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Note

Quantitation of bile acids and bile salts by a thin-layer chromatographic charring method

HONG K. KIM and DAVID KRITCHEVSKY

The Wistar Institute of Anatomy and Biology, 36th Street at Spruce, Philadelphia, Pa. 19104 (U.S.A.)

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The quantitation of bile acids or bile salts following thin-layer chromatography (TLC) has been achieved by spectrophotometry[1–3], densitometry[4,5] or a modified color reaction such as the Pettenkofer reaction[6]. Gas–liquid chromatographic (GLC) separation of bile acids usually requires derivatization and, to our knowledge, has not been successfully achieved with bile salts[7].

Our success in adapting the Marsh–Weinstein[8] charring technique to the quantitation of lipids in serum and tissue[9] prompted us to test the efficacy of this technique in the quantitation of bile salts and bile acids. Our findings are described in this note.

EXPERIMENTAL

Materials and methods

For the charring reaction, the silicic acid area carrying the individual material was scraped into a test-tube, 2 ml of concentrated sulfuric acid was added and the mixture was heated to 200 ± 2° for 15 min. The tubes were then immersed in water at room temperature for 15 sec and in ice for 5 min. After cooling, 3 ml of distilled water were added, and the solution was mixed, cooled in an ice-bath for 10 min and cooled at room temperature for another 10 min. The silicic acid was removed by centrifugation at 2000 *g* and the absorbance of the resulting supernatant was determined at 375 nm. Calibration graphs (Figs. 1 and 2) were constructed for pure bile acids and bile salts or for the silicic acid area obtained after TLC of the pure materials. Recovery of material after chromatography was over 90% when small (25-μg) samples were compared, but was never less than 85% even when 100-μg samples were used. Wollenweber *et al.*[10] reported a recovery of 82% of a standard following TLC.

For all chromatographic separations, we used plates which were pre-coated with silica gel G 250 μm; Analtech, Newark, Del., U.S.A.). The plates were developed in diethyl ether in order to remove contaminants, and they were reactivated immediately before use by heating at 115° for 30 min. Spots of the bile acids and bile salts were made visible either directly by exposure to iodine vapor[11], or by spraying one channel on each plate with concentrated sulfuric acid and heating to 115°.

When samples of bile were used, the bile (0.5 ml) was adjusted to pH 8.0 and extracted twice with 2 ml of diethyl ether in order to remove the neutral lipids. The

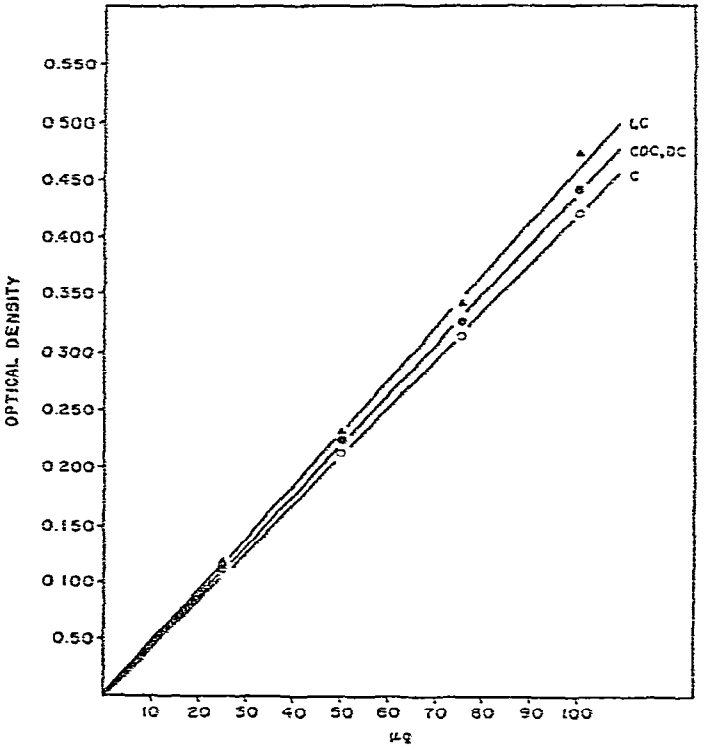

Fig. 1. Calibration graphs for free bile acids. LC = Lithocholic acid; CDC = chenodeoxych olic acid; DC = deoxycholic acid; and C = cholic acid.

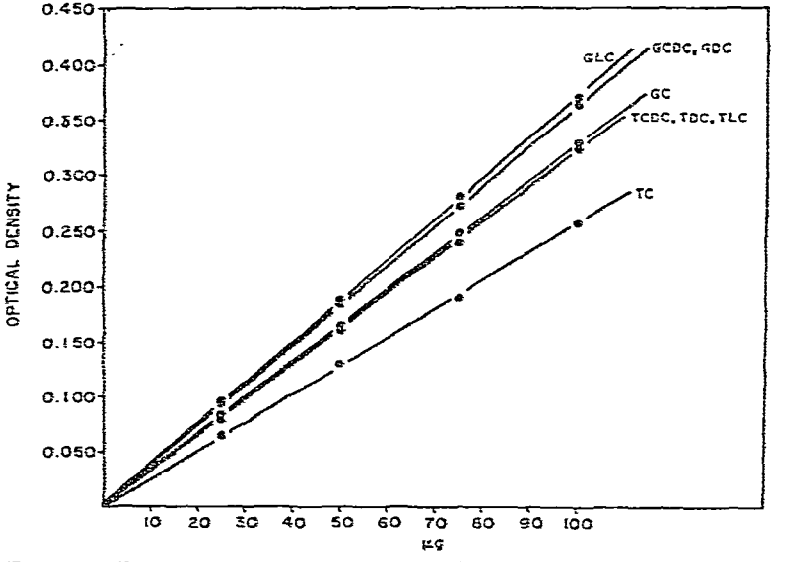

Fig. 2. Calibration graphs for bile salts. GLC = Glycolithocholic acid; GCDC = glycochenodeoxycholic acid; GDC = glycodeoxycholic acid; GC = glycocholic acid; TCDC = taurochenodeoxycholic acid; TLC = taurolithocholic acid; TC = taurocholic acid; and TDC = taurodeoxycholic acid.

aqueous layer was adjusted to pH 11 and proteins were removed by the addition of 10 ml of 95% ethanol. The ethanolic extract was filtered, evaporated to dryness in a stream of nitrogen and reconstituted to a volume of 1 ml in methanol. An aliquot portion of the methanol solution was applied to a TLC plate and the plate was developed in chloroform–methanol (9:1) in order to separate contaminants from the bile acids and bile salts which remained at the origin. The origin area was scraped from the plate, the bile acids and salts were eluted with 95% ethanol and the extract was dried under nitrogen. This material could be used directly for chromatography or could be hydrolyzed for extraction and analysis of the bile acids. The pure bile acids and bile salts used in this work were obtained from Supelco, Bellefonte, Pa., U.S.A. and Calbiochem, San Diego, Calif., U.S.A.

RESULTS AND DISCUSSION

Seven different solvent systems were tested for the separation of the free and the conjugated bile acids (Table I). Systems IV and V gave distinct separation between cholic and glycolithocholic acids. Use of these solvent systems permitted clean separation of the free bile acids, and of the glycine and taurine conjugates, and each of these classes could be eluted from the silica gel and re-chromatographed in another system to give a distinct separation of the individual compounds. The solvent system of Huang and Nichols[17] (isooctane–diisopropyl ether–glacial acetic acid–*n*-butanol–water, 10:5:5:3:1) gave excellent separation of chenodeoxycholic and deoxycholic acids, but the butanol caused interference with the charring procedure.

TABLE I

R_F VALUES OF FREE AND CONJUGATED BILE ACIDS SUBJECTED TO TLC IN DIFFERENT SOLVENT SYSTEMS

Solvent systems: I, benzene–acetic acid–water (10:10:1)[12]; II, isopentanol–acetic acid–water (18:5:3)[4]; III, isopentyl acetate–propionic acid–propanol–water (4:3:2:1)[13]; IV, isooctane–diisopropyl ether–isopropanol–acetic acid (2:1:1:1)[14]; V, ethyl acetate–cyclohexane–acetic acid (23:7:3)[15]; VI, isooctane–ethyl acetate–acetic acid (5:5:1)[15]; VII, isooctane–diisopropyl ether–acetic acid (2:1:1)[16].

Compound	*Solvent system*						
	I	*II*	*III*	*IV*	*V*	*VI*	*VII*
Free acids							
Lithocholic	0.84	0.85	0.84	0.72	0.74	0.57	0.46
Deoxycholic	0.74	0.78	0.76	0.61	0.54	0.42	0.33
Chenodeoxycholic	0.74	0.77	0.77	0.58	0.52	0.33	0.25
Cholic	0.61	0.70	0.67	0.42	0.21	0.13	0.10
Glycine conjugates							
Lithocholic	0.65	0.71	0.68	0.53	0.42	0.22	0.15
Deoxycholic	0.53	0.66	0.53	0.34	0.13	0.03	0.03
Chenodeoxycholic	0.54	0.67	0.53	0.32	0.13	0.03	0.03
Cholic	0.37	0.50	0.34	0.13	0.06	0	0
Taurine conjugates							
Lithocholic	0.23	0.35	0.21	0.01	0	0	0
Deoxycholic	0.16	0.26	0.11	0	0	0	0
Chenodeoxycholic	0.15	0.26	0.12	0	0	0	0
Cholic	0.05	0.11	0.05	0	0	0	0

Bile salts could be hydrolyzed with 6 *N* sodium hydroxide (3 h at 120°) in an autoclave, with 20% potassium hydroxide in ethylene glycol[18] or with the enzyme choloylglycine hydrolase[19] (EC 3.5.1.24). The enzyme gave 95% hydrolysis of glycocholic acid but only 85% hydrolysis of taurocholic acid, whereas treatment with alkali gave 94% hydrolysis of both conjugates. In view of this result and the probability that each bile salt exhibits a different K_m value for enzymic hydrolysis, we used alkaline hydrolysis throughout.

The recovery of added bile acids was tested in several ways. In one experiment, six different samples of human bile were analyzed for total cholic acid content and to each was added 50 μg of tauro- and glycocholic acids; the addition corresponded to 80.06 μg of cholic acid. The results are shown in Table II; the recovery ranged from 90 to 105% with average recovery being 78 $\pm$ 1.2 (S.E.) μg of cholic acid or 97.5%. In another test, aliquot portions of human bile were hydrolyzed, analyzed and then cholic (100 μg), chenodeoxycholic (50 μg) or deoxycholic acids (50 μg) were added. Recoveries were 103% for cholic acid, 98% for chenodeoxycholic acid and 95% for deoxycholic acids. The same quantities of the three acids were then added to other aliquot portions of the bile before hydrolysis. Recoveries of the added bile acids in this test were 97, 97 and 92% for cholic, chenodeoxycholic and deoxycholic acids, respectively.

TABLE II

RECOVERY OF ADDED CHOLIC ACID (80 μg) FROM BILE

Samples of human bile were analyzed for total cholic acid. To one aliquot portion of each sample, 40 μg each of taurocholic and glycocholic acids were added. Each sample was then subjected to hydrolysis and re-analyzed for cholic acid.

Sample	*Total cholic acid (μg)*		*Amount found (μg)*	*Amount recovered (μg)*
	Pre-addition	*Post-addition*		
1	280	360	356	76
2	460	540	540	80
3	192	272	264	72
4	192	272	276	84
5	232	312	316	84
6	152	232	224	72

This method was then compared with GLC analysis of bile samples which were supplied without disclosure of their content of bile acids. The results of this comparison are summarized in Table III. In three samples of dog bile, the average recoveries by GLC and TLC respectively were: deoxycholic acid, 26.6 and 25.7; chenodeoxycholic acid, 3.2 and 4.1; and cholic acid, 70.1 and 70.2%. For sheep bile the comparative values were: deoxycholic acid, 8.0 and 8.8; chenodeoxycholic acid, 4.9 and 5.8; and cholic acid, 86.8 and 85.8%. In the analysis of human bile we found only trace amounts of deoxycholic acid, values for the other bile acids being as follows: lithocholic acid, 1.9 and 2.1; chenodeoxycholic acid, 37.2 and 36.4; and cholic acid, 59.3 and 61.7%. The two methods of analysis gave consistent values within experimental error. While GLC does permit identification of trace amounts of bile acids,

TABLE III

COMPARISON OF GC AND TLC FOR ANALYSIS OF BILE ACIDS

Source		*Bile acid (%)*							
		Lithocholic		*Deoxycholic*		*Chenodeoxycholic*		*Cholic*	
		GLC	*TLC*	*GLC*	*TLC*	*GLC*	*TLC*	*GLC*	*TLC*
Dog	1	—	—	26.8	26.0	3.2	3.6	69.8	70.3
	2	—	—	26.9	25.3	3.3	4.4	69.5	70.3
	3	—	—	26.2	25.7	3.0	4.3	70.9	70.0
Sheep	1	—	—	8.4	8.7	5.2	5.5	85.7	85.8
	2	—	—	7.8	8.9	5.0	5.6	87.1	85.5
	3	—	—	7.9	8.7	4.6	6.2	87.5	85.1
Human	1	2.0	2.5	1.2	trace	37.0	36.3	59.4	61.2
	2	1.9	2.1	1.2	trace	37.3	35.8	59.2	62.3
	3	1.8	1.7	1.2	trace	37.2	37.2	59.3	61.5

we feel that the TLC charring method may be very useful for the analysis of large numbers of bile samples.

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REFERENCES

1 B. Frosch, *Arzneim.-Forsch.*, 15 (1965) 178.
2 B. Frosch and H. Wagener, *Klin. Wochenschr.*, 42 (1966) 901.
3 B. A. Kottke, J. Wollenweber and C. A. Owen, Jr., *J. Chromatogr.*, 21 (1966) 439.
4 S. Hara, H. Tanaka and M. Takeuchi, *Chem. Pharm. Bull.*, 12 (1964) 626.
5 G. Semenuk and W. T. Beher, *J. Chromatogr.*, 21 (1966) 27.
6 M. A. Eastwood, D. Hamilton and L. Mowbray, *J. Chromatogr.*, 65 (1972) 407.
7 P. Eneroth and J. Sjovall, in P. P. Nair and D. Kritchevsky (Editors), *The Bile Acids*, Vol. 1, Plenum Publ., New York, 1971, p. 121.
8 J. B. Marsh and D. B. Weinstein, *J. Lipid Res.*, 7 (1966) 574.
9 D. Kritchevsky, L. M. Davidson, H. K. Kim and S. Malhotra, *Clin. Chim. Acta*, 46 (1973) 63.
10 J. Wollenweber, B. A. Kottke and C. A. Owen, Jr., *J. Chromatogr.*, 24 (1966) 99.
11 D. Kritchevsky and M. R. Kirk, *Arch. Biochem. Biophys.*, 35 (1952) 346.
12 S. Hara, M. Takeuchi, M. Tachibana and G. Chihara, *Chem. Pharm. Bull.*, 12 (1964) 483.
13 A. F. Hofmann, *J. Lipid Res.*, 3 (1962) 127.
14 J. A. Gregg, *J. Lipid Res.*, 7 (1966) 579.
15 P. Eneroth, *J. Lipid Res.*, 4 (1963) 11.
16 K. A. Mitropoulos and N. B. Myant, *Biochem. J.*, 103 (1967) 472.
17 T. Huang and B. L. Nichols, *J. Chromatogr.*, 101 (1974) 235.
18 E. Evrard and G. Janssen, *J. Lipid Res.*, 9 (1968) 226.
19 P. P. Nair, M. Gordon and J. Reback, *J. Biol. Chem.*, 242 (1967) 7.