Single-Step Analysis of Individual Conjugated Bile Acids in Human Bile Using ^{1}H NMR Spectroscopy

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ABSTRACT: ^{1}H and ^{13}C NMR spectra of intact human bile were assigned using one-dimensional (^{1}H and ^{13}C) and two-dimensional (^{1}H-^{1}H and ^{1}H-^{13}C) experiments. Individual conjugated bile acids—glycocholic acid, glycodeoxycholic acid, glycochenodeoxycholic acid, taurocholic acid, taurodeoxycholic acid, and taurochenodeoxycholic acid—were identified. The bile acids were quantified accurately and individually in a single step by using distinct and characteristic amide signals. Making use of ^{13}C NMR, the study also suggests a way to analyze unconjugated bile acids separately, if present. Chemical shift assignments and rapid single-step analysis of individual conjugated bile acids from intact bile presented herein may have immense utility in the study of bile acid metabolism and deeper understanding of hepatobiliary diseases.

Paper no. L9954 in *Lipids 41*, 591–603 (June 2006).

Bile acids have been implicated in the digestion and absorption of fats and in several other important physiological functions, including cholesterol homeostasis and bile flow, help in the excretion or recirculation of drugs, vitamins, and endogenous and exogenous toxins (1,2). Recently, new biological properties of bile acids as signaling molecules and as regulators of their own synthesis have also been reported (3). Abnormal composition of the conjugated bile salts in bile may lead to various hepatobiliary and gastrointestinal diseases (4–6). Dietary fat malabsorption and gallstone formation are the common causes of abnormal bile composition in the bile (4,6). Conditions that prevent the synthesis of bile acids or their excretion cause cholestasis or impaired bile flow. Several human diseases arising from altered levels of bile acids underscore the essential role of bile acids in metabolism (7). Bile acids are considered to be involved in the development of biliary tract carcinoma, although the underlying mechanisms are yet to be established (8). They have long been implicated in the etiology of colorectal cancer, but epidemiologic evidence remains elusive (9). Deoxycholic acid is reported to cause higher risk for pressure ulcers in elderly bedridden patients (10). It has been suggested that the bile acids in the feces act as promoters of colon cancer, and among the bile acids, deoxycholic acid is said to have strong influence. The ratio of deoxycholic acid to cholic acid in feces is reported to have a diagnostic significance in colon cancer (11). Several reports indicate that bile acids cause DNA damage and act as carcinogens (12). Aggravation of taurochenodeoxycholic acid induced liver damage, and hepatocyte apoptosis is reported due to the inhibition of phosphatidylinositol 3-kinase (13). From the experiments with immortalized mouse cholangiocytes using several bile acids, carcinogenic potential of glycochenodeoxycholic acid in biliary tract has been demonstrated (8). Bile acid synthetic defects that are uncommon genetic disorders were reported to be responsible for approximately 2% of persistent cholestasis in infants. These defects lead to liver diseases that may be life threatening but are treatable by replacement of deficient primary bile acids (14).

In view of the multitude of useful as well as deleterious roles played by the bile acids, measurement of their conjugation pattern is necessary for understanding the pathophysiology of these diseases. Several methods for bile acids analysis have been proposed (15–25). Of these, liquid chromatography–mass spectrometry (22–25) and liquid chromatography–electrospray tandem mass spectrometry (2) methods are reported to be useful for routine qualitative/quantitative analysis. These methods are not straightforward since they involve tedious steps such as extraction, hydrolysis, derivatization, and/or purification before analyses. A simple and convenient method for determining identity and quantity of the individual bile acids has not been reported. Nuclear magnetic resonance (NMR) spectroscopy offers immense potential in this direction. However, major difficulties of NMR for such applications arise from severe overlap of signals of a large number of bile components. As a part of our efforts to unravel the signals from complex bile spectra for single-step analysis of several bile components, we reported ^{1}H and ^{13}C chemical shifts, under physiological conditions, for several conjugated and unconjugated bile acids that are supposedly found in bile/gastrointestinal fluids (26) and, subsequently, identified conjugated bile acid marker signals in human bile (27). In continuation, we present here assignments, identification, and

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Abbreviations: DQF-COSY, double quantum filtered correlated spectroscopy; GCA, glycocholic acid; GCDCA, glycochenodeoxycholic acid; GDCA, glycodeoxycholic acid; HMBC, heteronuclear multiple bond correlation; HSQC, heteronuclear single quantum correlation; NOE, nuclear Overhauser enhancement; TCA, taurocholic acid; TCDCA, taurochenodeoxycholic acid; TDCA, taurodeoxycholic acid; TOCSY, total correlated spectroscopy; TSP, sodium salt of trimethylsilylpropionic acid-d_4.

single-step analysis of all major conjugated bile acids in human bile individually using simple ^{1}H NMR experiments.

EXPERIMENTAL PROCEDURE

Chemicals and human bile specimens. Sodium salts of glycocholic acid (GCA), glycodeoxycholic acid (GDCA), glycochenodeoxycholic acid (GCDCA), taurocholic acid (TCA), taurodeoxycholic acid (TDCA), taurochenodeoxycholic acid (TCDCA), deuterium oxide (D_2O), and sodium salt of trimethylsilylpropionic acid-d_4 (TSP) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Discarded specimens of human gallbladder bile from patients undergoing cholecystectomy (laparoscopic or open) for symptomatic gallstone disease were used for the study.

NMR experiments. All ^{1}H and ^{13}C NMR experiments of bile specimens were performed on Bruker Biospin Avance 400-, 700-, and/or 800-MHz NMR spectrometers using a 5-mm broadband or broadband inverse probe head (400 MHz) or cryoprobe heads (700 and 800 MHz), all equipped with shielded *z*-gradient accessories. Unless otherwise mentioned, all bile samples were prepared by diluting gallbladder bile by a factor of 10 using distilled water and NMR experiments were performed by using similar parameters as mentioned in our previous article in this issue.

One-dimensional (1D) ^{1}H NMR experiments. The 1D ^{1}H NMR experiments were performed with and/or without homonuclear decoupling (of glycine and taurine CH_2 protons of conjugated bile acids) by using a single-pulse sequence with suppression of water signal by presaturation during relaxation delay of 6 s. For homonuclear decoupled spectra, Gausssian multiplication window function was used with line and Gaussian broadening of –1.0 and 0.1 Hz, respectively.

1D ^{13}C NMR experiments. The 1D ^{13}C spectra were obtained by using one-pulse sequence, spin echo Fourier transform (SEFT), and quaternary carbon detection (QCD) (28) sequences with proton decoupling using WALTZ-16 composite pulse sequence. Typical parameters used were as follows: spectral width: 24,000, 42,000, and 48,000 Hz (at 400-, 700-, and 800-MHz spectrometers, respectively); data points: 32 K; acquisition time: 0.68, 0.39, and 0.34 s (at 400, 700, and 800 MHz, respectively); recycle delay: 3 s; number of transients: 2,000; spectrum size: 32 K points; and line broadening for exponential data multiplication: 3 Hz.

Assignment of ^{1}H and ^{13}C NMR spectra. Assignments of ^{1}H and ^{13}C signals of bile spectra were made by using the combination of 1D and 2D spectra. The signals in the 2D spectra were broad and weak due to the short transverse relaxation times of the signals arising from the aggregation of major bile components. The library of ^{1}H and ^{13}C chemical shifts (26), partial assignments on bile in aqueous media (27), and the assignments of bile dispersed in a polar organic solvent aided identification of signals in the complex spectra and distinguishing bile acids signals from lipids and cholesterol

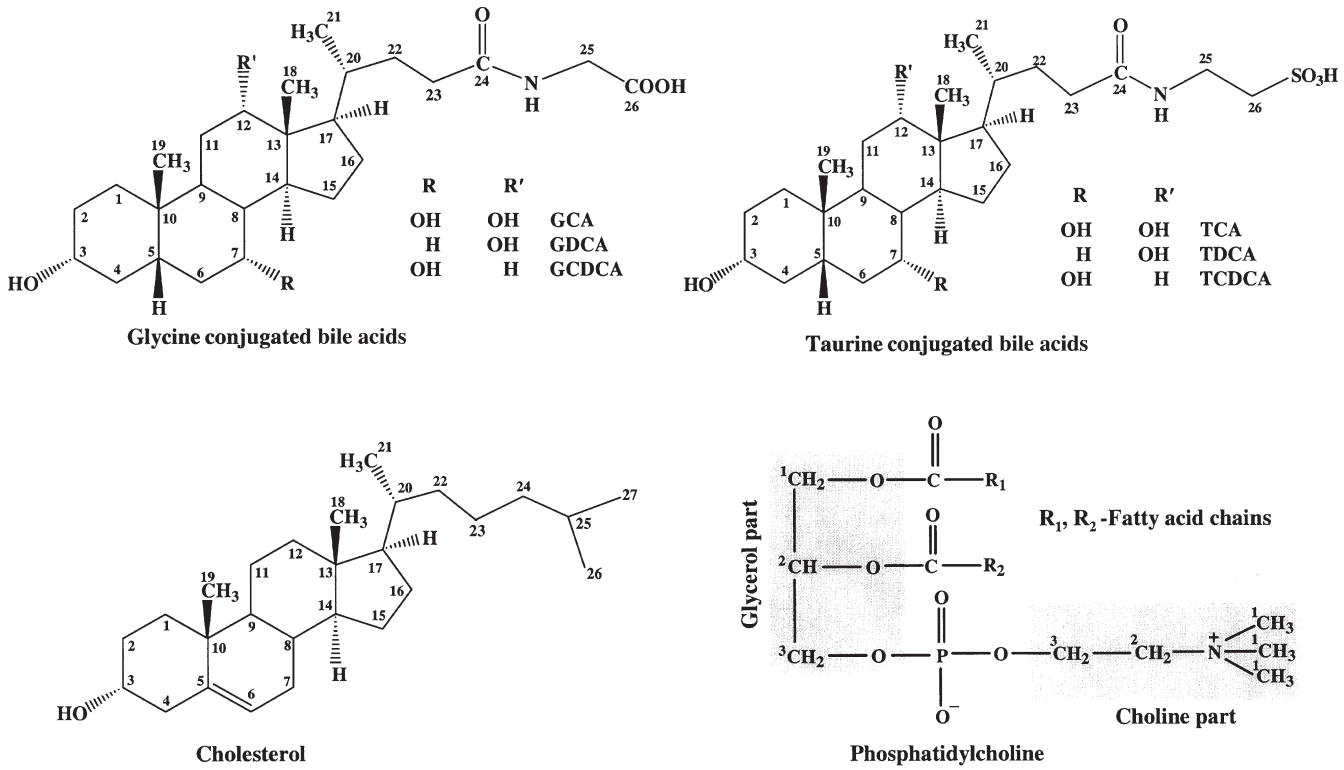

FIG. 1. Structures and numbering of protons and carbons of bile components identified in bile. The numbering of the proton(s) is the same as that of the corresponding directly attached carbons. The orientations of some substitutions in cholesterol and bile acids are marked with respect to the plane of the ring (cross-hatched) wedge: α substitution; bold wedge: β substitution).

signals (L9947 i.e. 29). Assignment from the combination of double quantum filtered correlated spectroscopy (DQF-COSY), total correlated spectroscopy (TOCSY), and multiplicity edited heteronuclear single quantum correlation; (HSQC) and heteronuclear multiple bond correlation (HMBC) spectra at 700 and 800 MHz confirmed assignments made at 400 MHz.

Identification of individual conjugated bile acids. In one-dimensional ^{1}H spectra, broad amide signals from conjugated bile acids, which invariably appear in the isolated region

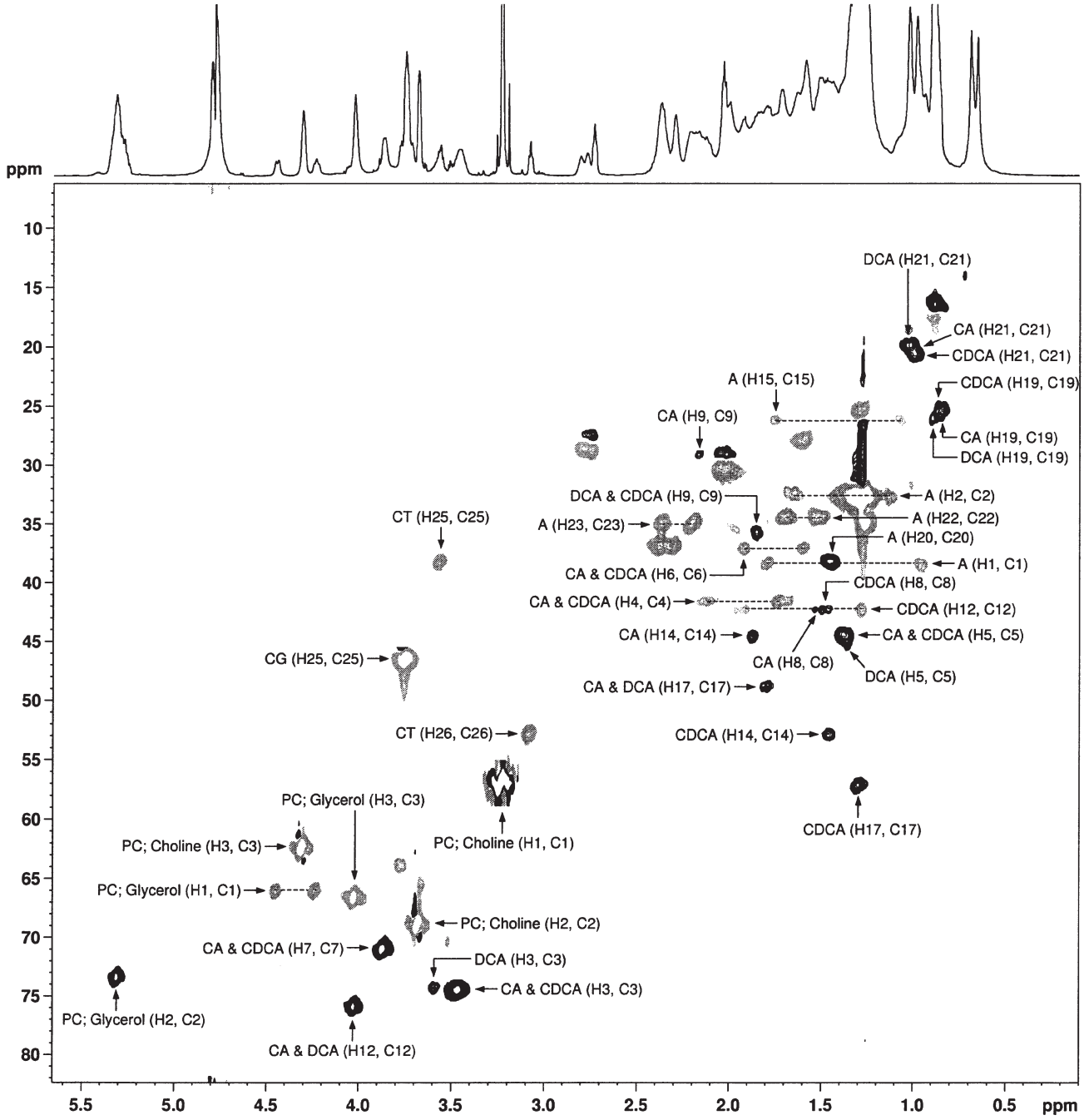

FIG. 2. Sensitivity-enhanced multiplicity edited ^{1}H-^{13}C HSQC spectrum of typical bile (50 µL diluted to 500 µL with water) obtained on a Bruker Biospin Avance 700-MHz spectrometer, along with the assignments of the cross peaks of protons and the attached carbons. For clarity, CH and CH_3 cross peaks are shown as dark continuous contours whereas CH_2 cross peaks are shown in light discontinuous contours. Wherever the chemical shifts of methylene protons (CH_2) are different (nondegenerate), two distinct cross peaks were observed corresponding to H_α and H_β protons (each pair of cross peaks is joined by dashed lines). PC, phosphatidylcholine; CT, conjugated taurine; CG, conjugated glycine; CA, glycine/taurine-conjugated cholic acid; DCA, glycine/taurine-conjugated deoxycholic acid; CDCA, glycine/taurine-conjugated chenodeoxycholic acid. A: All glycine/taurine conjugated bile acids. The numbering of protons/carbons is as shown for the structures in Figure 1. A trace of 1D ^{1}H spectrum is shown on the 2D spectrum.

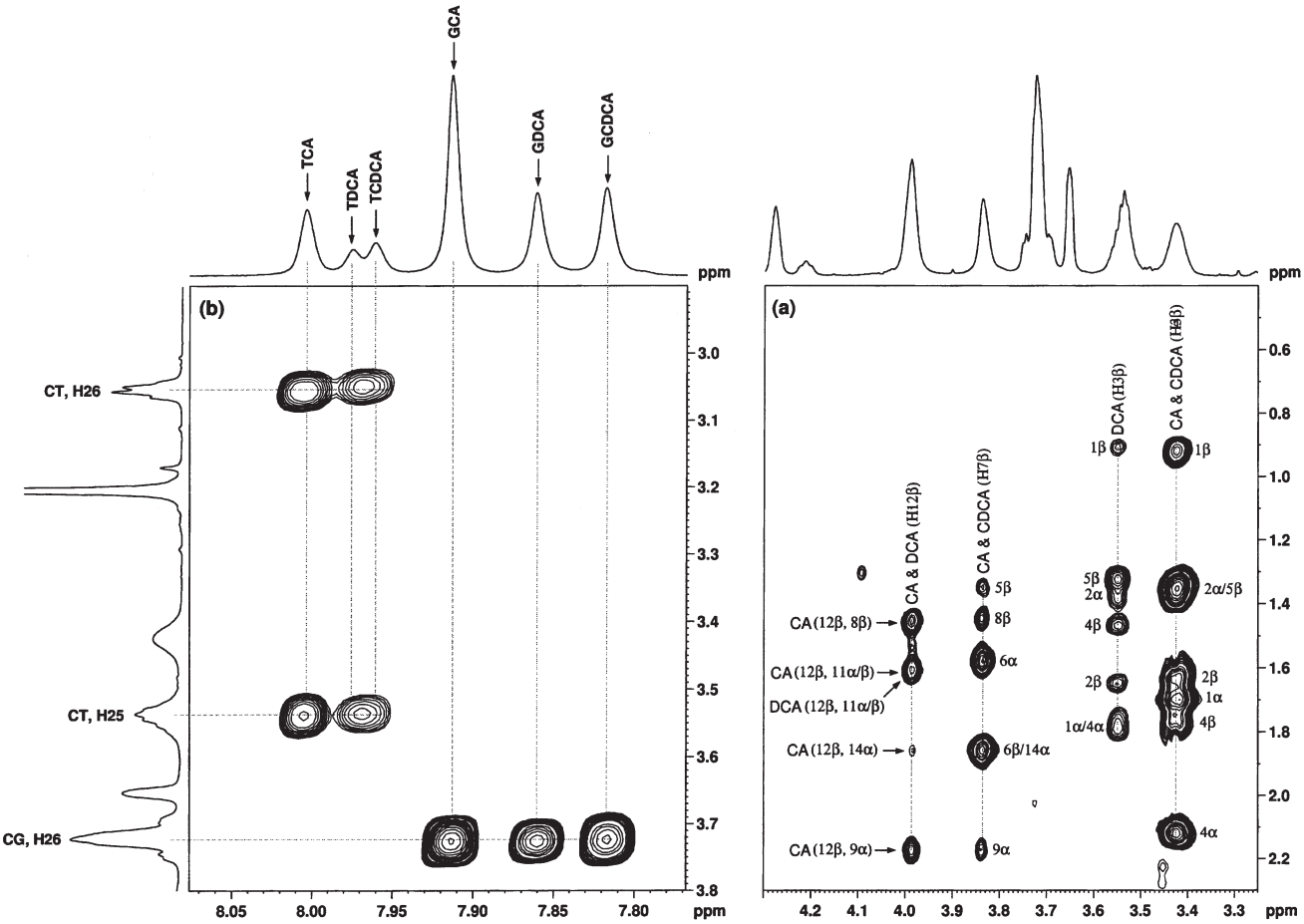

FIG. 3. Parts of TOCSY spectrum of typical bile (50 µL diluted to 500 µL with water) obtained on a Bruker Biospin AVANCE 800-MHz spectrometer. CT, conjugated taurine; CG, conjugated glycine; GCA, glycocholic acid; GDCA, glycodeoxycholic acid; GCDCA, glycochenodeoxycholic acid; TCA, taurocholic acid; TDCA, taurodeoxycholic acid; TCDCA, taurochenodeoxycholic acid; CA, glycine/taurine-conjugated cholic acid; DCA, glycine/taurine-conjugated deoxycholic acid; CDCA, glycine/taurine-conjugated chenodeoxy cholic acid. The numbering of protons is as shown in Figure 1. A trace of 1D ^{1}H spectrum is shown on the 2D spectrum.

(7.77 to 8.03 ppm) of the bile spectra, were nearly overlapping with each other. Experiments were performed to improve the resolution so as to identify the individual conjugated bile acids. The one-dimensional ^{1}H experiments were performed by using homonuclear decoupling sequence with decoupler offset set at 3.59 ppm, which is in between the chemical shifts of methylene protons (25-CH_2) of conjugated glycine (3.75 ppm) and conjugated taurine (3.56 ppm). From the analysis of the combination of spectra from 1D decoupled DQF-COSY and TOCSY experiments, two groups of amide signals were identified. Each group had three signals, one group corresponded to glycine-conjugated bile acids, and the other corresponded to taurine-conjugated bile acids. By comparison of these resolved amide and other ^{1}H and ^{13}C signals of bile acids with the library of chemical shifts of standard conjugated bile acids (26), the three amide signals of glycine were assigned to GCDCA (7.84 ppm), GDCA (7.88 ppm), and GCA (7.93 ppm) and the three amide signals of taurine-conjugated bile acids were assigned to TCDCA (7.98 ppm), TDCA (8.00 ppm), and TCA (8.02 ppm). To further confirm the assignments of the amide signals, ^{1}H spectra of bile were recorded separately at 800 MHz with simultaneously decoupling both 25-CH_2 signals of conjugated glycine and taurine, before and after the addition of each standard conjugated bile acid.

Bile acids recovery test. After identifying the six bile acids individually (GCA, GDCA, GCDCA, TCA, TDCA, and TCDCA) through their characteristic amide marker signals, NMR experiments were performed to test the accuracy and precision of quantitative analysis for each bile acid. The pH of a bile sample (4.0 mL diluted to 9.0 mL) was brought down (27) in the range 6 ± 0.5 using 6 N HCl, and, using this solution, six duplicate set of solutions (each 500 µL) were prepared in 5-mm NMR tubes. A reusable coaxial capillary containing quantitative reference, TSP (dissolved in 35 µL of D_2O), was inserted into the tube and ^{1}H spectra were recorded at 800 MHz by decoupling 25-CH_2 protons of conjugated glycine and taurine. The quantity of TSP in the capillary was

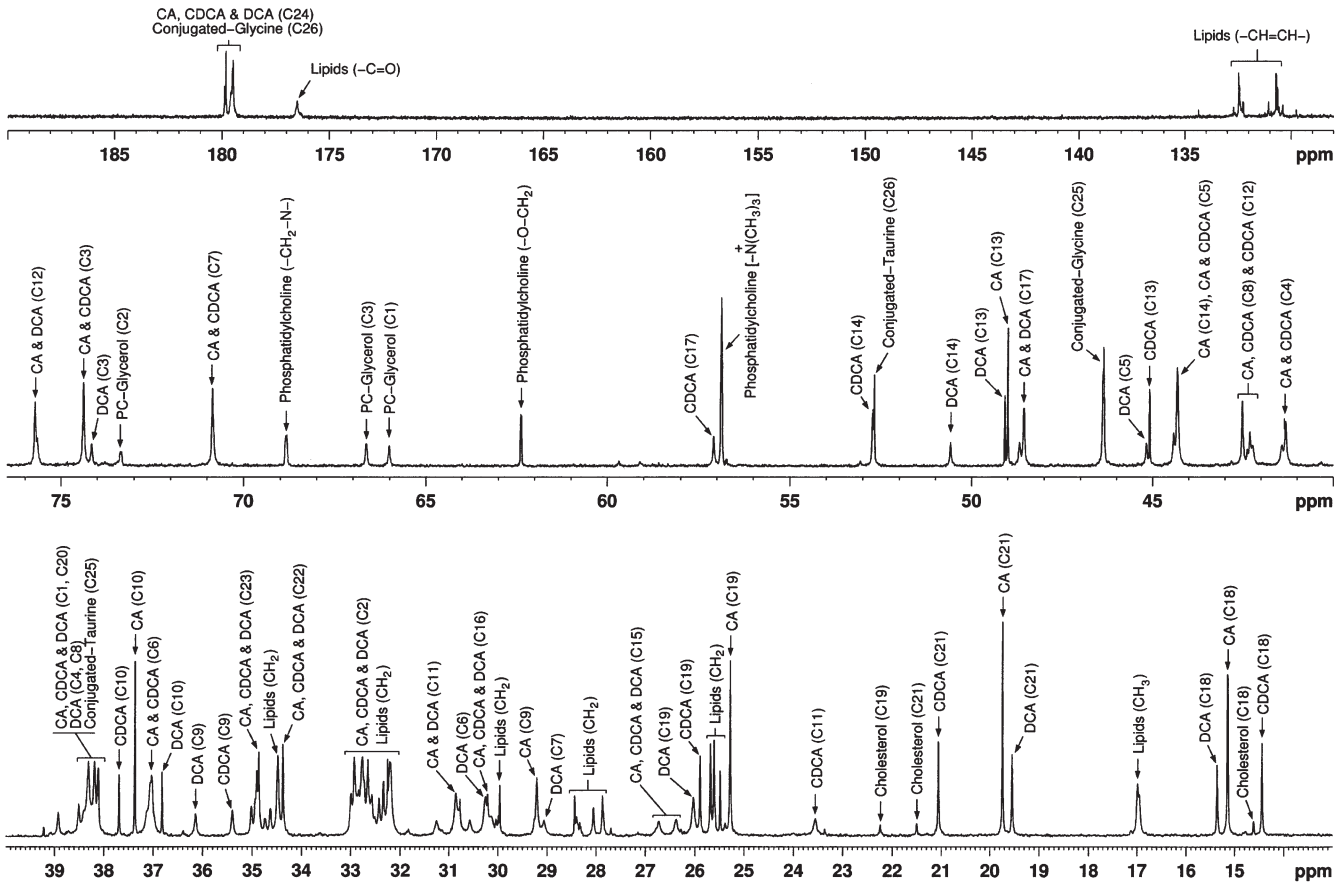

FIG. 4. Parts of ^{13}C spectrum of typical bile obtained on a Bruker Biospin Avance 800-MHz spectrometer with the assignments of most of the signals. PC, phosphatidylcholine; CA, glycine/taurine-conjugated cholic acid; DCA, glycine/taurine-conjugated deoxycholic acid; CDCA, glycine/taurine-conjugated chenodeoxycholic acid. The numbering of protons/carbons is as shown for the structures in Figure 1.

precalibrated by ^1H NMR experiment against a signal from a known concentration of a standard sample (30). Integral areas of the amide signals of all the six conjugated bile acids were determined relative to TSP by deconvolution of the signals using Xwinnmr software version 3.5 (Bruker Biospin Fällanden, Switzerland). Subsequently, known quantities of standard bile acids, GCA (1.04 mg), GDCA (0.84 mg), GCDCA (0.87 mg), TCA (0.87 mg), TDCA (0.84 mg), or TCDCA (0.75 mg), were added to the diluted bile solutions taken in separate sets of NMR tubes. ^1H NMR spectra were recorded again, under identical conditions, and the integrals of the amide signals were determined from the resultant spectra relative to the TSP signal. The quantity of each standard conjugated bile acid recovered through NMR was calculated from the corresponding amide integral (difference of amide integral before and after the addition of bile acid) and compared with the actual quantity of the added bile acid. In each case, deconvolution of the amide signals was repeated five times independently and the mean quantity of recovered bile acid along with the standard deviation was determined.

Quantitative estimation of bile acids. To demonstrate single-step analysis of individual conjugated bile acids, NMR experiments were performed on gallbladder bile specimens from 16 patients. The pH of each bile specimen was brought down in the range 6 ± 0.5 by addition of 1–2 μL of 6 N HCl. A reusable coaxial capillary tube containing TSP in D_2O was inserted into the NMR tube before recording the spectra. ^1H spectra at 800 MHz were obtained with decoupling 25-CH_2 protons of conjugated glycine and taurine. Integral areas of all the six amide signals were determined relative to TSP by deconvolution. Subsequently, quantities of all the six bile acids were determined, individually, using a computer program that takes into account integral area and molecular weight of each conjugated bile acid and the reference (TSP). In each case, deconvolution of the amide signals was repeated five times independently and the mean quantities along with the standard deviation were determined for each bile acid.

To determine whether decoupling of glycine and taurine methylene protons (25-CH_2) would alter the intensities of amide protons due to nuclear Overhauser enhancement (NOE) (if any), one-dimensional ^1H NMR experiments were performed on one of the bile specimens by suppressing the water signal using presaturation with 2-s acquisition time (during which homonuclear decoupling was also performed) and 45° excitation pulse. Spectra were recorded with and without decoupling of methylene protons by varying the re-

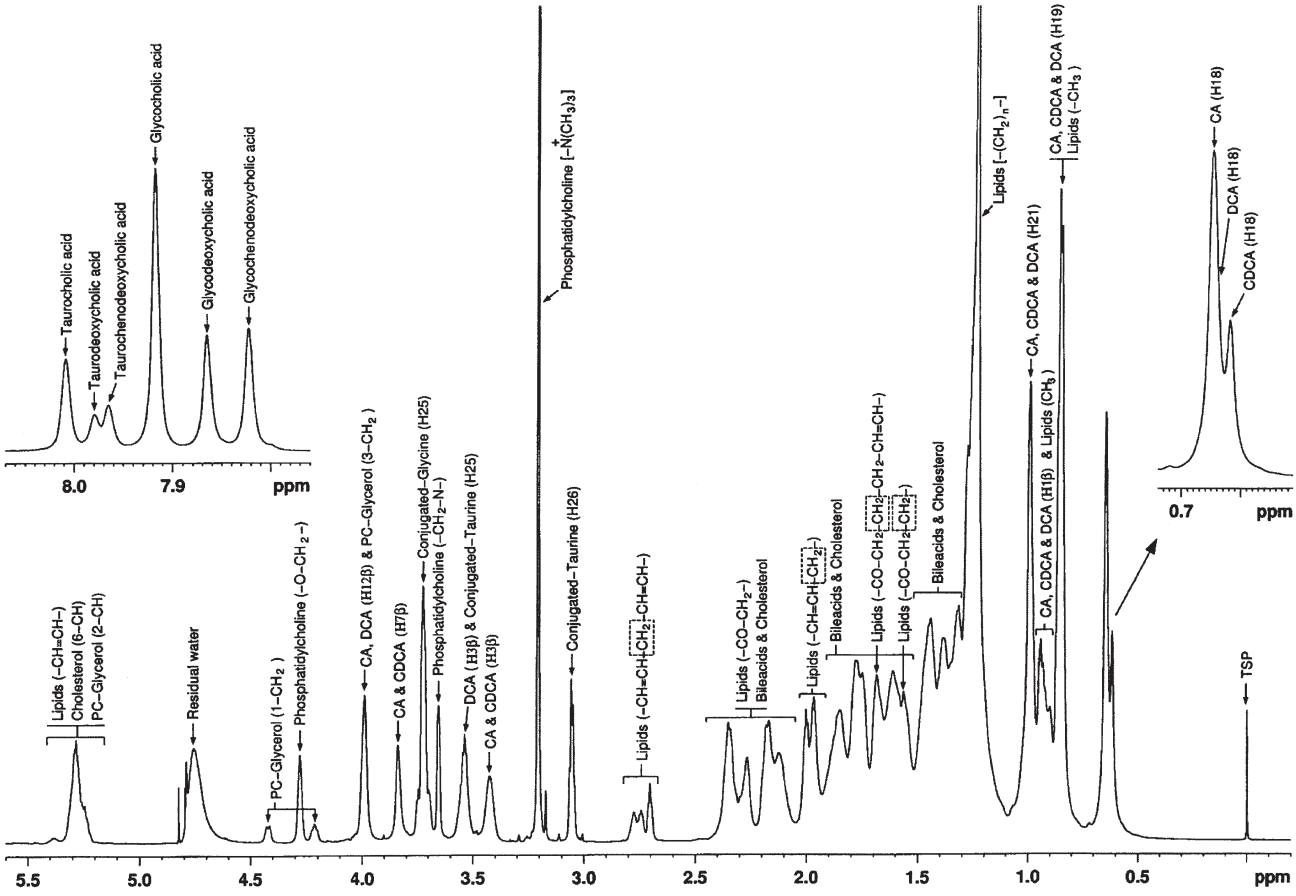

FIG. 5. Parts of ^{1}H spectrum of typical bile obtained on a Bruker Biospin Avance 800-MHz spectrometer with the assignments of most of the signals. PC, phosphatidylcholine; CA, glycine/taurine-conjugated cholic acid; DCA, glycine/taurine-conjugated deoxycholic acid; CDCA, glycine/taurine-conjugated chenodeoxycholic acid. The numbering of protons/carbons is as shown for the structures in Figure 1. Inset shows six amide signals, each corresponding to conjugated bile acid as indicated, obtained after decoupling 25-CH_2 protons of conjugated glycine and taurine.

cycle delay from 1 to 10 s in steps of 1 s. The integrals of all the amide signals at different recycle delay were obtained and compared.

RESULTS

Figure 1 shows the structures of bile components that are identified in intact gallbladder bile. Bile specimens from different patients were qualitatively consistent in terms of the signal pattern, but greatly varied in intensities of the signals from individual bile components. The cross peaks, particularly in heteronuclear 2D spectra, were weak in intensity due to short transverse relaxation times of the signals arising from aggregation of the bile components under natural conditions. Signals from cholesterol, lipids, and bile acids were identified from the analysis of two-dimensional spectra from DQF-COSY, TOCSY, multiplicity edited HSQC and HMBC experiments at 400, 700, and 800 MHz. Due to the structural similarity of various conjugated bile acids, most of the ^{1}H and ^{13}C signals of bile acids overlap, as shown in the typical edited HSQC and TOCSY spectra in Figures 2 and 3, respectively. Distinct chemical shifts of carbons C9, C10, C13, C18, C19, C21 of CA; C3, C5, C9, C10, C13, C14, C18, C19, C21 of DCA; and C9, C10, C11, C13, C17, C18, C19, C21 of CDCA, as shown in Figure 4, arising from the substitution effect (26) at position 7 and/or 12 (Fig. 1) enabled clear identification of CA, DCA, and CDCA moieties of the conjugated bile acids. Influence of glycine or taurine conjugation on almost all $^{1}H/^{13}C$ chemical shifts of bile acid moieties was minimal except in the vicinity of conjugation (Figs. 4 and 5). However, amide signals were distinctly observed for each conjugated bile acid. These amide signals appear very close to each other (7.77–8.03 ppm) at low magnetic fields (400 MHz) (Fig. 6a). However, at higher magnetic fields (700/800 MHz), four of six signals were resolved (Fig. 6d). Each amide signal is a triplet arising from indirect spin-spin coupling between amide (NH) and 25-CH_2 protons of conjugated glycine or taurine (Fig. 1). Upon removal of indirect spin-spin couplings by simultaneously decoupling the methylene protons of conjugated glycine (3.75 ppm) and conjugated taurine (3.56 ppm), amide signals further get resolved and all six bile acids signals were separated at 700 and 800 MHz (Fig. 6e). At 400 MHz, amide signals of TCDCA and TDCA were still overlapping (Fig. 6b).

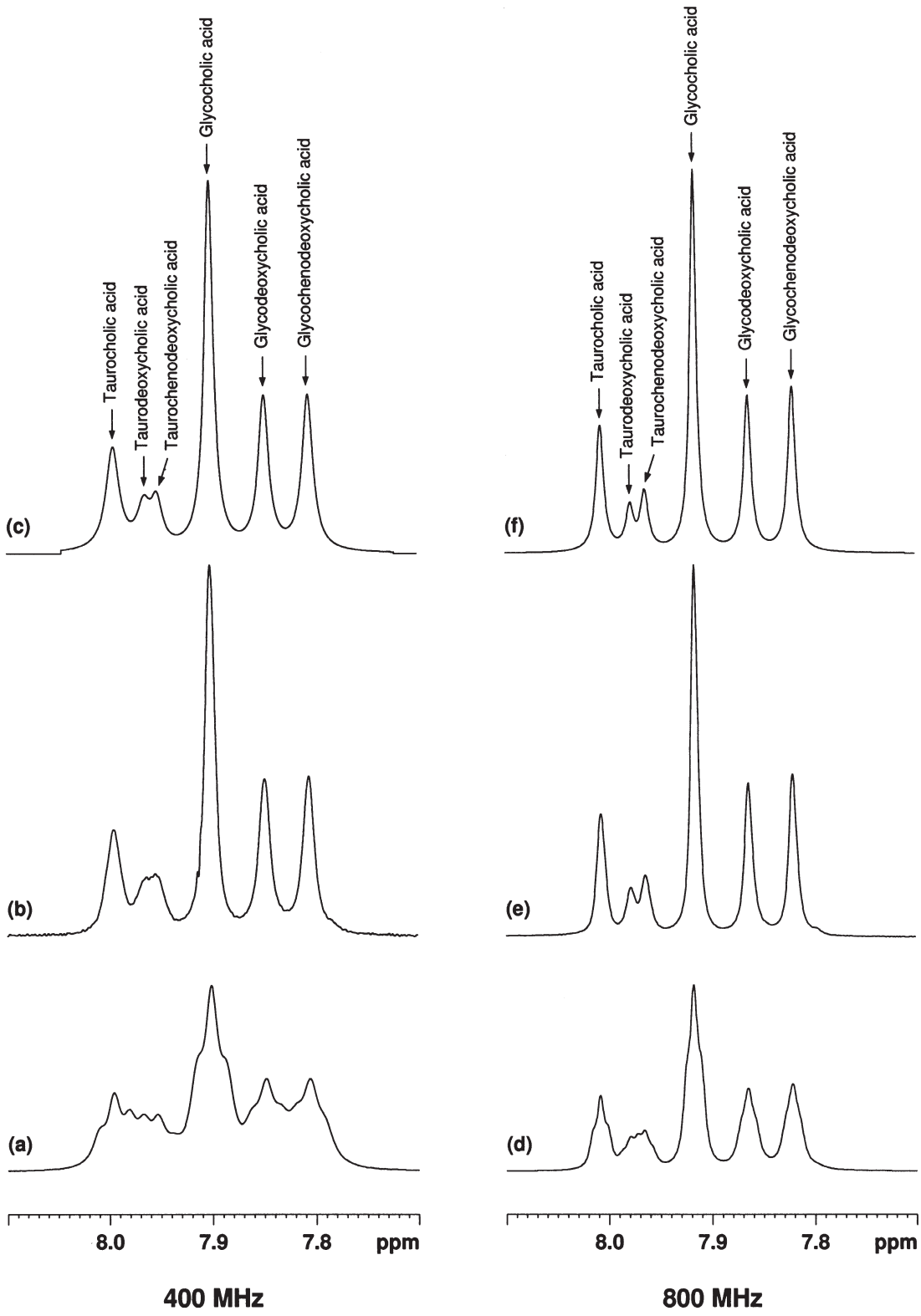

FIG. 6. Amide regions of bile spectra showing characteristic signals of glycochenodeoxycholic acid (GCDCA), glycodeoxycholic acid (GDCA), glycocholic acid (GCA), taurochenodeoxycholic acid (TCDCA), taurodeoxycholic acid (TDCA), and taurocholic acid (TCA). The spectra were obtained at 400 and 800 MHz without (a and d) and with (b and e) decoupling of 25-CH_2 protons of conjugated glycine and taurine. At 400 MHz, two of the amide signals (taurochenodeoxycholic acid and taurodeoxycholic acid) are still overlapping (b). All six amide signals are distinctly visible in the spectrum recorded at 800 MHz (d). The regions shown in c and f are the deconvoluted (Lorentzian) decoupled spectra b and e, respectively.

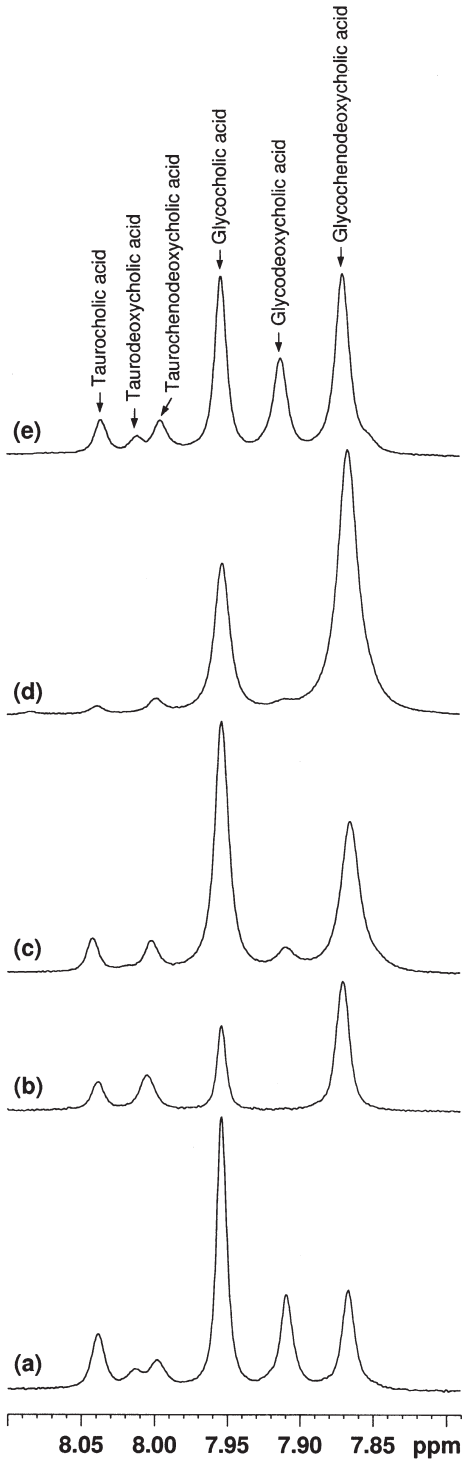

FIG. 7. Parts of 800-MHz ^{1}H NMR spectra of gallbladder bile (50 µL diluted to 500 µL with water) from five typical patients with gallbladder disease highlighting the variation of the relative intensities of the bile acids within as well as between the bile specimens (see also Table 4). All the spectra were plotted under identical conditions for direct comparison of the relative quantities of various bile acids. The bile samples were from patients (a) with chronic cholecystitis (CC); (b) with xanthogranulomatous cholecystitis (XGC); (c) normal (with no diagnosed gallstone disease); (d) with xanthogranulomatous cholecystitis (XGC); and (e) with chronic cholecystitis (CC).

Experiments performed before and after the addition of authentic conjugated bile acids confirmed the assignment of individual conjugated bile acids in bile. The ^{1}H and ^{13}C chemical shifts for the glycine- and taurine-conjugated bile acids in bile measured using 1D and 2D spectra are shown in Tables 1 and 2.

A typical best fit spectrum obtained after deconvolution of the decoupled amide region of a bile spectrum (Fig. 6e) is shown in Figure 6f. Integrals of individual amide signals in the best fit spectra were used for determining absolute quantities of individual conjugated bile acids. As shown in Table 3, the bile acids thus determined showed excellent agreement between the quantity of bile acid added and that recovered using ^{1}H NMR. Rapid and single-step analyses of individual conjugated bile acids were demonstrated on 16 human gallbladder bile specimens and the quantities of six bile acids thus determined are shown in Table 4. Significant differences in the quantities of bile acids within the bile and among various bile specimens were observed. Typical spectra for five gallbladder bile specimens, highlighting the variation in the absolute and relative quantities of individual bile acids in each bile specimen, are shown in Figure 7. Experiments performed to determine if there is any NOE buildup on the amide protons showed no change in integral of the amide protons in any of the spectra obtained with or without decoupling of the methylene protons of conjugated glycine and taurine.

DISCUSSION

Although NMR spectroscopy has tremendous advantages over conventional analytical methods, until today, its applications to human bile has been limited due to the complexity of the spectra arising from aggregation of major bile components such as cholesterol, phospholipids, and bile acids, which lead to broad signals. Several bile acids that are reported to be a part of major components of bile have very close structural resemblance, resulting in the severe overlap of most of the bile acid signals with each other, thus making the spectra further complicated to analyze. In the present study, assignment of ^{1}H and ^{13}C spectra of intact bile was greatly aided by the following sequential studies, which were aimed at devising a simple single-step method for simultaneously analyzing a large number of bile components that are detected by NMR: (a) establishment of the library of ^{1}H and ^{13}C chemical shifts by using various 1D and 2D experiments for several conjugated and unconjugated bile acids which are thought to be the components of human bile (26); (b) identification of bile acids and their distinct marker signals in human bile by using 1D and 2D experiments with the knowledge of the accurate chemical shifts of standard bile acids; in this study, amide signals were identified in human bile as conjugated bile acids markers (27); (c) assignment of ^{1}H and ^{13}C spectra of intact bile by using NMR experiments at 400 and 700 MHz after dispersing the bile in a polar organic solvent, which drastically reduced the signal line width due to increased transverse relaxation times and improved the spectral resolution (29).

TABLE 1
^{1}H and ^{13}C Chemical Shifts of Glycocholic Acid, Glycochenodeoxycholic Acid, and Glycodeoxycholic Acid from Intact Human Gallbladder Bile[a]

		Glycocholic acid			Glycochenodeoxycholic acid			Glycodeoxycholic acid		
			Proton			Proton			Proton	
No.	Type	Carbon	α	β	Carbon	α	β	Carbon	α	β
1	CH_2	38.30	1.80	0.96	38.30	1.80	0.96	38.30	1.80	0.96
		(37.66)	(1.80)	(1.00)	(38.10)	(1.83)	(0.96)	(38.17)	(1.81)	(0.98)
2	CH_2	32.24	1.38	1.65	32.24	1.38	1.65	32.24	1.40	1.65
		(32.06)	(1.39)	(1.66)	(32.49)	(1.38)	(1.64)	(32.02)	(1.42)	(1.69)
3	CH	74.38	—	3.46	74.38	—	3.46	74.15	—	3.58
		(74.43)		(3.49)	(74.41)		(3.47)	(74.21)		(3.63)
4	CH_2	41.31	2.12	1.70	41.31	2.12	1.70	38.18	1.81	1.53
		(41.20)	(2.07)	(1.70)	(41.27)	(2.08)	(1.69)	(37.98)	(1.81)	(1.54)
5	CH	44.31	—-	1.38	44.39	—	1.38	45.17	—	1.36
		(43.90)		(1.44)	(44.12)		(1.40)	(44.93)		(1.44)
6	CH_2	37.03	1.60	1.91	37.03	1.60	1.91	30.25	1.35	1.84
		(36.68)	(1.55)	(1.97)	(36.98)	(1.56)	(1.96)	(30.00)	(1.34)	(1.85)
7	CH	70.85	—	3.87	70.85	—	3.87	29.06	1.39	1.23
		(71.13)		(3.89)	(70.99)		(3.87)	(28.99)	(1.45)	(1.23)
8	CH	42.51	—	1.51	42.51	—	1.43	38.52	—	1.37
		(42.06)		(1.59)	(42.13)		(1.45)	(38.87)		(1.44)
9	CH	29.21	2.16	—	35.40	1.84	—	36.14	1.84	—
		(29.18)	(2.12)		(35.32)	(1.81)		(36.22)	(1.85)	
10	C	37.36	—	—	37.69	—	—	36.82	—	—
		(37.18)			(37.63)			(36.77)		
11	CH_2	30.85	1.58	1.58	23.55	1.52	1.27	30.85	1.58	1.58
		(30.54)	(1.59)	(1.59)	(23.46)	(1.50)	(1.26)	(31.14)	(1.55)	(1.55)
12	CH	75.72	—	4.03	42.30	1.26	1.97	75.72	—	4.03
		(75.94)		(4.05)	(42.18)	(1.25)	(1.98)	(75.85)		(4.05)
13	C	48.99	—	—	45.08	—		49.08	—	—
		(48.95)			(45.14)			(49.06)		
14	CH	44.31	1.87	—	52.72	1.45	—	50.58	1.58	—
		(44.36)	(1.83)		(52.76)	(1.42)		(50.60)	(1.61)	
15	CH_2	26.38	1.74	1.07	26.38	1.74	1.07	26.72	1.74	1.07
		(25.73)	(1.71)	(1.12)	(26.30)	(1.71)	(1.07)	(26.60)	(1.67)	(1.07)
16	CH_2	30.20	2.01	1.35	30.20	2.01	1.35	30.20	2.01	1.35
		(30.04)	(1.96)	(1.29)	(30.71)	(1.96)	(1.27)	(30.42)	(1.95)	(1.27)
17	CH	48.56	1.78	—	57.09	1.29	—	48.56	1.78	—
		(49.21)	(1.74)		(57.68)	(1.25)		(49.13)	(1.77)	
18	CH_3	15.14	0.69		14.44	0.65		15.36	0.68	
		(14.91)	(0.71)		(14.45)	(0.67)		(15.42)	(0.72)	
19	CH_3	25.28	0.84		25.90	0.87		26.04	0.89	
		(24.90)	(0.91)		(25.57)	(0.92)		(25.83)	(0.94)	
20	CH	38.29	1.45		38.29	1.45		38.29	1.45	
		(37.95)	(1.44)		(38.16)	(1.46)		(38.36)	(1.44)	
21	CH_3	19.73	1.02		21.05	0.98		19.54	1.02	
		(19.50)	(1.00)		(20.91)	(0.97)		(19.47)	(1.02)	
22	CH_2	34.38	1.49, 1.69		34.38	1.49, 1.69		34.38	1.49, 1.69	
		(34.37)	(1.43, 1.77)		(34.42)	(1.44, 1.72)		(34.37)	(1.46, 1.74)	
23	CH_2	34.86	2.37, 2.21		34.86	2.37, 2.21		34.86	2.37, 2.21	
		(35.29)	(2.36, 2.23)		(35.18)	(2.34, 2.17)		(35.33)	(2.39, 2.21)	
24	C	179.81	—		179.48	—		179.48	—	
		(180.02)			(179.64)			(179.67)		
25	CH_2	46.36	3.75		46.36	3.75		46.36	3.75	
		(46.13)	(3.74)		(46.20)	(3.73)		(46.19)	(3.75)	
26	C	179.59	—		179.59	—		179.59	—	
		(179.54)			(179.43)			(179.44)		
27	NH	—	7.93		—	7.84		—	7.88	
			(7.86)			(7.76)			(7.83)	

[a]The chemical shift values of the standard bile acids (26) are given in parentheses. Numbering for carbons/protons is as given in Figure 1.

All amide signals do not get resolved even at higher magnetic fields (Fig. 6d). This necessitated performing experiments with decoupling of methylene protons (25-CH_2) of glycine and taurine to obtain resolved signals. At lower magnetic fields (400 MHz), TDCA and TCDCA signals still overlap even after decoupling (Fig. 6b), whereas at higher mag-

TABLE 2
^{1}H and ^{13}C Chemical Shifts of Taurocholic Acid, Taurochenodeoxycholic Acid, and Taurodeoxycholic Acid from Intact Human Gallbladder Bile[a]

		Taurocholic acid			Taurochenodeoxycholic acid			Taurodeoxycholic acid		
			Proton			Proton			Proton	
No.	Type	Carbon	α	β	Carbon	α	β	Carbon	α	β
1	CH_2	38.30	1.80	0.96	38.30	1.80	0.96	38.30	1.80	0.96
		(37.74)	(1.80)	(0.99)	(38.09)	(1.83)	(1.00)	(38.20)	(1.80)	(0.97)
2	CH_2	32.24	1.38	1.65	32.24	1.38	1.65	32.24	1.40	1.65
		(32.06)	(1.38)	(1.65)	(32.48)	(1.37)	(1.66)	(32.02)	(1.40)	(1.67)
3	CH	74.38	—	3.46	74.38	—	3.46	74.15	—	3.58
		(74.39)		(3.47)	(74.46)		(3.48)	(74.24)		(3.62)
4	CH_2	41.31	2.12	1.70	41.31	2.12	1.70	38.18	1.81	1.53
		(41.19)	(2.08)	(1.70)	(41.30)	(2.08)	(1.70)	(38.12)	(1.80)	(1.52)
5	CH	44.31	—	1.38	44.31	—	1.38	45.17	—	1.36
		(43.95)		(1.43)	(44.10)		(1.41)	(44.93)		(1.42)
6	CH_2	37.03	1.60	1.91	37.03	1.60	1.91	30.25	1.35	1.84
		(36.74)	(1.55)	(1.94)	(36.96)	(1.57)	(1.96)	(30.01)	(1.31)	(1.84)
7	CH	70.85	—	3.87	70.85	—	3.87	29.06	1.39	1.23
		(71.04)		(3.87)	(71.07)		(3.87)	(28.99)	(1.42)	(1.22)
8	CH	42.51	—	1.51	42.51	—	1.43	38.52	—	1.37
		(42.16)		(1.55)	(42.11)		(1.48)	(38.89)		(1.42)
9	CH	29.21	2.16	—	35.40	1.84	—	36.14	1.84	—
		(29.18)	(2.14)		(35.32)	(1.82)		(36.24)	(1.82)	
10	C	37.36	—	—	37.69	—	—	36.82	—	—
		(37.22)			(37.64)			(36.78)		
11	CH_2	30.85	1.58	1.58	23.55	1.52	1.27	30.85	1.58	1.58
		(30.62)	(1.57)	(1.57)	(23.46)	(1.50)	(1.26)	(31.21)	(1.53)	(1.53)
12	CH	75.72	—	4.03	42.30	1.26	1.97	75.72	—	4.03
		(75.84)		(4.03)	(42.19)	(1.28)	(1.96)	(75.85)		(4.03)
13	C	48.99	—	—	45.08	—	—	49.08	—	—
		(48.96)			(45.17)			(49.07)		
14	CH	44.31	1.87	—	52.72	1.45	—	50.58	1.58	—
		(44.33)	(1.84)		(52.80)	(1.41)		(50.63)	(1.59)	
15	CH_2	26.38	1.74	1.07	26.38	1.74	1.07	26.72	1.74	1.07
		(25.78)	(1.72)	(1.09)	(26.29)	(1.72)	(1.08)	(26.60)	(1.65)	(1.05)
16	CH_2	30.20	2.01	1.35	30.20	2.01	1.35	30.20	2.01	1.35
		(30.09)	(1.95)	(1.26)	(30.73)	(1.97)	(1.27)	(30.42)	(1.92)	(1.22)
17	CH	48.56	1.78	—	57.09	1.29	—	48.56	1.78	—
		(49.10)	(1.74)		(57.71)	(1.24)		(49.16)	(1.75)	
18	CH_3	15.14	0.69		14.44	0.65		15.36	0.68	
		(14.99)	(0.69)		(14.43)	(0.67)		(15.42)	(0.70)	
19	CH_3	25.28	0.84		25.90	0.87		26.04	0.89	
		(24.99)	(0.90)		(25.54)	(0.92)		(25.84)	(0.92)	
20	CH	38.29	1.45		38.29	1.45		38.29	1.45	
		(37.96)	(1.41)		(38.13)	(1.44)		(38.33)	(1.42)	
21	CH_3	19.73	1.02		21.05	0.98		19.54	1.02	
		(19.51)	(0.99)		(20.91)	(0.96)		(19.51)	(0.99)	
22	CH_2	34.38	1.49, 1.69		34.38	1.49, 1.69		34.38	1.49, 1.69	
		(34.38)	(1.41, 1.73)		(34.45)	(1.43, 1.72)		(34.39)	(1.42, 1.71)	
23	CH_2	34.86	2.37, 2.21		34.38	2.37, 2.21		34.86	2.37, 2.21	
		(35.39)	(2.32, 2.18)		(35.27)	(2.31, 2.15)		(35.53)	(2.33, 2.16)	
24	C	179.81	—		179.48	—		179.48	—	
		(180.05)			(179.85)			(179.79)		
25	CH_2	38.11	3.56		38.11	3.56		38.11	3.56	
		(37.88)	(3.56)		(37.94)	(3.56)		(37.95)	(3.56)	
26	C	52.68	3.08		52.68	3.08		52.68	3.08	
		(52.69)	(3.07)		(52.70)	(3.07)		(52.69)	(3.07)	
27	NH	—	8.02		—	7.98		—	8.00	
			(7.96)			(7.93)			(7.92)	

[a]The chemical shift values of the standard bile acids (26) are given in parentheses. Numbering for carbons/protons is as given in Figure 1.

netic fields, all the signals get resolved (Fig. 6e). We have shown that decoupling of methylene protons of conjugated glycine and taurine does not cause any change in intensity of the amide protons and thus it rules out interference from the NOE buildup on the amide protons during decoupling of the adjacent methylene protons. In fact, with a 45° excitation

pulse, all the amides proton magnetization reaches equilibrium even at a recycle delay of 3 s (including acquisition time). Thus the relaxation times of the amide protons are favorable for rapid analysis of individual bile acids. The longer recycle delay of 6 s used in this study was, however, necessitated due to longer relaxation time of the reference signal (TSP) used for quantitative analysis.

It is clear from Tables 1 and 2 that most bile acid signals severely overlap with each other except amide proton signals. In our previous study, we identified an unresolved bunch of amide signals to be markers of total glycine- and taurine-conjugated bile acids in bile (27). In this study, from the rigorous analysis of bile spectra under natural conditions, we have unraveled them into six individual conjugated bile acids. In the whole bile ^{1}H NMR spectrum, amide signals are the only ones that are distinct signatures of individual conjugated bile acids. Under physiological conditions of bile (pH = 7.0 to 7.7), amide signal intensities are attenuated due to the chemical exchange and hence they do not represent true concentration of the bile acids. Earlier we have made a detailed study under variable pH conditions for human bile as well as for several standard conjugated bile acids and shown that the attenuation of amide signals arising from chemical exchange can be suppressed by reducing the pH to slightly lower than the physiological value (6.0 ± 0.5) (27). Therefore, quantitative analysis of individual bile acids was performed after the addition of 1–2 μL of hydrochloric acid to bile solutions so as to bring down the pH in the range of 6.0 ± 0.5 and, as expected, the quantities thus determined show good agreement with the known amounts in the standard addition experiments (Table 3). Alternatively, bile solution pH may be adjusted by the addition of aqueous buffer (pH = 6.0) instead of diluting the bile using distilled water. This may allow better control of the pH and hence the pH-dependent signal intensity variations, if any.

TABLE 3
Quantity of Bile Acids Recovered from Gallbladder Bile Through Increase in Amide Signal Integral in 1H NMR Versus the Quantity Added (Each Experiment Was Performed in Duplicate)[a]

Bile acid	Quantity added to bile (mg)	Quantity recovered by NMR (mg) (mean ± SD)	Error (%) Mean ± SD
Glycocholic acid	1.04	0.99 ± 0.003	4.77 ± 0.28
		1.00 ± 0.003	3.61 ± 0.28
Glycochenodeoxycholic acid	0.87	0.81± 0.01	3.95 ± 1.20
		0.81 ± 0.01	3.92 ± 1.04
Glycodeoxycholic acid	0.84	0.82 ± 0.002	1.73 ± 0.20
		0.82 ± 0.001	2.82 ± 0.14
Taurocholic acid	0.87	0.85 ± 0.001	2.01 ± 0.11
		0.86 ± 0.011	0.49 ± 1.27
Taurochenodeoxycholic acid	0.75	0.74 ± 0.007	0.78 ± 0.94
		0.77 ± 0.005	2.21 ± 0.66
Taurodeoxycholic acid	0.84	0.83 ± 0.003	1.75 ± 0.32
		0.87 ± 0..005	3.49 ± 0.54

[a]Mean and standard deviation for each recovered quantity of the bile acid were obtained from deconvolution of the amide signal five times, independently.

TABLE 4
Typical Concentrations of Six Conjugated Bile Acids Obtained Individually from Single-Step ^{1}H NMR Analysis in Gallbladder Bile of 16 Patients[a]

Serial Number	Glycocholic acid	Glycochenodeoxycholic acid	Glycodeoxy cholic acid	Taurocholic acid	Taurochenodeoxycholic acid	Taurodeoxycholic acid	Type of disease
1	3.97 ± 0	3.86 ± 0.01	0.82 ± 0.01	1.21 ± 0.01	1.05 ± 0.1	ND	CC
2	13.65 ± 0.08	7.89 ± 0.11	6.31 ± 0.05	3.28 ± 0.08	1.96 ± 0.05	1.07 ± 0.01	CC
3	18.76 ± 0.04	19.91 ± 0.09	5.83 ± 0.04	5.57 ± 0.06	5.74 ± 0.03	1.55 ± 0.01	CC
4	13.02 ± 0.03	9.29 ± 0.03	ND	0.58 ± 0.01	0.34 ± 0.01	ND	CC
5	17.23 ± 0.03	7.91 ± 0.08	7.11 ± 0.05	4.33 ± 0.05	2.29 ± 0.05	1.36 ± 0.01	CC
6	33.62 ± 0.24	23.18 ± 0.55	11.36 ± 0.19	6.05 ± 0.22	5.09 ± 0.23	1.59 ± 0.04	CC
7	18.36 ± 0.04	9.29 ± 0.02	3.06 ± 0.03	5.55 ± 0.01	2.71 ± 0	0.42 ± 0.01	CC
8	39.75 ± 0.19	30.57 ± 0.05	21.69 ± 0.12	6.52 ± 0.01	5.04 ± 0.16	3.37 ± 0.02	CC
9	29.05 ± 0.11	36.34 ± 0.06	17.67 ± 0.05	5.73 ± 0.05	6.27 ± 0.04	2.22 ± 0.01	CC
10	6.11 ± 0.03	6.79 ± 0.07	3.74 ± 0.01	1.36 ± 0.03	1.25 ± 0.02	0.59 ± 0.01	CC
11	28.16 ± 0.04	45.79 ± 0.22	ND	6.33 ± 0.14	9.47 ± 0.12	ND	CC
12	11.72 ± 0.01	15.36 ± 0.11	6.64 ± 0.02	2.40 ± 0.01	2.46 ± 0.01	1.21 ± 0.02	CC
13	62.65 ± 0.23	18.27 ± 0.11	8.80 ± 0.12	17.92 ± 0.09	5.36 ± 0.08	1.05 ± 0.03	Normal
14	28.39 ± 0.03	9.48 ± 0.06	10.36 ± 0.05	8.45 ± 0.05	4.08 ± 0.02	3.86 ± 0.01	CC
15	27.82 ± 0.06	22.56 ± 0.09	12.31 ± 0.03	4.08 ± 0.03	3.76 ± 0.03	1.60 ± 0.01	CC
16	19.56 ± 0.03	15.54 ± 0.14	7.74 ± 0.08	4.71 ± 0.09	4.88 ± 0.01	1.24 ± 0.01	CC

[a]Mean and standard deviation for each bile acid were obtained from deconvolution of the amide signal five times independently. ND = Not detected; CC = chronic cholecystitis (gallstone disease); Normal = no diagnosed gallstone disease.

As seen in Table 4, in all 16 bile specimens analyzed, three each of glycine- and taurine-conjugated bile acids (GCA, GDCA, GCDCA, TCA, TDCA, and TCDCA) were observed, except in three bile specimens where GDCA and/or TDCA were not detected (Table 4 serial Nos. 1, 4, and 11). Further, although there was a large variation between the relative and absolute quantities of bile acids, the ratio of median values of taurine- to glycine-conjugated bile acids was 1:4 for primary bile acids (CA and CDCA) whereas the ratio was 1:5.7 for the secondary bile acid (DCA). These results show the potential of NMR for determining the individual conjugated bile acids rapidly in a single step. Furthermore, it is even possible to analyze unconjugated bile acids, if present. This can be achieved from the presence or absence of carbonyl carbon signal(s) near 187 ppm (26). The chemical shift region of the carboxylic carbons for unconjugated bile acids, which normally does not contain any other bile component signals, serves as a marker. In the ^{13}C spectrum of typical bile shown in Figure 4, the absence of such signals (near 187 ppm) clearly indicates the absence of any unconjugated bile acids (under the detection limits of present experimental conditions). Although variations in the bile acids, as shown in Table 4, does not seem to show any direct correlation with the diagnosis, detailed and systematic studies are required to relate these variations to the underlying pathophysiology of the hepatobiliary diseases.

In summary, we present here ^{1}H and ^{13}C NMR chemical shift assignment of intact bile under natural conditions and identified six major conjugated bile acids. Accurate and rapid analysis of all the conjugated bile acids in a single step presented herein is significantly advantageous over other methods. Moreover, the method is simple and does not need any sample processing. This may have significant impact on the studies of bile acid synthesis, metabolism, and associated hepatobiliary and gastrointestinal diseases. Studies using the method presented herein are currently in progress on bile specimens from a large number of patients with gallbladder disease, to have better understating of the diseases of biliary origin.

ACKNOWLEDGMENTS

Financial support from Department of Science and Technology, Government of India; and the use of the 700 MHz NMR facility at NMR Research Centre, Indian Institute of Science, Bangalore, and the 800 MHz NMR facility at Tata Institute of Fundamental Research, Mumbai, are gratefully acknowledged.

REFERENCES

1. Hoffman, A.F. (1994) *Bile Acids in The Liver: Biology and Pathobiology,* 3rd ed. (Arias, I.M., Boyer, J.L., Fausto, N., Jakoby, W.B., and Schachter, D.A., eds.), pp. 677–718, Raven Press, New York.
2. Perwaiz, S., Tuchweber, B., Mignault, D., Gilat, T., and Yousef, I.M. (2001) Determination of Bile Acids in Biological Fluids by Liquid Chromatography-Electrospray Tandem Mass Spectrometry, *J. Lipid Res. 42,* 114–119.
3. Fabiani, D.E., Mitro, N., Godio, C., Gilardi, F., Caruso, D., and Crestani, M. (2004) Bile Acid Signaling to the Nucleus: Finding New Connections in the Transcriptional Regulation of Metabolic Pathways, *Biochimie 86,* 771–778.
4. Moseley, R.H. (1995) *Bile Secretion in Textbook of Gastroenterology,* 2nd ed. (Yamada, T., Alpers, D.H., Owyang, C., Powell, D.W., and Silverstein, F.E., eds.), Vol. 1, pp. 383–404, J. B. Lippincott, Philadelphia.
5. Greim, H., (1976) Bile Acids in Hepato-Biliary Diseases, in *The Bile Acids: Chemistry, Physiology and Metabolism. Pathophysiology* (Kritchevsky, D., and Nair, P.P., eds.), Vol. 3, pp. 53–80, Plenum Press, New York.
6. Lee, S.P., and Kuver, R. (1995) *Gallstones in Textbook of Gastroenterology,* 2nd ed. (Yamada, T., Alpers, D.H., Owyang, C., Powell, D.W., and Silverstein, F.E., eds.), pp. 2187–2212, J.B. Lippincott, Philadelphia.
7. van Mil S.W., Houwen, R.H., and Klomp, L.W. (2005) Genetics of Familial Intrahepatic Cholestasis Syndromes, *J. Med. Genet. 42,* 449–463.
8 Komichi, D., Tazuma, S., Nishioka, T., Hyogo, H., and Chayama, K. (2005) A Nuclear Receptor Ligand Down-Regulates Cytosolic Phospholipase A2 Expression to Reduce Bile acid-Induced Cyclooxygenase-2 Activity in Cholangiocytes: Implications of Anticarcinogenic Action of Farnesoid X Receptor Agonists, *Dig. Dis. Sci. 50,* 514–524.
9. Hagiwara, T., Kono, S., Yin, G., Toyomura, K., Nagano, J., Mizoue, T., Mibu, R., Tanaka, M., Kakeji, Y., Maehara, Y., Okamura, T., Ikejiri, K., Futami, K., Yasunami, Y., Maekawa, T., Takenaka, K., Ichimiya, H., and Imaizumi, N. (2005) Genetic Polymorphism in Cytochrome P450 7A1 and Risk of Colorectal Cancer: The Fukuoka Colorectal Cancer Study, *Cancer Res. 65,* 2979–2982.
10. Kanoh, M., Tadano, T., Tanba, T., Katayama, H., Shimizu, T., Sato, Y., Shibuya, M., Ushio, H., Matsumoto, M., Kojima, Y., Sakamoto, K., and Kamano, T. (2005) Histopathologic Changes in Serum Bile Acid Fractions in Pressure Ulcer Patients, *Hepatogastroenterology 52,* 1015–1018.
11. Matsumoto, M., Takei, H., Kanoh, M., Kamano, T., and Maeda, M. (2004) Development of an Enzyme-Linked Immunosorbent Assay for Fecal Bile Acid, *Rinsho Byori 52,* 891–896.
12. Bernstein, H., Bernstein, C., Payne, C.M., Dvorakova, K., and Garewal, H. (2005) Bile Acids as Carcinogens in Human Gastrointestinal Cancers, *Mutat. Res. 589,* 47–65.
13. Rust, C., Bauchmuller, K., Fickert, P., Fuchsbichler, A., and Beuers, U. (2005) Phosphatidylinositol 3-Kinase Dependent Signaling Modulates Taurochenodeoxycholic Acid-Induced Liver Injury and Cholestasis in Perfused Rat Livers, *Am. J. Physiol. Gastrointest. Liver Physiol. 289,* G88–G94.
14. Bove, K.E., Heubi, J.E., Balistreri, W.F., and Setchell, K.D., (2004) Bile Acid Synthetic Defects and Liver Disease: A Comprehensive Review, *Pediatr. Dev. Pathol. 7,* 315–334.
15. Wildgrube, H.J., Stockhausen, H., Petri, J., Fussel, U., and Lauer, H. (1986) Naturally Occurring Conjugated Bile Acids Measured by High Performance Liquid Chromatography in Human, Dog, and Rabbit Bile, *J. Chromatogr. 35,* 207–213.
16. Bloch, C.A., and Watkins, J.B. (1978) Determination of Conjugated Bile Acids in Human Bile and Duodenal Fluid by Reverse Phase High-Performance Liquid Chromatography, *J. Lipid Res. 19,* 510–513.
17. Tietz, P.S., Thistle, J.L., Miller, L.J., and LaRusso, N.F. (1984) Development and Validation of a Method for Measuring the Glycine and Taurine Conjugates of Bile Acids in Bile by High Performance Liquid Chromatography, *J. Chromatogr. 336,* 249–257.
18. Budai, K., and Javitt, N.B. (1997) Bile Acid Analysis in Biological Fluids: A Novel Approach, *J. Lipid Res. 38,* 1906–1912.
19. Guldutuna, S., You, T., Kurts, W., and Leuschner, U. (1993) High-Performance Liquid Chromatographic Determination of

Free and Conjugated Bile Acids in Serum, Liver Biopsies, Bile, Gastric Juice and Feces by Fluorescene Labeling, *Clin. Chim. Acta 214,* 195–207.
20. Sjovall, J., and Setchell, K.D.R. (1988) Techniques for Extraction and Group Separation of Bile Acids, in *The Bile Acids: Chemistry, Physiology, and Metabolism* (Setchell, K.D.R., Kritchevsky, P., and Nair, P.P., eds.), pp. 1–42, Plenum Press, New York.
21. Dionne, S., Tuchweber, B., Plaa, G., and Yousef, I.M. (1994) Phase I and Phase II Metabolism of Lithocholic Acid in Hepatic Acinar Zone 3 Necrosis. Evaluation in Rats by Combined Radiochromatography and Gas-Liquid Chromatography-Mass Spectrometry, *Biochem. Pharmacol. 48,* 1187–1197.
22. Gelpi, E. (1995) Biomedical and Biochemical Applications of Liquid Chromatography-Mass Spectroscopy, *J. Chromatogr. A 703,* 59–80.
23. Setchell, K.D.R., and Vessel, C.H. (1989) Thermo Spray Ionization Liquid Chromatography-Mass Spectroscopy: A New and Highly Specific Technique for the Analysis of Bile Acids, *J. Lipid Res. 30,* 1459–1469.
24. Libert, R., Hermans, D., Draye, J., Van Hoof, F., Sokal, E., and Hoffmann, E. (1991) Bile Acids and Conjugates Identified in Metabolic Disorders by Fast Atom Bombardment and Tandem Mass Spectrometry, *Clin. Chem. 37,* 2102–2110.
25. Eckers, C., New, A.P., East, P.B., and Haskins, N.J. (1990) The Use of Tandem Mass Spectroscopy for the Differentiation of Bile Acid Isomers and for the Identification of Bile Acids in Biological Extracts, *Rapid Commun. Mass Spectr. 10,* 449–453.
26. Ijare O.B., Somashekar, B.S., Jadegoud, Y., and Nagana Gowda, G.A. (2005) ^{1}H and ^{13}C NMR Characterization and Stereochemical Assignments of Bile Acids in Aqueous Media, *Lipids 40,* 1031–1041.
27. Ijare, O.B., Somashekar, B.S., Nagana Gowda, G.A., Sharma, A., Kapoor, V.K., and Khetrapal, C.L. (2005) Quantification of Glycine and Taurine Conjugated Bile Acids in Human Bile Using ^{1}H NMR Spectroscopy, *Magn. Reson. Med. 53,* 1441–1446.
28. Nagana Gowda, G.A. (2001) One-Dimensional Pulse Technique for Detection of Quaternary Carbons, *Magn. Reson. Chem. 39,* 581–585.
29. Nagana Gowda, G.A., Somashekar, B.S., Ljare, O., Sharma, A., Kapoor, V.K., and Khetrapal, C.L. (2006) One-Step Analysis of Major Bile Components in Human Bile Using ^{1}H NMR Spectroscopy, *Lipids 41*, 577–589.
30. Somashekar B.S., Ijare, O.B., Nagana Gowda, G.A., Ramesh, V., Gupta, S., and Khetrapal, C.L. (2006) Simple Pulse-Acquire NMR Methods for the Quantitative Analysis of Calcium, Magnesium and Sodium in Human Serum, *Spectrochim. Acta Part A 65,* 254–260.

[Received March 2, 2006; accepted June 24, 2006]