Quantitative analysis of unconjugated and conjugated bile acids in duodenal fluid by densitometry after paper electrophoresis

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Abstract A new paper electrophoretic method for the separation of bile acids into five groups, (*1*) unconjugated, (*2*) glycine conjugates and (*3*) taurine conjugates, and (*4*) and (*5*) the respective monosulfates, is described. Rapid and accurate qualitative and quantitative estimations of each group are obtained by densitometry after internal standardization and phosphomolybdate color development. The technique can be done in the routine clinical laboratory and is useful for the detection of diseases affecting the enterohepatic circulation of bile acids.

Supplementary key words glycine/taurine ratio · bile acid sulfates

Estimation of bile acids in biological fluids is important for the recognition and management of a variety of hepatic and intestinal disorders. Currently available procedures for quantitative estimation of bile acids require a combination of analytical methods, such as preliminary purification by thin-layer chromatography, elution, and quantitative determination enzymatically, or hydrolysis followed by spectrophotometry or gas–liquid chromatography.

Using readily available paper electrophoresis equipment, we have devised a simple electrophoretic separation of bile acids into five groups: unconjugated, glycine conjugated, taurine conjugated, and the sulfate esters of glycine and taurine conjugated bile acids. The relative proportions of each bile acid group present in a sample can be determined by densitometry, and the absolute amount of any given species is found by the change in proportions after the addition of a known standard. The new method compares closely in accuracy with bile acid analysis by gas–liquid chromatography; it is applicable to specimens of intestinal contents or bile; and the method greatly facilitates the study of patients with abnormalities in bile acid metabolism and excretion.

MATERIALS AND METHODS

Unconjugated and conjugated bile acids were purchased from Supelco, Bellefonte, Pa., and Maybridge Research Chemicals, Cornwall, U.K. Glycolithocholic acid sulfate and taurolithocholic acid sulfate were a gift of Dr. Robert Palmer. The sulfate ester of 3β-hydroxy-5-cholenyl taurine was prepared using standard techniques (1). Standard solutions were prepared by dissolving weighed amounts of standards in either water or methanol. Cholesterol was obtained from J. T. Baker Chemical Co., Phillipsburg, N.J., and was dissolved in petroleum ether. Lecithin was obtained from General Biochemicals, Chagrin Falls, Ohio, and was dissolved in absolute ethanol.

Aliquots of standards, bile, and intestinal contents were applied in 10–25-μl amounts across the width of a Beckman no. 320046 paper strip and within 3 cm of the end of the paper placed in contact with the cathode. Alternatively, the samples can be applied at the midpoint of the paper. Separation of unconjugated bile acid from cholesterol and lecithin is not complete when samples are applied at the midpoint, and much better resolution is obtained by application close to the cathode.

Paper electrophoresis was done in a Spinco cell (Durrum type). The electrolyte solution consisted of 10 ml of pyridine and 70 ml of glacial acetic acid diluted to 2500 ml with water (pH 3.7). After electrophoresis at 500 V (12–14 mA) for 5 hr at 4°C, the air-dried strips were stained by passing each rapidly through a solution consisting of 5 g of phosphomolybdic acid dissolved in a

mixture of 95 ml of glacial acetic acid and 5 ml of concentrated sulfuric acid (2). The strips were allowed to air dry and then were placed in a vacuum desiccator in the dark. Although color was visible after 2 hr and the intensity did not change thereafter, color was allowed to develop overnight since this was most convenient. A variety of methods using phosphomolybdic acid for identification of bile acids have been described, but this method for color development was found to be the most reproducible.

Densitometry of the stained and developed paper electrophoretic strips was performed using a Densicord (Photovolt Corp., New York) with 610-nm red filter. The relative densities of the unconjugated and conjugated bile acids were measured by weighing the densitometry recording paper corresponding to the area under each curve and expressing each peak as a proportion of the total area. Alternatively, an automatic integrator can be utilized in conjunction with densitometry. Formulas for calculations of bile acid concentrations are indicated in the Appendix. Precision of the densitometric method was evaluated by running duplicate samples of bile acid standards ranging in glycine/taurine ratios from 1 to 5 for five consecutive runs. The coefficient of variation was found to be in the range of 2–8% in these experiments. The method of gas–liquid chromatographic estimation of total bile acids was previously described (3).

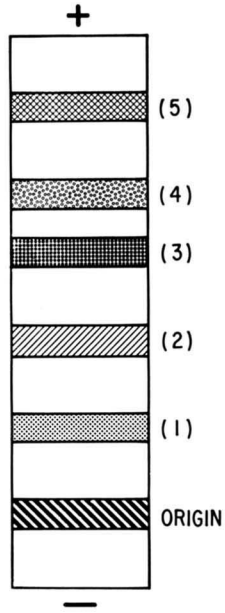

FIG. 1. Separation of unconjugated and conjugated bile acids by paper electrophoresis. A representation of a stained paper electrophoretic strip is shown. At the origin are found cholesterol, lecithin, and bile pigments. Progressing towards the anode are found: (*1*) cholic, chenodeoxycholic, deoxycholic, and lithocholic acids; (*2*) glycocholic, glycochenodeoxycholic, glycodeoxycholic, and glycolithocholic acids; (*3*) taurocholic, taurochenodeoxycholic, taurodeoxycholic, and taurolithocholic acids; (*4*) glycolithocholate sulfate; and (*5*) taurolithocholate sulfate and the sulfate ester of 3β-hydroxycholenyl taurine.

RESULTS

Separation of bile acids by electrophoresis

The separation of bile acids into five groups is shown in Fig. 1. At the origin are found lecithin, cholesterol, and pigment derived from bilirubin. Band *1* comprises lithocholic, deoxycholic, chenodeoxycholic, and cholic acids. The glycine conjugates of these bile acids are found in band *2*, and their taurine conjugates in band *3*. The separation of bile acid conjugates in duodenal fluid of a normal individual compared with standards is shown in Fig. 2.

At pH 3.7 it was found that the number of hydroxyl groups on the steroid ring did not detectably affect migration. Sulfation of the bile acids imparts an additional negative charge so that band *4* (Fig. 1) represents the sulfate ester of glycolithocholate, and band *5* (Fig. 1) the sulfate esters of taurolithocholate and 3β-hydroxy-5-cholenyl taurine. Other monosulfate esters could not be obtained.

Estimation of relative amounts of glycine and taurine conjugates

Aliquots of known mixtures of glycine and taurine conjugates were applied to paper, and the electro-

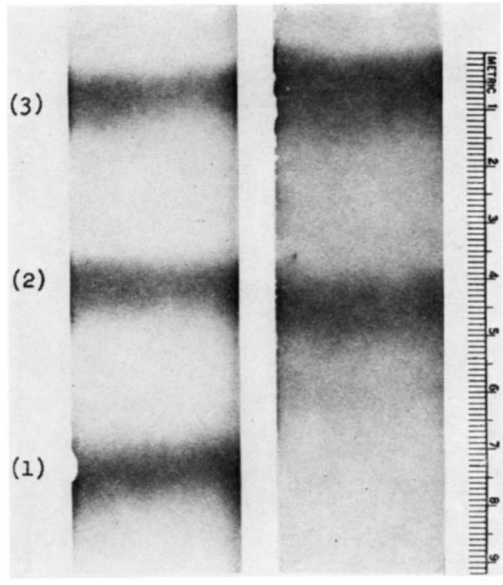

FIG. 2. Paper electrophoresis of bile acid standards and duodenal fluid. After electrophoresis for 5 hr at 500 V, the strips were stained with phosphomolybdate. The strip on the left contains the following standards: (*1*) cholic acid, (*2*) glycocholic acid, and (*3*) taurocholic acid. On the right is a duodenal sample demonstrating the presence of two bands corresponding to glycine and taurine conjugated bile acids.

TABLE 1. Estimation of relative proportions of glycine and taurine conjugates and unconjugated bile acids

	Expected Ratio	Observed Ratio
A. Glycocholate/Taurocholate	0.20	0.20
$r = 0.99$	0.40	0.37
$P < 0.001$	0.60	0.62
	0.80	0.85
	1.00	0.95
	1.25	1.13
	1.67	1.72
	2.50	2.42
	5.00	4.91
B. $\frac{\text{Glycocholate + Glycodeoxycholate}}{\text{Taurocholate + Taurodeoxycholate}}$	1.00	0.90
	1.33	1.43
$r = 0.90$	1.92	2.20
$P < 0.02$	2.00	1.86
	2.00	1.96
C. $\frac{\text{Glycocholate}}{\text{Taurocholate}} + \frac{\text{Glycocholate}^a}{\text{Taurocholate}}$	0.50	0.48
	0.67	0.64
$r = 0.99$	1.00	1.03
$P < 0.001$	1.125	1.149
	1.33	1.31
	1.50	1.40
	2.00	1.91
	3.33	3.35
D. $\frac{\text{Glycine + Taurine Conjugates}}{\text{Cholic Acid}}$	0.50	0.62
	1.00	0.95
$r = 0.99$	2.00	2.05
$P < 0.001$	4.00	3.80

[a] Equal volumes of two separate samples applied to same point of origin on paper strip.

phoretic separation was performed. After color development and densitometry, the area under each curve was cut out, the paper was weighed, and the relative proportion of each conjugated bile acid was expressed as the glycine/taurine ratio. As shown in Table 1, mixtures of the different conjugated and unconjugated bile acids varying in ratio from 0.20 to 5.00 were recovered in the expected proportions ($r > 0.9$). In 24 of 26 determinations, the expected value differed less than 10% from the observed value. In two instances the observed value differed by less than 20% from the expected value.

Estimation of the absolute amounts of bile acids

A highly significant linear correlation ($r = 0.99$) was found (Fig. 3) between the amounts of cholesterol and unconjugated and conjugated bile acids applied to paper and their densities. These studies were done by applying 5–100 μg of the various compounds to the paper and developing all the strips for the same length of time and under identical conditions. With this technique, color development is proportional to the absolute amount of bile acid, and the densitometric estimation is directly convertible to mass.

However, there was considerable daily variation in the intensity of color development so that calibration curves prepared on one day were not accurate for subsequent analyses. Therefore, two methods of internal standardization are possible. One possible method (not tried) is to apply a known amount of bile acid to the dried strip after electrophoresis and before color development. If the site of application is on the cathodal side of the origin, superimposition of the standard on areas containing other bile acids will be avoided. Alternatively, the procedure employed in this study was to apply a known amount of glycine and/or taurine conjugated bile acid to the same site as the sample, prior to electrophoresis. Thus, two strips with the same amount of sample, and one having standards added, are run simultaneously. The internal standard causes a shift in the proportion of each group of bile acids present on the paper strip. By comparing the change in proportion of each bile acid group caused by addition of the known standard, it is possible to calculate (see Appendix) the absolute amount of each group of bile acids. The calculation is preferable to that of subtracting the corresponding peaks from two strips, since the latter introduces the error of variation in intensity of color between strips as a result of staining and development. Quantitative estimation of each group of bile acids present in the upper intestinal contents of five patients is shown in Table 2. The total bile acid concentration calculated by the ratio method (see Appendix) is compared with the value obtained by gas–liquid chromatography. The two methods are in excellent agreement.

Detection of bile acid sulfates

Application of standards of glyco- and taurolithocholate sulfates to the paper strip prior to electrophoresis yielded migration patterns distinct from the other groups of bile acids (Fig. 1). The quantity of material available did not permit a complete study of the relationship between mass and densitometric estimation over the range used for the other bile acids. However, the color density obtained with 10 μg of sulfate conjugates was comparable to that obtained with 10 μg of other bile acids. In the duodenal contents of the five patients indicated in Table 2 and in concentrated urine specimens from three patients with liver disease who excreted bile acids in urine, no color development was noted in areas corresponding to the standard bile acid sulfates. As expected, the monosulfate ester of 3β-hydroxy-5-cholenyl taurine migrated at the same rate as taurolithocholate sulfate.

DISCUSSION

This study was undertaken because of the need for a rapid and accurate method for separating and quantitating unconjugated and conjugated bile acids in the evalua-

tion of patients suspected of having intestinal diseases. It has been shown that the identification of an increased glycine/taurine ratio of conjugated bile acids may be a sensitive early indication of disease of the terminal ileum (4). In normal man, the ratio of glycine to taurine conjugated bile acids ranges from 1 to 5 (mean approximately 3) (5). In diseases of the terminal ileum such as regional enteritis (6) or in the presence of a significant ileal resection (7, 8), there is an increase in bile acid turnover as a result of fecal loss. As a consequence, an increased proportion of glycine conjugates is synthesized by the liver (6–8). The change in ratio can occur in duodenal fluid before any abnormalities are detected on radiological evaluation of the ileum (4). Therefore, the measurement of the glycine/taurine ratio in fluid aspirated from the duodenum may be helpful in the early diagnosis of disease of the terminal ileum. The electrophoretic method described in this report offers a simple, accurate means of determining the relative proportions of these conjugates, and its application would, therefore, seem useful in the diagnostic evaluation of patients with prolonged diarrhea.

Malabsorption of fat in the blind-loop syndrome is believed to occur as a consequence of bacterial deconjugation of bile salts in the upper small intestine (9, 10). Quantitation by electrophoresis of the proportion of unconjugated bile acids, in the manner reported here, provides a rapid method for the detection of increased intestinal bacterial activity.

The detection of the sulfate esters of glyco- and taurolithocholate in human bile (11) has led to the possibility that esterification of other bile acid conjugates may also be an important pathway for their metabolism. Thus far, the only compounds available for study are the sulfates produced by esterification of monohydroxy bile acid conjugates. The electrophoretic migration of sulfates at rates faster than the respective conjugates is consistent with the additional negative charge of the molecule. Since the number of hydroxy groups does not appear to affect the migration of unconjugated and conjugated bile acids, it seems likely that the monosulfates of the predominant dihydroxy and trihydroxy bile acid conjugates in intestinal contents would also be detected by this electrophoretic method. Their absence in intestinal contents suggests that sulfation of these compounds is not a normal major pathway of bile acid metabolism.

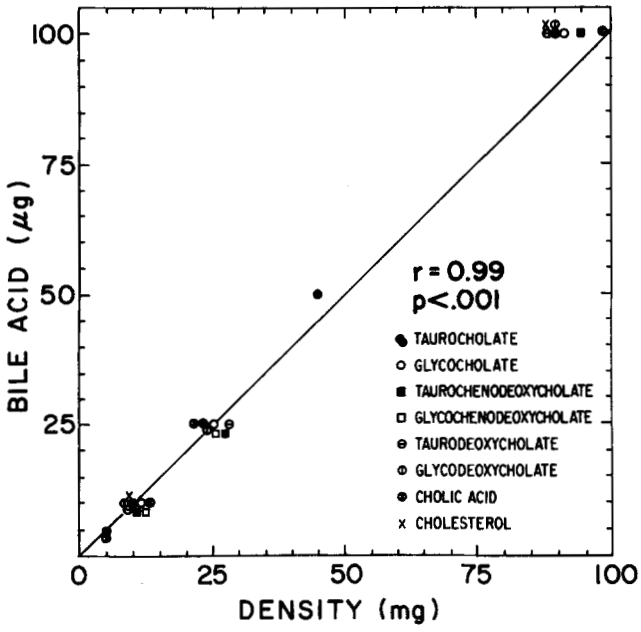

FIG 3. Relationship between bile acid mass and densitometry. By comparing a range of amounts of bile acids from 5 to 100 μg with the densities obtained from the stained paper electrophoretic strip expressed in arbitrary density units, a highly significant linear correlation for the various bile acids was observed between the relative amounts and the densitometrically determined proportions.

With regard to di- and trihydroxy bile acid conjugates, it is possible that sulfation in vivo could lead to di- and trisulfate esters. Since these compounds are not available, it is not possible to be certain of their location after electrophoresis. However, it is likely that the additional negative charge(s) would result in migration at a rate faster than the monosulfate ester of taurolithocholate. Further support for this view can be obtained from the knowledge that the electrophoretic mobilities of phenol-

TABLE 2. Estimation of absolute concentration of bile acids in duodenal contents

Diagnosis	Glycine/ Taurine Ratio	Bile Acid Concentration[a]				Total Bile Acid Concentration[b]
		Glycine	Taurine	Unconjugated	Total	
		mg/ml				*mg/ml*
1. Prolonged diarrhea	9.4	4.10	0.40		4.50	4.80
2. Regional ileitis	11.0	1.87	0.17		2.05	2.41
3. Blind loop	2.1	0.80	0.37	1.92	3.09	2.82
4. Chronic hepatitis	5.3	1.02	0.20		1.22	1.40
5. Functional bowel	3.4	0.73	0.21		0.94	1.10

[a] Determined by the method reported in the text.
[b] Determined by gas–liquid chromatography.

tetrabromphthalein and its mono-, di-, and tetrasulfonate derivatives are different and increase in proportion to the number of sulfonic acid groups (12). Therefore, the presence of detectable bands, after phosphomolybdate development, closer to the anode than taurolithocholate sulfate would suggest the presence of other sulfated compounds.

Thus, a paper electrophoretic method is reported which, in conjunction with densitometry, offers the following readily available information: the relative proportion of unconjugated and glycine and taurine conjugated bile acids, the absolute concentration of bile acids in biological fluids, and the identification of bile acid sulfates.

APPENDIX

General Equation for Calculating Absolute Amount of Bile Acid

a_1 = amount of unknown bile acid

a_t = total amount of bile acids on paper strip

$\frac{a_1}{a_t}$ = proportion of unknown bile acid = r_1

s = standard added to a_1

$\frac{a_1 + s}{a_t + s}$ = new proportion of unknown bile acid = r_2

$$r_2/r_1 = \frac{a_1 + s}{a_t + s} \Big/ \frac{a_1}{a_t}$$

$$r_2/r_1 = \frac{a_1/a_t + s/a_t}{a_t/a_t + s/a_t} \Big/ \frac{a_1}{a_t}$$

Solve for a_t

$$a_t = \frac{s(1 - r_2)}{(r_2 - r_1)}$$

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