papers and notes on methodology

High pressure liquid chromatographic analysis of conjugated bile acids in human bile: simultaneous resolution of sulfated and unsulfated lithocholyl amidates and the common conjugated bile acids

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Abstract A reversed phase high pressure liquid chromatography (HPLC) system capable of simultaneously separating four lithocholyl species (sulfated and unsulfated forms of lithocholylglycine and lithocholyltaurine) as well as the eight other major conjugated bile acids present in human bile is described. The system uses a C_{18} octadecylsilane column and isocratic elution with methanol phosphate buffer, pH 5.35. Relative bile acid concentration is determined by absorbance at 200 nm. Retention times relative to chenodeoxycholylglycine are reported for the four lithocholic acid forms, the glycine and taurine amidates of the four major bile acids present in human bile (cholic, chenodeoxycholic, ursodeoxycholic, and deoxycholic), and for their corresponding unconjugated forms. Retention times are also reported for the glycine and taurine amidates as well as the unconjugated form of the C_{23} nor-derivatives of these bile acids. Maximal absorbance of bile acid amidates is at 200 nm and is very similar for the (unsulfated) glycine and taurine amidates. Sulfated lithocholyl amidates exhibit molar absorptivities at 200 nm which are 1.4 times greater than that of non-sulfated lithocholyl amidates. Unconjugated bile acid absorbance at 200 nm or 210 nm is 20 to 30 times less than that of corresponding peptide conjugates. The method has been applied to samples of gallbladder bile obtained from 14 healthy subjects to define the pattern of conjugated bile acids present in human bile.—**Rossi, S. S., J. L. Converse, and A. F. Hofmann.** High pressure liquid chromatographic analysis of conjugated bile acids in human bile: simultaneous resolution of sulfated and unsulfated lithocholyl amidates and the common conjugated bile acids. *J. Lipid Res.* 1987. **28:** 589–595.

Supplementary key words conjugated bile acids • sulfated lithocholyl amidates • nor-bile acid amidates

The present method of choice for identification and relative quantitation of conjugated bile acids in biological fluids is high pressure liquid chromatography (HPLC) using reversed phase systems. Isocratic (1–5) and gradient (6, 7) systems for characterizing the major taurine and glycine amidates have been described. None of the systems reported to date are capable of discretely separating the two sulfated and two unsulfated lithocholic acid amidates, while simultaneously resolving the other glycine and taurine conjugated bile acids which are generally present in human bile.

Relative quantitation of each of the four lithocholic acid forms present in bile could provide valuable information on the metabolism of lithocholic acid in health, disease, or when the composition of the circulating bile acids is altered by bile acid administration. In the National Cooperative Gallstone Study, about 3% of patients receiving oral doses of chenodeoxycholic acid for gallstone dissolution developed biochemical or morphological evidence of liver toxicity (8). Such toxicity might arise from enterohepatic accumulation of lithocholic acid, which would be anticipated to occur in patients with defective hepatic sulfation of lithocholic acid (9, 10). To detect such defective sulfation, a chromatographic method was needed to resolve the four lithocholic acid species present in human bile.

In this report, we describe an isocratic HPLC technique which resolves not only the sulfated and unsulfated lithocholic acid conjugates, but also the major natural glycine- and taurine-conjugated bile acids. In addition to good resolution, determination of extinction coefficients for each of the conjugated bile acids enabled us to define the true relative proportion of the conjugated bile acids in 14 healthy subjects. We also report the behavior of C_{23} nor-bile acid conjugates in this system, as part of a larger investigation concerning the unusual choleretic and metabolic properties of these novel bile acids (11, 12).

Abbreviations: HPLC, high pressure liquid chromatography; CDCG, chenodeoxycholylglycine.

MATERIALS AND METHODS

Reagents

All reagents were HPLC-grade and were purchased from Fisher Scientific (Fairlawn, NJ), including NaOH (Kjeldahl Nitrogen Determination Grade, Fisher No. 410-4).

Bile acid standards

Conjugated bile acid standards were purchased from Calbiochem (La Jolla, CA) or Steraloids (Wilton, NH) or synthesized in this laboratory. Glycine or taurine conjugates of bile acids were prepared using a modification of the method of Tserng, Hachey, and Klein (13). The sulfates of lithocholylglycine and lithocholyltaurine were prepared by chlorosulfonation of their corresponding glycine or taurine amidates (14). An extremely pure sample of sodium dodecyl sulfate was provided by K. J. Mysels. Natural bile acids (C_{24}) were converted to their C_{23} nor-derivatives by the Barbier-Wieland procedure (15). Butane sulfonic acid and *n*-butyric acid were obtained from Aldrich Chemical Company, Milwaukee, WI.

Chromatographic instrumentation

A Beckman Altex Model 324 M liquid chromatograph (Beckman Instruments, Fullerton, CA) was used. The chromatograph was fitted with an Altex Ultrex C_{18} (octadecylsilane) column, 25 cm × 4.6 mm I.D., 5 μm particle size. A 300-mg guard column containing Ultrex C_{18} material was placed in line between the injector and analytical column (Upchurch Scientific, Oak Harbor, WA). A Hitachi variable wavelength ultraviolet spectrophotometer Model 100-40 was used for detection (Hitachi Instruments, Tokyo, Japan). Peak characterization was performed by slope integration on a Shimadzu CR1A recorder (Shimadzu Instruments, Columbia, MD).

Solvent

The solvent system was methanol–0.01 M KH_2PO_4 75:25. To each liter of this mixture, 4.2 ml of 5 N NaOH was added. The pH was then adjusted, using a glass electrode, to apparent pH 5.35 by addition of 85% H_3PO_4 (about 1.0 ml/l). Solvent was filtered through a 0.45-μm nylon filter (No. 5-8067, Supelco, Inc., Bellefonte, PA) prior to use. The solvent flow rate of 0.7 ml/min produced operating pressures of 2200–2400 psi.

Sample preparation

Human gallbladder bile (0.1 ml, < 5 μmol of total bile acids) was diluted with 8 ml of 0.1 N NaOH prior to isolation of the bile acids by adsorption to a hydrophobic cartridge, as described by Rossi, Clayton, and Hofmann (16). Diluted bile was passed through 500-mg reverse phase C_{-18} (Bond Elut, Analytichem International, Harbor City, CA) cartridges under vacuum. After rinsing with 20 ml of water, bile acids were eluted with 8 ml of methanol. Methanol eluates were reduced in volume to 1 ml under N_2 and filtered through a 0.45-μm nylon membrane (No. 5-8067, Supelco, Inc.) prior to injection of 5 μl. This volume was sometimes modified, particularly for samples that contained low levels of lithocholyltaurine or lithocholylglycine. Retention times for standards were obtained twice daily by injecting 5 μl of solvent containing 1 μmol of bile salt/ml of solvent.

Extinction coefficient determination

Molar absorptivities in HPLC solvent were determined with 1.00 mM bile acid solutions on a Uvikon 860 dual beam scanning spectrophotometer (Kontron Instruments, Hayward, CT). Bile acid concentration was verified by enzymatic endpoint analysis with 3α,3β-hydroxysteroid dehydrogenase (17). The wavelength scan range was from 190

TABLE 1. Relative retention times (R_t) for the glycine and taurine amidates as well as the unconjugated form of the bile acids occurring in human bile (R_t = 1.00 for chenodeoxycholylglycine)

Nuclear Substituent(s)	Relative Retention Time[a]		
	Taurine Conjugate	Glycine Conjugate	Unconjugated Bile Acid
3αOH (Lithocholic acid)	1.25	1.93	5.65
$3\alpha OSO_3^-$ (Sulfolithocholic acid)	0.37	0.52	1.05
3αOH, 7αOH (Chenodeoxycholic acid)	0.70	1.00[b]	2.30
3αOH, 7βOH (Ursodeoxycholic acid)	0.33	0.42	0.84
3αOH, 12αOH (Deoxycholic acid)	0.79	1.17	2.67
3αOH, 6βOH, 7βOH (β-Muricholic acid)[c]	0.32	0.43	0.97
3αOH, 7βOH, 12αOH, (Ursocholic acid)	0.23	0.28	0.38
3αOH, 7αOH, 12αOH (Cholic acid)	0.46	0.62	1.26

[a]The average retention time of an inert injection marker was 3.6 min under usual chromatographic conditions.
[b]Retention standard: this peak had a retention time averaging 19.5 min under usual chromatographic conditions.
[c]An important constituent of rat bile, included for comparison.

TABLE 2. Relative retention times (R_t values) for C_{24} and C_{23} (nor) bile acids (R_t = 1.00 for glycochenodeoxycholic acid)

Bile Acid Nucleus[a]	Taurine Conjugate		Glycine Conjugate		Unconjugated	
	C_{23} (nor)	C_{24}	C_{23} (nor)	C_{24}	C_{23} (nor)	C_{24}
Lithocholic	0.61	1.25	1.05	1.93	3.00	5.65
Chenodeoxycholic	0.39	0.70	0.54	1.00	1.29	2.30
Ursodeoxycholic	0.25	0.33	0.30	0.42	0.51	0.84
Deoxycholic	0.49	0.79	0.71	1.17	1.58	2.67
Cholic	0.29	0.46	0.65	0.62	0.72	1.26

[a]For chemical structure, see Tables 1 and 3.

nm to 400 nm. All conjugated bile acids exihibited an absorption maximum at 200 nm, whereas maximal light absorption for unconjugated bile acids peaked at either 200, 205, or 210 nm, depending on the bile acid.

Human bile samples

Human bile samples kindly donated by Dr. R. Thomas Holzbach of the Cleveland Clinic were obtained from organ donor subjects (brain-dead because of acute head trauma) at the time of organ collection or by gallbladder puncture at laparotomy from subjects without biliary disease.

RESULTS

Identification and resolution

Fig. 1A illustrates the separation efficiency of this system for a complex mixture of bile acid standards. With a run time of under 40 min, satisfactory resolution was obtained for all the common natural conjugated bile acids. Elution profiles of human bile samples likewise indicated excellent resolution among the major bile acids, even when large amounts of sample were placed on column to facilitate quantitation of minor components Fig. 1B, C.

The retention times of several major and minor bile acid conjugates, as well as their unconjugated precursors, relative to chenodeoxycholylglycine (CDCG), are presented in **Table 1**. Table 1 also includes the retention time of 7α,12α-dihydroxy-5β-cholanoyl glycine, which was used as an internal standard, as suggested by Muraca and Ghoos (18). CDCG was chosen as the retention index because it elutes closest to the midpoint of this chromatographic system and is generally the dominant bile acid amidate present in human bile (19). **Table 2** presents data for the relative mobility of C_{23} (nor) bile acids and their amidates. A decrease in the length of the side chain from five carbons to four carbons markedly decreased retention times for these synthetic bile acids, as anticipated.

Absorptivity of conjugated bile acids

Table 3 presents data on the molar absorbance of bile acids and their corresponding glycine and taurine conjugates at 200 nm and 210 nm, the two most commonly employed wavelengths for ultraviolet detection of bile acids. Glycine- and taurine-amidated bile acids exhibited much greater absorbance (at both wavelengths) than did their corresponding unconjugated derivatives. In this buffer system, molar absorptivity was similar for glycine and taurine amidates, although the molar absorptivity at 200 nm of some taurine amidates was about 5% greater than that of the corresponding glycine amidate. To confirm the greater molar absorptivity of the sulfonic acid group as compared to the carboxylic acid group, the molar absorptivity of butane sulfonic acid was compared with that of *n*-butyric acid. As anticipated, the molar absorptivity of the sulfonic acid was 400% greater than that of the carboxylic acid (data not shown). Sulfation of the 3-position in the steroid nucleus appeared to enhance absorptivity at 200 nm. This increase in absorptivity was partially attributable to additional absorbance from the "ethereal" linkage of the sulfate group, as reported for glycine versus taurine conjugates of cholic acid (20). To show that the sulfate group was responsible for the increase in absorptivity, the absorptivity of dodecylsulfate and butyl sulfate was examined; both compounds showed significant absorbance at 200 nm (data not shown). Since molar absorbance was greater for the sulfated bile acid amidates, the peak area was computed using two extinction coefficients: one for the sulfated amidates and a second for the non-sulfated amidates.

Sensitivity, precision, and linearity

Using these methods, the minimum detectable amount of a single bile acid amidate (taurocholate) was 5.0 nmol/20 μl of injection volume. Standard curves for representative conjugated, unconjugated, and all the nor- and sulfated-bile acids were produced by plotting peak area versus concentration (data not shown). The curves were linear from 0.01 to 0.50 μmol, and correlation coefficients of linear regression ranged from 0.97 to 0.99. These values are in good agreement with previously reported values for the working range of HPLC methods of conjugated bile acid analysis (4). Day-to-day precision was identical among bile acid species and averaged 3.7%. The average percent error in retention time between standard bile acid amidates and

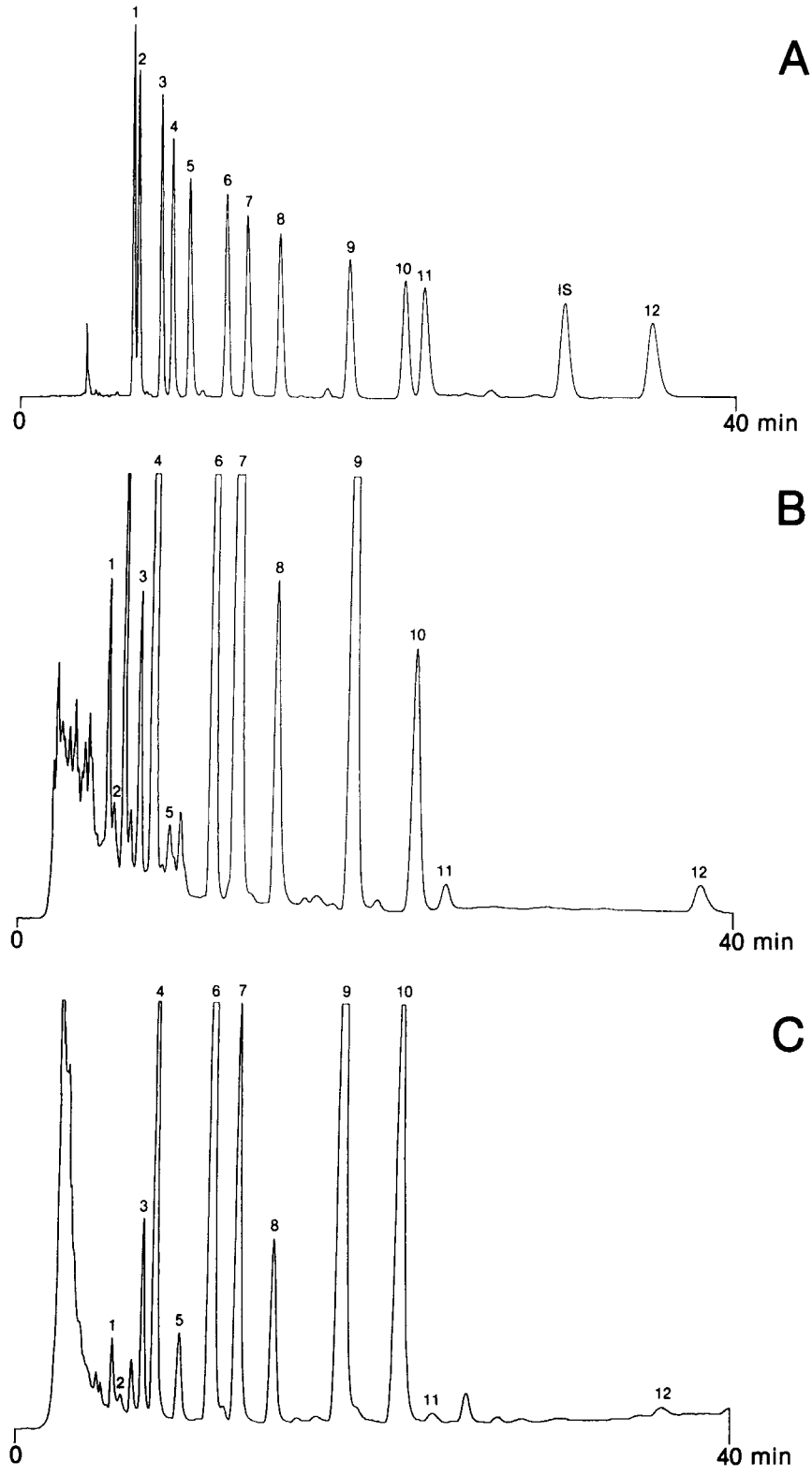

Fig. 1. A. Chromatogram of a standard mixture of synthetic conjugated bile acids. Peaks: IS, internal standard (7α,12α-5β-cholanoyl glycine); 1, ursodeoxycholyl taurine; 2, sulfolithocholyl taurine; 3, ursodeoxycholyl glycine; 4, cholyltaurine; 5, sulfolithocholyl glycine; 6, cholylglycine; 7, chenodeoxycholyl taurine; 8, deoxycholyl taurine; 9, chenodeoxycholyl glycine; 10, deoxycholyl glycine; 11, lithocholyl taurine; 12, lithocholyl glycine. B. Distribution of bile acid conjugates in normal human bile obtained by gallbladder puncture at laparotomy. C. Profile of amidated bile acids in bile from patient exhibiting abnormal liver function concurrent with oral administration of chenodeoxycholic acid for treatment of biliary calculi.

TABLE 3. Molar absorptivity[a] for the glycine or taurine amidates as well as the unconjugated form of bile acids present in human bile

Nuclear Substituent (Trivial Name)	Absorbance (200 nm/210 nm)		
	Taurine Conjugate	Glycine Conjugate	Unconjugated Bile Acid
		μM/cm at 25°C	
3αOH (Lithocholic)	2.2/0.6	2.1/1.0	0.06/0.07
$3\alpha OSO_3^-$ (Sulfolithocholic)	3.1/1.0	3.1/0.9	0.19/0.15
3αOH, 7αOH (Chenodeoxycholic)	2.1/0.8	2.1/0.7	0.09/0.08
3αOH, 7βOH (Ursodeoxycholic)	2.2/0.7	2.1/0.9	0.07/0.07
3αOH, 12αOH (Deoxycholic)	2.5/0.8	2.4/1.0	0.09/0.09
3αOH, 6βOH, 7βOH (β-Muricholic)[b]	2.2/0.7	2.0/0.6	0.11/0.10
3αOH, 7βOH, 12αOH, (Ursocholic)	2.2/0.7	2.1/0.7	0.11/0.09
3αOH, 7αOH, 12αOH (Cholic)	2.1/0.6	2.1/0.9	0.12/0.07

The optical density of HPLC solvent (against water) (1 cm light path) is 0.79 at 200 nm and 0.16 at 210 nm. Absorptivities were determined with HPLC solvent as reference.

[a]Extinction coefficient.

[b]An important constituent of rat bile, included for comparison.

those in the 14 human gallbladder bile samples was 1.1 (range 0.0–5.6%).

Gallbladder bile analysis

The distribution of amidated bile acids in bile from 14 healthy human subjects is given in **Table 4**. Based on chromatograms that were characterized at 200 nm wavelength, no detectable unconjugated bile acids were observed. Cholic and chenodeoxycholic acid amidates comprised the major proportion of bile acids, along with deoxycholic acid conjugates. Percent amidation with glycine was quite constant (60–70%), even for bile acids present in lesser amounts, such as lithocholic and sulfolithocholic acids. In contrast, sulfation was highly variable. A small, yet significant, portion of the detected peaks remain unidentified, pending further analysis by mass spectrometry. Conjugates of the 7β epimer of cholic acid, ursocholic acid, were present at levels less than 1% of the total bile acid content (data not shown), in agreement with published reports on biliary bile acid composition in gallstone patients (21).

DISCUSSION

The method reported here appears to be a satisfactory technique for measurement of the relative composition of the 12 major conjugated bile acids present in human bile. This method differs from other methanol–phosphate buffer systems by using a more acidic pH, and indeed, exact control of buffer pH was essential for high resolution. The present technique is similar to a number of other isocratic methods, but offers advantages of resolving the four lithocholyl conjugates, as well as the other eight conjugated bile acids generally present in human bile, in a relatively short period of time (40 min). As expected from theoretical considerations (20, 22), amidation, as well as sulfation, significantly decreased retention time.

The pH employed was chosen to obtain the desired retention time for glycine amidates and sulfolithocholyl glycine. Experiments with a non-ionizing bile acid (the serinol conjugate of chenodeoxycholic acid) indicated that changes in mobile phase pH between pH 5 and pH 6 had no influence on the properties of the stationary phase. Thus, the use of a more acidic mobile phase pH resulted in decreased ionization of glycine-amidated bile acids and a corresponding increase in their retention times.

Although absorbance for taurine conjugates slightly exceeded that of the glycine conjugates, the difference was small (< 5%). In our judgment, this difference is too small to invalidate the commonly employed assumption that a constant extinction coefficient can be used for calculation of the relative concentration of glycine- versus taurine-amidated bile acids in samples analyzed by ultraviolet absorption spectrophotometry. However, unconjugated bile acids exhibited extinction coefficients that differed markedly from their amidated counterparts. Maximal absorbance for amidated bile acid conjugates occurred at 200 nm. At this wavelength bile acid conjugates exhibited a 20- to 30-fold greater molar absorptivity than their corresponding unconjugated derivatives. Although the magnitude of difference was not foreseen (20), some discrepancy was anticipated, based on extinction coefficient differences between amide bond and carboxyl end group functionalities alone (23). Sulfolithocholylglycine and sulfolithocholyltaurine had greater absorbance at 200 nm, presumably due to significant absorbance of the S–O chromophore at this wavelength. While this work was in progress, a similar observation was made by Hedenborg and Norman (24).

The data in Table 4 are among the first complete analyses of major bile acids present in human bile. Data for this table were calculated using a constant extinction coefficient

TABLE 4. Conjugated bile acid composition of 14 healthy subjects[a]

Patient and File (age, sex)[b]	% Cholic	% Chenodeoxycholic	% Deoxycholic	% Ursodeoxycholic	% Lithocholic[c]	% Sulfolithocholic[d]	% Unknown[e]
CD-814	40.6	34.0	15.7	2.4	0.7	0.4	6.2
(14 F)	(84.0)	(84.9)	(86.7)	(62.8)	(85.2)	(47.2)	
CD-817	31.7	40.7	12.6	5.0	1.7	0.9	7.4
(10 M)	(48.2)	(42.5)	(51.5)	(52.5)	(62.2)	(51.2)	
CD-825	27.0	53.0	15.3	1.0	0.6	0.3	2.8
(35 M)	(74.6)	(72.5)	(67.6)	(63.5)	(57.3)	(53.7)	
CD-836	35.3	40.5	13.9	3.4	0.3	0.5	6.1
(20 M)	(72.2)	(68.1)	(58.6)	(75.6)	(69.1)	(55.0)	
CD-839	31.7	48.4	12.3	1.2	0.5	0.4	5.5
(12 F)	(76.8)	(73.9)	(74.7)	(81.0)	(57.1)	(52.8)	
CD-855	34.0	31.4	23.6	2.4	1.0	0.2	7.4
(54 M)	(73.7)	(73.1)	(80.6)	(25.1)	(67.2)	(92.1)	
CD-856	35.4	35.6	19.6	1.3	0.6	0.4	7.1
(34 F)	(64.5)	(62.4)	(63.2)	(61.5)	(52.9)	(57.2)	
CD-862	48.2	26.3	16.1	1.6	0.5	0.8	6.5
(19 M)	(78.2)	(73.6)	(76.2)	(73.1)	(73.5)	(41.1)	
CD-887	51.7	30.4	7.1	1.1	0.2	0.5	9.0
(18 M)	(73.2)	(70.8)	(43.3)	(67.6)	(77.1)	(89.3)	
CD-890	27.1	38.9	17.3	5.0	0.5	0.7	10.5
(18 M)	(58.5)	(55.9)	(53.7)	(68.9)	(0)	(41.8)	
GPAL-Co	48.1	41.7	5.8	0.9	0.1	0.02	3.4
(F)	(68.7)	(66.7)	(60.9)	(69.4)	(62.5)	(100)	
GPAL-Ho	23.6	26.8	41.2	0.4	1.3	0.6	6.1
(F)	(79.7)	(82.0)	(84.0)	(85.4)	(83.2)	(64.1)	
GPAL-Mi	33.4	49.3	11.5	2.80	0.8	0.6	1.6
(M)	(82.3)	(74.1)	(77.0)	(100)	(66.3)	(100)	
GPAL-Pe	43.3	42.1	9.4	1.3	0.4	1.3	2.2
(M)	(48.0)	(43.1)	(43.8)	(42.1)	(37.3)	(53.4)	
Mean ± SD	36.5 ± 8.3	38.5 ± 8.0	15.8 ± 8.4	2.1 ± 1.4	0.7 ± 0.4	0.6 ± 0.3	5.8 ± 2.5
	(70.2 ± 11.1)	(67.4 ± 12.2)	(65.8 ± 14.0)	(66.3 ± 17.7)	(60.8 ± 20.7)	(64.2 ± 20.6)	

[a]Values in parentheses are % amidation with glycine.
[b]Abbreviations: CD, cadaver donor; GPAL, gallbladder puncture at laparotomy (ages not available on these patients); M, male; F, female.
[c]Total lithocholic: % sulfated = 35.8 ± 14.0 (= % sulfolithocholic/% lithocholic + % sulfolithocholic).
[d]Lithocholyl glycine: % sulfated = 36.8 ± 16.4; lithocholic taurine: % sulfated = 34.2 ± 19.5.
[e]Unknown denotes peaks eluting after ursodeoxycholyltaurine, which did not correlate with known standards. Secondary ion mass spectrometry analyses indicate that some of the unknown peaks which elute prior to cholyltaurine are bile acid amidates.

for all bile acid amidates, except the sulfolithocholyl species. The biliary bile acid composition obtained by this HPLC system gave values in agreement with those reported in healthy subjects using gas-liquid chromatography (19, 25). The proportion of the three major bile acids (cholic, chenodeoxycholic, and deoxycholic) that are amidated with glycine is rather constant for a given subject. However, this proportion is somewhat more variable for ursodeoxycholic, lithocholic, and sulfolithocholic acid, which are present at much lower levels in bile. The results also indicate that glycine and taurine amidates of lithocholic acid are sulfated about equally, although the percentage sulfation varies widely. The proportion of all lithocholate species in bile that were sulfated also varied widely from sample to sample, but on the average about one-third of the lithocholate species were sulfated.

In bile, conjugated oxo-bile acids as well as epimers of the natural bile acids are also present in trace proportions (21, 26). We are currently synthesizing such standards in order to have a complete tabulation of retention times for all major and minor bile acids. However, definitive identification of minor peaks in chromatograms will require a complementary technique, such as coupled HPLC-mass spectrometry since it cannot be assumed that all peaks detected at 200 nm are bile acids.

The method reported here should be useful for defining changes in the proportion and chemical forms of lithocholic acid in animal model studies (cf. 27), as well as in patients ingesting chenodeoxycholic acid or ursodeoxycholic acid for gallstone dissolution (28, cf. 29).

This work was supported in part by NIH grants AM-21506, AM-32130, and AM-31461, as well as a grant-in-aid from the Falk Foundation. We are grateful to Dr. R. Thomas Holzbach for providing the gallbladder bile samples essential to this study and to Dr. Enrique Michelotti, Dr. Claudio Schteingart, as well as Ms. Huong-Thu Ton-Nu for the synthesis of many novel bile acid standards. Dr. Joseph Steinbach assisted in the statistical evaluation of chromatographic data, and Ms. Devorah Gurantz critiqued the manuscript. Ms. Vicky Huebner prepared and edited the manuscript.

Manuscript received 9 June 1986 and in revised form 22 December 1986.

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