notes on methodology

Separation of individual sulfated bile acid conjugates as calcium complexes using reversed-phase partition thin-layer chromatography

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Summary A method for separating individual monosulfated primary bile acid conjugates by reversed-phase partition thin-layer chromatography on octadecyl-bonded silica gel is described. The solvent system is acetonitrile containing calcium, probably as calcium carbamate. Excellent resolution of the 3- and 7-monosulfated glycine conjugates, as well as 3- and 7-monosulfated taurine conjugates of cholic and chenodeoxycholic acids is reported. A convenient class separation of sulfated from nonsulfated primary bile acid conjugates by adsorption thin-layer chromatography on low-polarity silica gel is also described.—**Raedsch, R., A. F. Hofmann, and K-Y. Tserng.** Separation of individual sulfated bile acid conjugates as calcium complexes using reversed-phase partition thin-layer chromatography. *J. Lipid Res.* 1979. **20:** 796–800.

Supplementary key words bile acid sulfates · conjugated bile acids

For over a century, it has been known that bile acids are conjugated with glycine or taurine, but other modes of conjugation, such as sulfation or glucuronidation, have received little attention. In 1967, Palmer (1) identified lithocholic sulfates in human and rat bile. Since then, it has been well established that sulfation of primary bile acids becomes an important biotransformation in patients with obstructive hepatobiliary disease (2–5), in whom excretion of bile acids in urine becomes the predominant mechanism for cholesterol elimination (6, 7). In the past few years, glucuronidation of bile acids has also been shown to become a major pathway in patients with cholestasis (8, 9).

Bile acid sulfates are usually separated from nonsulfated bile acids by column chromatography (10). The bile acid sulfate fraction is then further separated into monosulfate, disulfate, and trisulfate fractions (3). The sulfate group is then removed by a solvolysis procedure (e.g., 11, 12) and individual steroid moieties are quantitated by gas–liquid chromatography. Such an analytical approach provides no direct information on the position of the sulfate group. It is possible to oxidize the nonsulfated groups before solvolysis and obtain indirect information on the position of the sulfate group (13), but even this approach provides no information on whether the sulfated bile acids are amidated with glycine or with taurine.

We felt that it would be desirable to develop a method for chromatographic separation of individual conjugated bile acid sulfates. Successful separation of nonsulfated bile acid glycine or taurine conjugates by reversed-phase partition chromatography has recently been reported by Goto et al. (14) and Bloch and Watkins (15), and we thought that such an approach might be successful when applied to sulfates of bile acid conjugates. We report here the successful separation of individual monosulfated conjugates of the glycine and taurine conjugates of the two primary bile acids in man, using reversed-phase partition thin-layer chromatography (TLC). We also report a convenient class separation of sulfated from nonsulfated conjugated primary bile acids by conventional adsorption TLC.

MATERIALS AND METHODS

In this report we have referred to glycine and taurine conjugates of bile acids as "amidates", following the suggestion of Nair, Gordon, and Reback (16), and have used the term "unconjugated" to mean bile acids containing neither sulfate nor glycine nor taurine.

Sulfates of bile acid conjugates were synthesized by one of us (KYT) using methodology reported in detail elsewhere (17–19). Recently, the synthesis of a number of individual sulfated bile acid amidates has also been reported by Parmentier and Eyssen (20). Precoated silicic acid plates were used for adsorption TLC (K 1, low polarity, 0.25 mm layer thickness, 10 × 20 cm, Whatman, Inc.) and for reversed-phase partition TLC (KC 18, 0.25 mm layer thickness, 5 × 20 cm, Whatman, Inc. and Merck silica gel 60 silanized, 0.25 mm layer thickness, 20 × 20 cm,

Abbreviations: TLC, thin-layer chromatography; chn, chenodeoxycholic; chl, cholic; gly, glycine; tau, taurine; sul, sulfate; mixt, mixture.

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Merck, W. Germany). All plates were used without further activation. Bile acids were detected using phosphomolybdic acid in ethanol (10 g/100 ml). Calcium chloride was purchased from Mallinckrodt (anhydrous, porous, 4 Mesh), and Baker (anhydrous, granular, 4 Mesh); ammonium carbonate (powder) was obtained from the same companies.

For a class separation of individual bile acids, the solvent system reported by Cass et al. (21) for lithocholyl sulfates was used with low-polarity silica gel. This silica gel differs from that used by Cass et al. (21) because its polarity is less and it contains no calcium sulfate as binder. The solvent system was chloroform–methanol–acetic acid–water 65:24:15:9 (v/v/v/v); this system is a modification of numerous other solvent systems reported in the literature, e.g., that by Wagner, Hörhammer, and Wolff (22) for the TLC separation of phospholipids.

For reversed-phase partition TLC of individual sulfated amidates, the solvent system was prepared

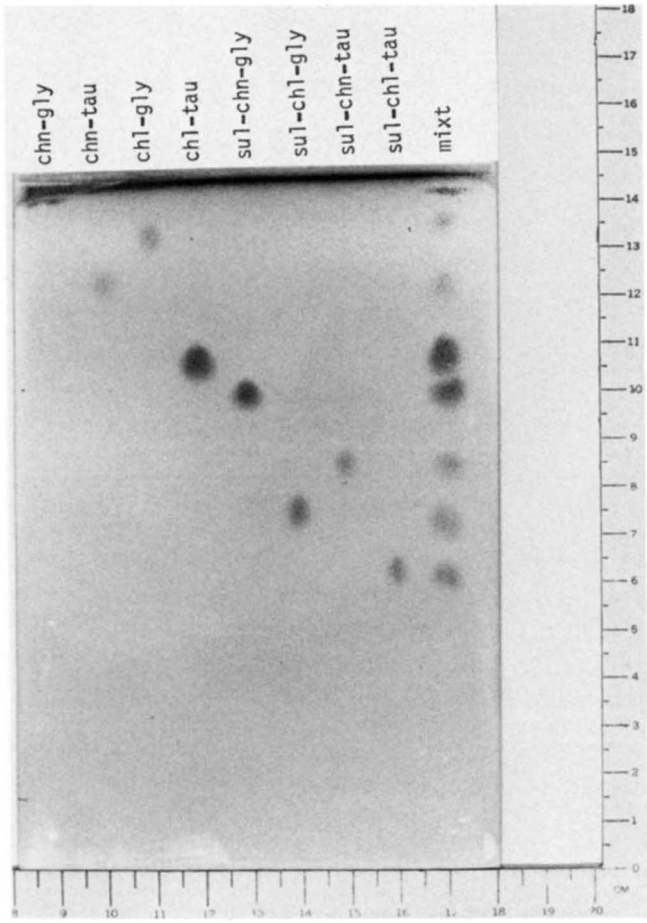

Fig. 1. Separation of unsulfated bile acid conjugates (left four spots) from sulfated bile acid conjugates (right five spots) by adsorption chromatography on low-polarity silicic acid. The solvent system and R_f values are given in Table 1.

TABLE 1. R_f values of bile acids during adsorption chromatography on low-polarity silicic acid[a]

Unconjugated bile acids	
Chenic	1.00
Deoxycholic	1.00
Unsulfated amidates (glycine conjugates)	
Chenodeoxycholylglycine	0.96
Cholylglycine	0.91
Deoxycholylglycine	0.95
Lithocholylglycine	0.96
Unsulfated amidates (taurine conjugates)	
Chenodeoxycholyltaurine	0.83
Cholyltaurine	0.72
Lithocholyltaurine	0.85
Deoxycholyltaurine	0.77
Monosulfated non-amidates	
Sulfo-chenodeoxycholic (3 or 7)	0.82
Sulfo-cholic (3 or 7 or 12)	0.73
Sulfo-lithocholic (3)	0.95
Monosulfated amidates (glycine conjugates)	
Sulfo-chenodeoxycholylglycine (3 or 7)	0.65
Sulfo-cholylglycine (3 or 7)	0.54
Sulfo-lithocholylglycine	0.73
Monosulfated amidates (taurine conjugates)	
Sulfo-chenodeoxycholyltaurine (3 or 7)	0.46
Sulfo-cholyltaurine (3 or 7)	0.37
Sulfo-lithocholyltaurine (3)	0.58
Disulfated amidates	
(di)-sulfo-chenodeoxycholylglycine (3,7)	0.35
(di)-sulfo-cholylglycine (3,7)	0.20

[a] A solvent system of chloroform–methanol–acetic acid–water 65:24:15:9 (v/v/v/v) was used.

at room temperature as follows. A 1 M solution of calcium chloride is mixed with an equal volume of a 1.5 M solution of ammonium carbonate. The suspension is filtered to remove precipitated calcium carbonate and calcium carbamate. The clear filtrate is mixed with acetonitrile, 32 parts filtrate to 68 parts acetonitrile. The two phases are shaken vigorously, and the upper phase is removed and used for chromatography. To determine its composition, we determined Ca^{2+} concentration by atomic absorption spectroscopy, salt content by direct weighing after evaporation of an aliquot, and water content by isotope dilution using ^3H_2O. All chromatographic procedures were carried out at room temperature.

RESULTS

Class separation of sulfated from nonsulfated amidates

With low-polarity silicic acid and the chloroform–methanol–acetic acid–water system, unconjugated bile acids and nonsulfated amidated bile acids have an $R_f \geq 0.72$, whereas sulfated amidates of cholic and chenodeoxycholic acids have an $R_f < 0.65$. This

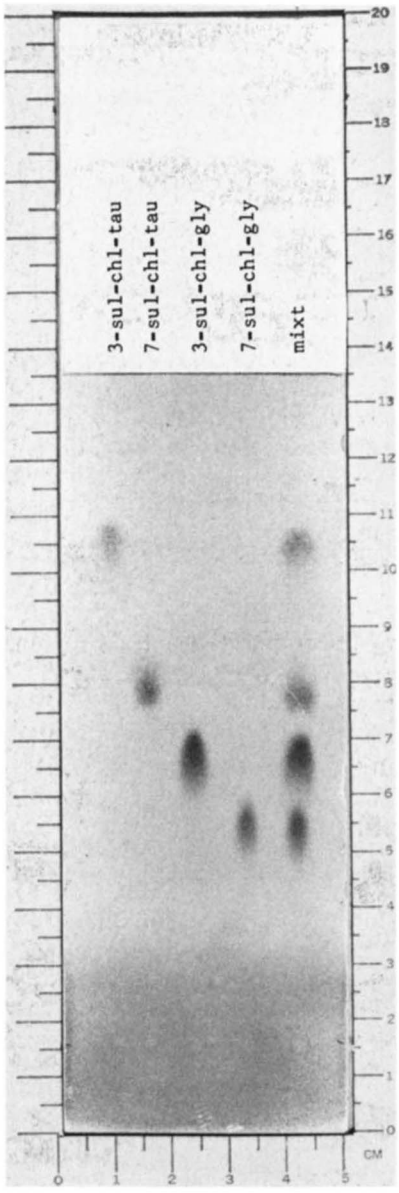

Fig. 2. Separation of individual monosulfates of cholyltaurine (left) and cholylglycine (right) by reversed-phase partition TLC as calcium complexes. Solvent system and R_f values are given in Table 2.

system thus resolves the nonsulfated and sulfated glycine or taurine amidates of the primary bile acids by class. **Fig. 1** shows a typical chromatogram and R_f values are given in **Table 1.**

Separation of individual nonsulfated bile acids

The reversed-phase partition TLC separation of individual sulfated bile acid amidates is shown in **Figs. 2** and **3,** with R_f values given in **Table 2.** This system gives a clear separation of all the monosulfates of the amidated and nonamidated forms of chenodeoxycholic and cholic acids, although there is overlap between the 7-sulfated chenodeoxycholyl amidates and the 3-sulfated cholyl amidates. R_f values are reported for chromatograms developed at room temperature. Developing the reversed-phase plates at lower temperatures (down to 5°C) resulted in no loss of resolution and even better spot definition. The R_f values were reproducible within a range of 0.1 at different temperatures.

To define the composition of the solvent system actually used for reversed-phase TLC, we determined

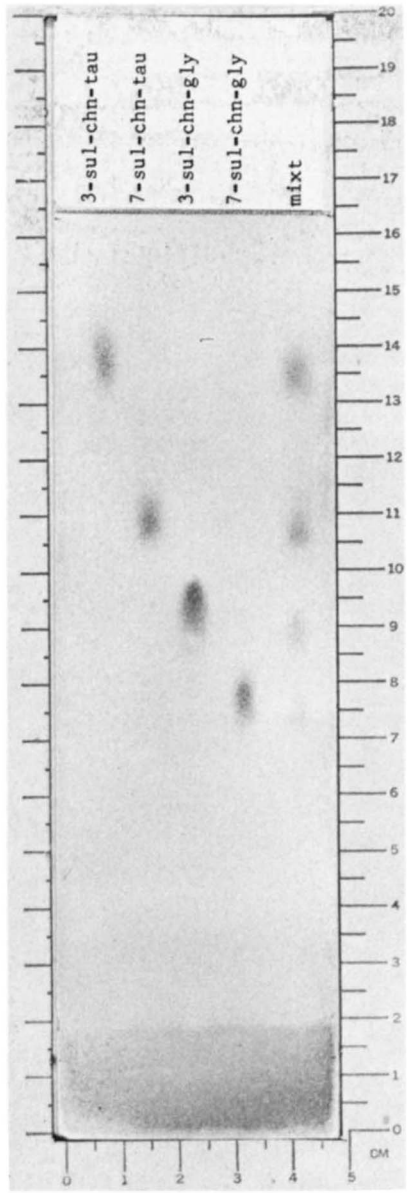

Fig. 3. Separation of individual monosulfates of chenodeoxycholyltaurine (left) and chenodeoxycholylglycine (right) by reversed phase partition TLC as calcium complexes. Solvent system and R_f values are given in Table 2.

TABLE 2. R_f values of bile acids during reversed-phase partition TLC on octadecyl-bonded silicic acid[a]

Monosulfated non-amidated	
3 sulfo-chenodeoxycholic	0.93
7 sulfo-chenodeoxycholic	0.85
3 sulfo-cholic	0.90
7 sulfo-cholic	0.83
12 sulfo-cholic	0.81
Monosulfated, glycine amidated bile acids	
3 sulfo-chenodeoxycholylglycine	0.54
7 sulfo-chenodeoxycholylglycine	0.43
3 sulfo-cholylglycine	0.41
7 sulfo-cholylglycine	0.35
3 sulfo-lithocholylglycine	0.65
Monosulfated taurine amidated bile acids	
3 sulfo-chenodeoxycholyltaurine	0.75
7 sulfo-chenodeoxycholyltaurine	0.61
3 sulfo-cholyltaurine	0.63
7 sulfo-cholyltaurine	0.56
Disulfated amidated (glycine or taurine) bile acids	
3,7 disulfo-chenodeoxycholylglycine	0.43
3,7 disulfo-chenodeoxycholyltaurine	0.60

[a] The solvent system was acetonitrile containing calcium carbamate, prepared as described in Methods.

calcium and total salt concentration, as well as water content. The calcium concentration was found to be 0.8 mg/ml, and the total salt content by weight was 3.2 mg/ml; the water content was 27% at room temperature. We speculated that the reversed-phase TLC separations resulted from the presence of calcium carbamate in the acetonitrile phase since this compound is known to be formed when calcium chloride and ammonium carbonate are mixed. To test this, we synthesized pure calcium carbamate using the method of MacLeod and Haskins (23). We then prepared a 2% solution (w/v) of this in anhydrous acetonitrile and tested this isotropic solution as a developing solvent. Excellent chromatograms were obtained with comparable separation of the individual bile acid sulfates, although with somewhat lower R_f values than those listed in Tables 1 and 2.

DISCUSSION

The two chromatographic systems reported here give a very satisfactory separation of the individual isomeric sulfated bile acid conjugates. After this work was completed, Parmentier and Eyssen (24) reported the behavior of many unconjugated and conjugated bile acid sulfates during partition TLC. They reported the separation of 3- and 7-monosulfates of chenodeoxycholylglycine and chenodeoxycholyltaurine, although they did not report separation of the 3- or 7-monosulfates of cholyl conjugates, as reference compounds were not available to them.

It was considered beyond the scope of our study to define the mechanism of separation. We suspect that the compounds are being chromatographed as calcium carbamate complexes, since if calcium was omitted from the solvent system, the sulfates were not resolved. In addition, a solution of calcium carbamate in acetonitrile gave a separation nearly identical to that obtained using acetonitrile equlibrated with the aqueous calcium chloride–ammonium carbonate solution.

The two chromatographic techniques reported here should permit definition of the pattern of sulfation of bile acid conjugates in patients with cholestasis. Indeed, in preliminary studies (25) we have found both 3α- and 7α-monosulfated bile acid conjugates present in the urine of cholestatic patients.

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