

Determination of individual bile acids in biological fluids by thin-layer chromatography and fluorimetry

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SYNOPSIS A method is described for the separation and determination of individual bile acids in serum and in intestinal contents, employing thin-layer chromatography and fluorimetry. The mean recovery from intestinal juice was 94% and from serum 82%.

Normal values for the individual bile acids of serum and examples of clinical applications of the method are given.

Several procedures have been described for determining individual bile acids in biological fluids, including column and paper chromatography (Rudman and Kendall, 1957; Sjövall, 1959; Wootton and Osborn, 1960), thin-layer chromatography (Kottke, Wollenweber, and Owen, 1966) and gas-liquid chromatography. Despite the refinement of gas-chromatography analysis for the estimation of free bile acids (Sandberg, Sjövall, Sjövall, and Turner, 1965; Roovers, Evrard, and Vanderhaeghe, 1968) the measurement of individual conjugated bile acids by this method requires preliminary separation of glycine and taurine conjugates and of free bile acids, followed by hydrolysis of the conjugates.

Requiring data on conjugated as well as free bile acids in large numbers of samples, we have developed a method using thin-layer chromatography and fluorimetry. Unlike ultraviolet spectrophotometry, fluorimetry provides a method for determining lithocholic acid; it is also of great sensitivity.

Thin-layer chromatography has been found to provide satisfactory resolution of the major conjugated and free bile acids for quantitation.

Methods

PURIFICATION OF SILICIC ACID

Untreated silicic acid (Kieselgel G. Merck & Co)

gives a high fluorescent blank. It is treated by allowing it to stand overnight in 20N sulphuric acid, then washing on a sintered glass funnel with distilled water to neutrality (Sandberg *et al.*, 1965). The silicic acid is dried in an oven at 120°C for 24 hours and then passed through a nylon sieve of 0.1 mm mesh size. It may be stored for at least three months.

PURIFICATION OF ANION EXCHANGE RESIN

The resin XN 1006 (A26) (Lennig Chemicals, Bedford Row, London, WC1) is washed on a sintered glass funnel successively with 10 volumes each of water, ethanol, hexane, ethanol, and water. It is stored in 0.2 M ammonium carbonate.

THIN-LAYER CHROMATOGRAPHY

To obtain sufficient separation, particularly of chenodeoxycholic acid and its stereoisomer, deoxycholic acid, chromatography is carried out on plates 50 cm in length. The width is 20 cm. These are spread with a slurry of 15 g silicic acid and 30 ml distilled water containing Rhodamine 6 G (BDH Ltd) 0.005% w/v. A Shandon Unoplan spreader is used, set to give a layer 0.3 mm in thickness. Plates are divided into seven lanes.

Solvents (BDH Ltd) are redistilled before use, with the exception of di-isopropyl ether

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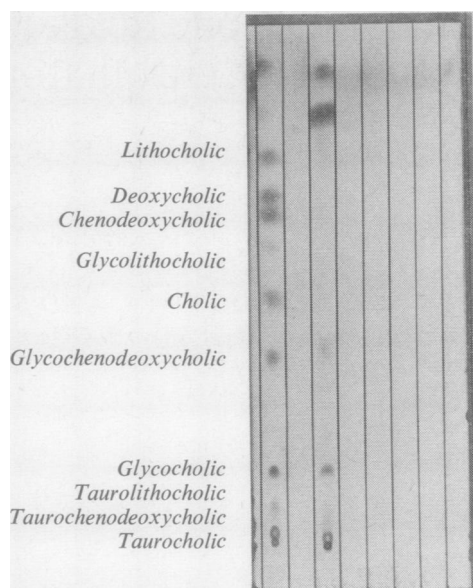


Fig. 1 Thin-layer chromatogram of standard bile acid mixture (left) and of bile acid fraction from serum of a patient with obstructive jaundice after running first and second solvent systems. Note incomplete resolution of taurine conjugates.

which is stored in the dark but used without prior treatment. Solvent covers the bottom of the tank, which is lined with filter paper to ensure saturation with solvent vapour. A small trough of freshly prepared solvent is placed in the tank; the thin-layer plate stands in this trough.

Samples of 50 μ l serum extract or 10 μ l intestinal content or bile are applied using a Hamilton syringe fitted with a 'point style 3' needle, avoiding disturbance of the silica layer. The sample is applied within an area 5-6 mm in diameter. One lane serves as a blank; to another lane is applied 20 μ l of a standard n-butanolic solution containing 1 μ g/ μ l of each of eight bile acids; these are taurocholic, taurochenodeoxycholic, glycocholic, and glycochenodeoxycholic acids (Maybridge Research Chemicals Tintagel, N. Cornwall), and cholic, chenodeoxycholic, deoxycholic, and lithocholic acids (Steraloids, Croydon, Surrey).¹

The plate is developed successively in three solvent systems. In the first solvent, chloroform, the less polar lipids, including cholesterol and triglycerides, move at or near the solvent front, while bile acids remain at the origin; a measure of purification is thus achieved. To shorten the running time to about two hours this may be

¹More recently we have included a tauroolithocholic acid standard and routinely determined this acid.

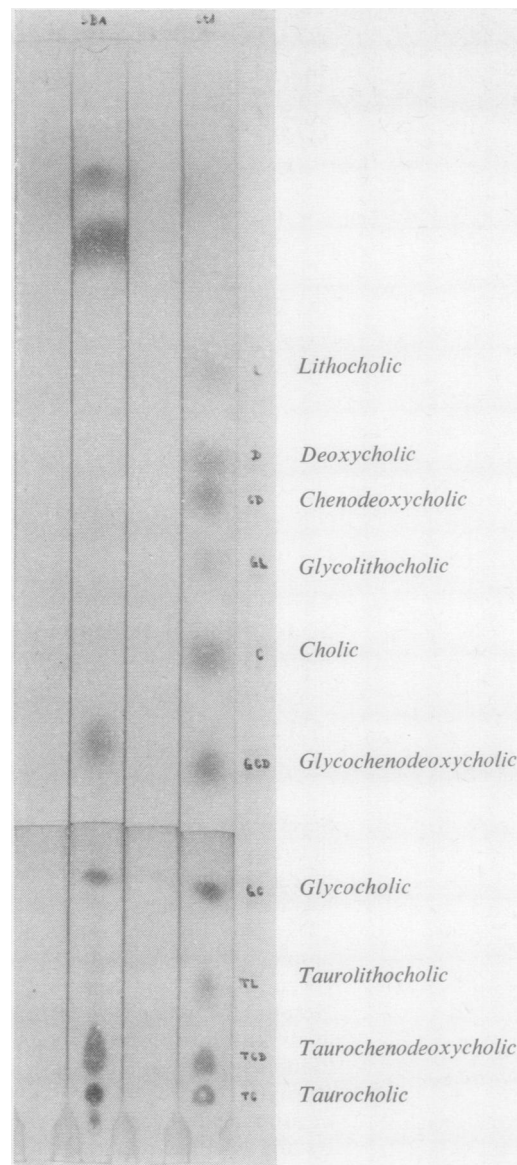


Fig. 2 Thin-layer chromatogram of bile acid standards (right hand lane) and bile acid extract from serum of patient with obstructive jaundice after third solvent. Spray reagent: 10% ethanolic phosphomolybdic acid.

carried out by the descending technique; the solvent is supplied to the plate by a wick of Whatman no. 1 paper, held in place by a thin glass plate, 20 \times 1 cm, which is fastened by a plastic-covered Terry clip at either side.

The second solvent consists of 2, 2, 4-trimethyl pentane/di-isopropyl ether/acetic acid/iso-propanol, 2:1:1:1 by volume (Gregg, 1966). Development is carried out by the ascending method overnight, ie, about 15 hours. Less satisfactory

separations were obtained by descending chromatography. Free bile acids and glycine conjugates are well separated, with complete resolution of deoxycholic and chenodeoxycholic acids, but taurine conjugates, which have a relatively low R_f , are not well separated (Fig. 1).

After air-drying, the plate is exposed for five minutes to iodine vapour and then viewed under ultraviolet light (350 m μ). This affords an extremely sensitive method for detecting several classes of organic substance (Milborrow, 1965), possibly dependent on the strong absorption of ultraviolet light by iodine reversibly bound to the spots. The free bile acids and glycochenodeoxycholic acid spots are marked, and corresponding areas are outlined in the blank lane.

After the iodine has completely sublimed, the third chromatographic system is used to separate the taurine conjugates. Each lane is 'wedged' (see Fig. 2) at the origin; this enhances the separation. A short ascending run is carried out with the system propionic acid/iso-amyl acetate/water/n-propanol, 3:4:1:2 by volume (Hofmann, 1961). Development is stopped when the solvent front has reached a point 2 cm below the glycochenodeoxycholic acid spots. Taurine conjugates and glycocholic acid are then located as before.

QUANTITATION

Sample, standard, and blank spots are scraped into 130 \times 17 mm glass-stoppered tubes. To each is added 2 ml concentrated sulphuric acid (Analytical Reagent, BDH Ltd) for analysis of serum, 4 ml for intestinal contents. The silica is thoroughly dispersed, using a vortex mixer, and the stoppered tubes are heated at 60°C for one hour in a water bath without further mixing.

Bile Acid	No. of Observations	Recovery (μ g)	Recovery (range %)	Recovery (mean %)
Taurocholic	7	7.7-10.8	77-108	92
Taurochenodeoxycholic	7	8.3-10.2	83-102	92
Glycocholic	7	8.4-10.0	84-100	94
Glycochenodeoxycholic	6	8.2-10.3	82-103	97
Cholic	7	9.1-10.4	91-104	97
Chenodeoxycholic	7	8.3- 9.8	83- 98	91
Deoxycholic	7	9.0-10.4	90-104	97
Lithocholic	7	9.3-10.4	93-104	98

Table 1 Recovery of 10 μ g quantities of bile acids added to samples of intestinal juice

Bile Acid	No. of Observations	Recovery (μ g)	Recovery (range %)	Recovery (mean %)
Taurocholic	6	14.2-17.0	71- 85	77
Taurochenodeoxycholic	6	14.0-16.2	70- 81	76
Glycocholic	6	15.6-18.6	78- 93	78
Glycochenodeoxycholic	6	13.6-15.8	68- 79	74
Cholic	6	14.8-20.8	74-104	90
Chenodeoxycholic	6	15.6-19.4	78- 97	87
Deoxycholic	6	13.8-17.8	69- 89	80
Lithocholic	6	14.8-18.4	74- 92	82

Table II Recovery of 20 μ g quantities of bile acids added to serum

They are then centrifuged at 3,000 rpm for 30 minutes at 4°C; at this temperature the fluorescence is stable for at least 24 hours.

Fluorimetry is carried out in a spectrophoto-fluorimeter (Aminco-Bowman), using a xenon arc. Peak excitation was found to be at 470 m μ and the fluorescence peak was at 490 m μ . Excitation has also been carried out at lower wavelengths with limited loss of sensitivity, eg, using the 436 m μ mercury line. The Locarte LFM/5 fluorimeter has also been found satisfactory; a zinc lamp is used, with an LF 11 primary filter. The fluorescent band is selected by an LF 7 filter, and a monochromator setting of 525 m μ was found optimal in our instrument. One millilitre of sulphuric acid is then used for both serum and intestinal content.

SERUM BILE ACIDS

A bile acid fraction is isolated from 2 to 10 ml samples of serum, essentially by the procedure of Sandberg *et al* (1965). The resin is slurried in 0.2 M ammonium carbonate and added to a 10 mm ID chromatography column to a height of 10 cm. It is washed successively with 500 ml volumes of 1N aqueous sodium hydroxide, of 1N sodium hydroxide in 80% ethanol, and of water until the wash is neutral. The sample of serum (2-10 ml depending on the expected bile acid concentration) is diluted 1:1 with water and the pH adjusted to 11 with 1N NaOH. The column is eluted with 20 ml 95% ethanol, 40 ml ethylene chloride-ethanol mixture (1:1 v/v), and 20 ml 80% ethanol which are discarded. The anionic fraction is then eluted with 150 ml 0.2 M ammonium carbonate in 80% ethanol and this is taken to dryness in a rotary evaporator. Bile acids are transferred by means of several washes of n-butanol to a pear-shaped 10 ml flask, and the solvent is again removed on the rotary evaporator. The residue is dissolved in 100-400 μ l n-butanol and an aliquot of 50 μ l is taken for thin-layer chromatography.

Samples of aspirate from the small intestine obtained from fasting subjects, or bile, are applied directly to the plate in amounts of 10 μ l.

Results

By sequential use of three chromatographic solvents satisfactory separations of taurocholic, tauroolithocholic, glycocholic, glycolithocholic, cholic, deoxycholic, chenodeoxycholic, and lithocholic acids are obtained (Fig. 2), as well as a substantial degree of purification from contaminating steroids and triglycerides. Taurine conjugates of deoxycholate and chenodeoxycholate are not resolved and are estimated together as taurochenodeoxycholate. Similarly glycine conjugates of dihydroxy-acids were

	Present Method	β -Hydroxysteroid Dehydrogenase Method
1	67.0	66.0
2	15.9	15.0
3	8.3	8.0
4	9.0	11.0
5	3.9	2.8
6	3.1	0

Table III Total serum bile acid concentrations (μ -mole/l)¹

¹Duplicate specimens were analysed by the present method and by the enzymic procedure of Iwata and Yamasaka (1964)

	Present Method	Sandberg et al (1965)	Roovers et al (1968)
Cholic acid	0.77-2.60	0.05-1.58	0.76-1.05
Chenodeoxycholic & deoxycholic acids	0.68-1.44	0.51-3.02	2.16-3.80
Total	2.32-3.68	0.73-4.60	2.92-4.35
Number of samples	12	15	3

Table V Normal range of serum bile acid concentrations expressed as free bile acids (μ -mole/l)

Bile Acid	Male						Female						Mean	Standard Deviation
	1	2	3	4	5	6	7	8	9	10	11	12		
Taurocholic	0.72	0.52	0.68	0.45	0.10	0.31	0.52	0.23	0.20	0.20	0.25	0.39	0.38	0.20
Taurochenodeoxycholic	0.36	0.40	0.36	0.30	0.28	0.24	0.32	0.30	0.44	0.20	0.20	0.20	0.30	0.08
Glycocholic	1.12	0.47	0.26	1.72	1.59	2.14	1.66	1.46	1.72	2.79	1.67	2.15	1.57	0.71
Glycochenodeoxycholic	0.60	0.83	0.89	1.41	0.71	1.27	0.85	0.96	0.31	1.05	0.60	0.80	0.86	0.30
Total	2.80	2.22	2.19	3.88	2.68	3.96	3.35	2.95	2.67	4.24	2.72	3.64	3.11	0.69
3-OH/2-OH ¹	1.91	0.80	0.75	1.27	1.71	1.62	1.86	1.34	2.56	2.39	2.40	2.64	1.77	0.65
GLY/TAU ²	1.60	1.41	1.13	4.17	6.05	6.20	2.98	4.57	3.17	9.60	5.05	5.17	4.27	2.44

Table IV Fasting serum bile acid concentrations (μ -mole/l) in normal subjects

¹Ratio of sum of trihydroxy-bile acid concentrations (taurocholic, glycocholic, and cholic acids) to dihydroxy-bile acid concentrations (taurochenodeoxycholic, taurodeoxycholic, glycochenodeoxycholic, glycodeoxycholic, deoxycholic, and chenodeoxycholic acids).

²Ratio of total glycine conjugates to total taurine conjugates.

estimated as glycochenodeoxycholate as their resolution was not sufficient for separate quantitation.

The mean recovery of bile acids added to intestinal juice (Table I) was 94%, ranging from 90 to 98% for individual bile acids. Recovery of bile acids added to serum and carried through the entire analytical procedure is shown in Table II; the mean was 82%.

The precision of the estimation was examination by conducting duplicate analysis of all the constituent bile acids of eight sera. The levels found varied from below 1 μ -mole/l to over 50 μ M and the 40 pairs of results have therefore been divided into three groups: (1) values below 3 μ -mole/l, ie, within the normal range; (2) values between 3 and 10 μ -mole/l; and (3) values greater than 10 μ -mole/l.

The standard deviation of a single estimation, calculated as

$$S = \sqrt{\frac{\sum d^2}{2N}}$$

where d = difference between duplicates and N = number of pairs was 0.27, 0.71, and 2.16 μ -mole/l respectively, giving a coefficient of variation of about 10%.

To test the specificity of the procedure, six duplicate specimens of serum were analysed for bile acids by the method of Iwata and Yamasaki (1964) which depends on oxidation of bile acids by the enzyme β -hydroxysteroid dehydrogenase. The findings are compared in Table III with

those obtained by the present method; there is reasonable agreement at higher bile acid concentrations, and the discrepancies at lower concentrations may be explicable on the lesser sensitivity of the enzymatic procedure as compared with fluorimetry.

To determine the lower limit of sensitivity of the method, the mean and standard deviation of the blank reading were calculated for 14 thin-layer plates. The standard deviation did not exceed 25% of the mean blank. Bile acid fluorescence which does not exceed 50% of the blank fluorescence on the same plate is therefore regarded as insignificant. When using a plasma sample of 10 ml and dissolving the bile acid fraction in 100 μ l n-butanol the lower limits of sensitivity are: taurocholic acid 0.12 μ -mole/l, taurochenodeoxycholic acid 0.12 μ -mole/l, glycocholic acid 0.28 μ -mole/l, glycochenodeoxycholic acid 0.27 μ -mole/l, cholic acid 0.31 μ -mole/l, chenodeoxycholic acid 0.19 μ -mole/l, deoxycholic acid 0.14 μ -mole/l, and lithocholic acid 0.36 μ -mole/l.

The range of bile acid concentrations in 12 samples of fasting peripheral blood serum (obtained from healthy laboratory personnel) is shown in Table IV. There was no evident sex difference. Free bile acids were not detectable. Our data, when recalculated as free cholic acid and free dihydroxy acids, correspond fairly closely with those reported by Sandberg *et al* (1965) and Roovers *et al* (1968), who used gas-liquid chromatography (Table V).

Bile Acid	Diagnosis			Polya Partial Gastrectomy		
	Case 1	Case 2		Case 3	Case 4	Case 5
	Duodenal Sample	Duodenal Sample	Jejunal Sample	Jejunal Sample	Jejunal Sample	Jejunal Sample
Taurocholic	0.08	0.12	—	1.1	0.37	0.83
Taurochenodeoxycholic	0.81	0.01	—	2.3	0.90	3.1
Glycocholic	6.5	2.80	0.20	6.2	1.00	4.6
Glycochenodeoxycholic	10.7	1.70	0.20	6.3	4.00	9.6
Cholic	6.7	0.20	2.20	0.21	0.16	0.44
Chenodeoxycholic	2.7	—	0.23	0.08	—	—
Deoxycholic	4.2	—	—	—	—	—
Lithocholic	—	—	—	—	—	—
Total bile acids	31.7	4.83	2.83	16.1	6.53	18.6
Free bile acids	13.6	0.20	2.43	0.29	0.16	0.44

Table VI Bile acids (m-mole/l) in small intestinal aspirates from patients with malabsorption

Bile Acid	Normal Range	Diagnosis							
		Hepatitis		Intrahepatic Cholestasis		Extrahepatic Obstructive Jaundice		Cirrhosis	Massive Intestinal Resection
		Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7	Case 8
Bilirubin (mg/100 ml)	0.1-0.8	11.00	4.5	3.0	1.6	10	5.2	1.2	0.6
Taurocholic	0.10-0.72	50.4	20.6	56.3	9.08	19.0	2.9	8.15	0.23
Taurochenodeoxycholic	0.20-0.44	19.5	34.2	56.7	9.65	17.3	78.4	2.81	0.30
Glycocholic	0.26-2.79	34.3	58.8	16.3	4.55	45.7	65.0	11.8	0.75
Glycochenodeoxycholic	0.31-1.41	20.7	35.0	25.9	2.99	2.61	73.0	28.8	0.51
Cholic	—	1.56	2.44	1.54	2.44	0.46	2.2	1.59	2.69
Chenodeoxycholic	—	—	—	0.71	4.19	0.46	—	0.41	6.39
Deoxycholic	—	—	—	—	—	—	—	—	—
Lithocholic	—	—	—	—	—	—	—	—	—
Total bile acids	2.2-4.2	140.5	151.0	157.4	32.9	84.9	280.5	54.6	10.9
3-OH/2-OH	0.75-2.64	2.15	1.18	0.89	0.95	3.29	0.87	0.65	0.51
GLY/TAU	1.13-9.6	0.79	1.71	0.37	0.40	1.31	0.97	3.7	2.4

Table VII Serum bile acids (μ -mole/l) in jaundice and the stagnant loop syndrome

ILLUSTRATIVE CLINICAL OBSERVATIONS

The concentration of conjugated and free bile acids in the intestinal contents of five patients with intestinal malabsorption is shown in Table VI.

Patients 1 and 2 had massive duodenal and jejunal diverticulosis with bacterial colonization of the small intestine. Both patients had severe steatorrhoea with faecal fat excretion of 22 to 30 g/day. In patient 1, duodenal juice contained the free bile acids, cholic, chenodeoxycholic, and deoxycholic acid; patient 2, who in addition had malnutrition and hypoproteinaemia, had lower levels of total bile acids in the jejunal sample in which the main bile acid was cholic acid (Tabaqchali, Hatzioannou, and Booth, 1968).

In patients 3, 4, and 5, who had undergone Polya partial gastrectomy and had minimal steatorrhoea, the bile acids were present in greater concentration, and almost entirely in the conjugated form. Lithocholic acid was not detectable in any of these specimens.

Serum bile acid measurements in patients with jaundice of various types, and in a patient who had had a massive intestinal resection, are shown in Table VII. All patients with hepatobiliary disease (1-7) had a marked increase in fasting serum bile acid concentrations, and

the bile acids were present mainly in the conjugated form. Patients 1 and 2 had viral hepatitis. Serial bile acid measurements were carried out in patient 1 (Fig. 3). Patient 3 had primary biliary cirrhosis, and patient 4 had intrahepatic cholestasis associated with ulcerative colitis. A striking finding in patients 3 and 4 was the decrease in the glycine/taurine ratio in serum bile acids. Patients 5 and 6 had extrahepatic

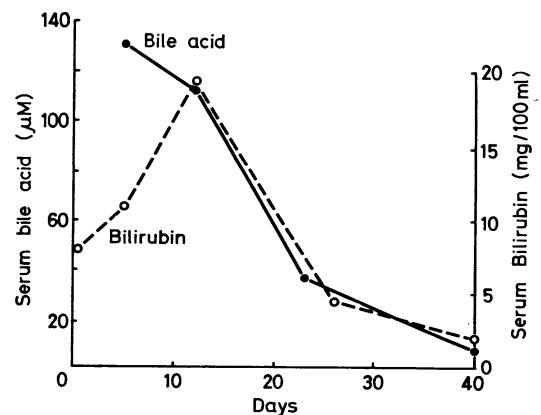


Fig. 3 Serial measurements of total serum bile acid concentrations in a patient with viral hepatitis.

obstructive jaundice due to carcinoma of the head of the pancreas, and patient 7 had alcoholic cirrhosis. The ratio of trihydroxy to dihydroxy bile acids varied considerably, and no consistent trend is as yet evident. Patient 8 had undergone massive intestinal resection following thrombosis of the mesenteric vessels, leaving only 14 in. of proximal jejunum which was anastomosed to the transverse colon. He also had bacterial colonization of the jejunal remnant. In contrast to the patients with hepatobiliary disease, the serum bile acids in this patient consisted chiefly of free acids. The free bile acids present were cholic and chenodeoxycholic acid. Deoxycholic and lithocholic acids were not found. The ratio of trihydroxy to dihydroxy bile acids was low, as we have observed in seven out of eight patients with intestinal resections or with the stagnant loop syndrome (Panveliwalla, 1968).

Discussion

The procedure described has been in use in this laboratory for two years; the accuracy appears to be good, and the precision is adequate. The sensitivity is such as to permit the determination even of subnormal concentrations.

In samples of 10 ml normal serum, only glycocholic, glycochenodeoxycholate, taurocholic, and taurochenodeoxycholate were detected, confirming the results of Sandberg *et al* (1965). In serum from jaundiced patients, not only may abnormal patterns of conjugates be shown, but free acids may be detected in measurable amounts.

By permitting the individual measurement of the conjugated and free bile acids of serum, the present relatively simple method may extend the value of bile acid studies in the investigation of hepatobiliary and intestinal disease.

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