr drying time between runs.

Fig. 1 illustrates a typical chromatographic separation of methylated standards and a representative methylated serum extract. There is sufficient resolution between conjugated bile acids, as limited by methyl glycolithocholate, and free bile acids, as limited by methylcholate, to prevent cross-contamination when the gel containing these bile acid groups is removed from the plate for subsequent extraction. The amounts of individual bile acids present in 4 ml of normal sera are too little to be well-visualized by staining. It is reasonable to assume that the stained bands observed in the chromatographed serum extract represent other serum lipids. Although cholesterol does not separate completely from methyl lithocholate, the bulk of serum lipids (presumably cholesteryl esters, triglycerides, and methylated fatty acids) migrate well above the bile acid region. When the bile acid standards are visualized by nonpermanent iodine vapor or water, areas on the gel corresponding to the unconjugated and conjugated classes of bile acids can be identified and the gel can be removed from the plate for subsequent extraction. Purification would be achieved because a major portion of serum lipids remains behind. After eluting bile

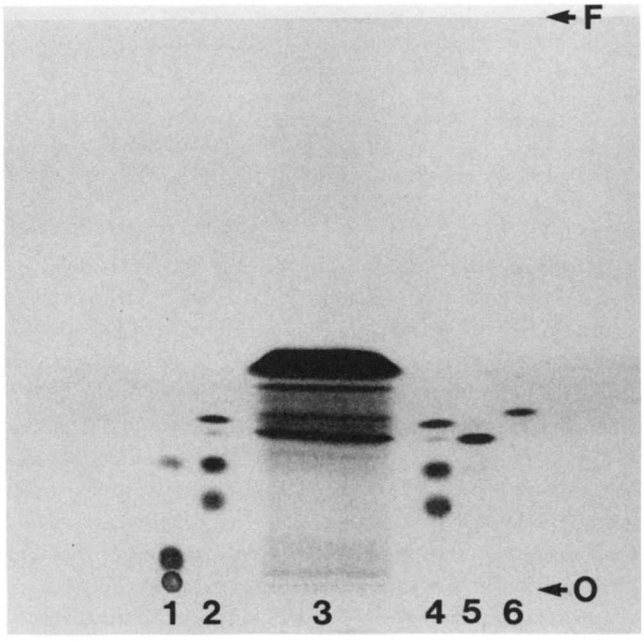


**Fig. 1.** Thin-layer chromatogram of methylated bile acids and a methylated serum extract. Chromatographic conditions are described in the text and in Table 1. Lane 1: (in ascending order) taurocholic acid, methyl glycocholate, methyl cholate (trace); lanes 2 and 4: methyl glycolithocholate, methyl cholate, methyl deoxycholate (trace), methyl lithocholate; lane 3, methylated extract from 4 ml of normal pooled sera; lane 5: methyl deoxycholate and methyl chenodeoxycholate; lane 6: cholesterol. The origin (O) and solvent front (F) are indicated by arrows. It is unlikely that the stained bands in the serum extract represent bile acids, but rather other serum lipids. Note that the bulk of serum lipid is less polar than methyl lithocholate.

acids from the gel, they are ready for further processing or for analysis by high pressure liquid chromatography or GLC–MS. Thus, classs separation of methylated free bile acids from bile acid conjugates may be useful as a preparative step in the analysis of bile acids.

*Manuscript received 21 July 1986 and in revised form 9 January 1987.*

## REFERENCES


1. Street, J. M., D. J. H. Trafford, and H. L. J. Makin. 1983. The quantitative estimation of bile acids and their conjugates in human biological fluids. *J. Lipid Res.* **24:** 491–511.
2. Huang, C. T. L., and B. Nichols. 1975. New solvent systems for the separation of free and conjugated bile acids. II. Separation of free bile acids as a group. *J. Chromatogr.* **109:** 427–431.
3. Chavez, M. N., and C. L. Krone. 1976. Silicic acid thin-layer chromatography of conjugated and free bile acids. *J. Lipid Res.* **17:** 545–547.
4. Nakagaki, M., and F. Nakayama. 1979. Class separation of bile lipids by thin-layer chromatography. *J. Chromatogr.* **177:** 343–348.
5. Hanson, R. F., J. N. Isenberg, G. C. Williams, D. Hachey, P. Szczepanik, P. D. Klein, and H. L., Sharp. 1975. The metabolism of 3$\alpha$, 7$\alpha$, 12$\alpha$-trihydroxy-5$\beta$-cholestan-26-oic acid in two siblings with cholestasis due to intrahepatic bile duct anormalies. *J. Clin. Invest.* **56:** 577–587.
6. Salen, G. S., S. Shefer, T. Setoguchi, and E. H. Mosbach. 1975. Bile alcohol metabolism in man. Conversion of 5$\beta$-cholestane-3$\alpha$, 7$\alpha$, 12$\alpha$, 25-tetrol to cholic acid. *J. Clin. Invest.* **56:** 226–231.
7. Norman, A., and B. Strandvik. 1973. Excretion of bile acids in extrahepatic biliary atresia and intrahepatic cholestasis of infancy. *Acta Paediat. Scand.* **62:** 253–263.
8. Spears, R., D. Vukusich, S. Mangat, and B. S. Reddy. 1976. New improved method for separation of fecal bile acids by thin-layer chromatography. *J. Chromatogr.* **116:** 184–187.
9. Sundarum, G. S., H. Singh, and H. S. Sodhi. 1971. Thin-layer chromatographic separation of chenodeoxycholic and deoxycholic acids. *Clin. Chim. Acta.* **34:** 425–429.
10. Einarsson, K., K. Hellström, and M. Kallner. 1974. Bile acid kinetics in relation to sex, serum lipids, body weights and gallbladder disease in patients with various types of hyperlipoproteinemia. *J. Clin. Invest.* **54:** 1301–1311.
11. Watanabe, H. 1975. Effect of 17$\alpha$-ethinylestradiol on biliary excretion of bile acids. *Biochim. Biophys. Acta.* **399:** 79–84.
12. Cantafora, A., M. Angelico, A. F. Attili, L. Ercoli, and L. Capocaccia. 1979. An improved gas-chromatographic method for the determination of sulfated and unsulfated bile acids in serum. *Clin. Chim. Acta.* **95:** 501–508.
13. Galeazzi, R., and N. B. Javitt. 1977. Bile acid excretion: the alternate pathway in the hamster. *J. Clin. Invest.* **60:** 693–701.
14. Watkins, J. B., E. Goldstein, R. Coryer, E. R. Brown, and A. Eraklis. 1979. Sulfation of bile acids in the fetus. *In* The Liver: Quantitative Aspects of Structure and Function. R. Preisig and J. Bircher, editors. Editio Cantor, Aulendorf, Federal Republic of Germany. 249–254.
15. Cohen, B. I., K. Budai, and N. B. Javitt. 1981. Solvolysis of chendeoxycholic acid sulfates. *Steroids.* **37:** 621–626.
16. Ali, S. S., and N. B. Javitt. 1970. Quantitative estimation of bile salts in serum. *Can. J. Biochem.* **78:** 1054–1057.
17. Lillington, J. M., D. J. H. Trafford, and H. L. J. Makin. 1981. A rapid and simple method for the esterification of fatty acids and steroid carboxylic acids prior to gas–liquid chromatography. *Clin. Chim. Acta.* **111:** 91–98.
18. Shaw, R., and W. H. Elliot. 1978. Bile acids. LV. 2,2-Dimethoxypropane: an esterifying agent preferred to diazomethane for chenodeoxycholic acid. *J. Lipid Res.* **19:** 783–787.
19. Street, J. M., D. J. H. Trafford, and H. L. J. Makin. 1986. Capillary gas-liquid chromatography of glycine-conjugated bile acids without prior hydrolysis. *J. Lipid Res.* **27:** 208–214.
20. DeMark, B. R., G. T. Everson, P. D. Klein, R. B. Showalter, and F. Kern, Jr. 1982. A method for the accurate measurement of isotope ratios of chenodeoxycholic and cholic acids in serum. *J. Lipid Res.* **23:** 204–210.