Magnetic Resonance in Medicine 53:1441–1446 (2005)

Quantification of Glycine and Taurine Conjugated Bile Acids in Human Bile Using ^{1}H NMR Spectroscopy

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A simple method for quantification of conjugated bile acids in human bile using ^{1}H NMR spectroscopy is presented. Bile acids in human bile are essentially conjugated with either glycine or taurine. The amide NH resonances from the conjugated bile acids are invariably devoid of interfering signals in ^{1}H NMR spectra. Under physiologic conditions of human bile (pH ~7.0 to 7.7), amide signal intensities are attenuated due to the chemical exchange and hence quantitative estimation is precluded. In the present study, the quantity of total glycine and taurine conjugated bile acids could be obtained accurately by suppressing the amide exchange by reducing the pH slightly lower than physiologic value (6.0 ± 0.5). Further, the quantity of glycine conjugated bile acids can be calculated accurately by subtracting the quantity of taurine conjugated bile acids from the total conjugated bile acids as determined from the present method. Magn Reson Med 53:1441–1446, 2005. © 2005 Wiley-Liss, Inc.

Key words: ^{1}H NMR; human bile; glycine and taurine conjugated bile acids; quantification

Major constituents of human bile are phospholipids, bile acids, and cholesterol. Primary bile acids such as cholic acid and chenodeoxycholic acid are synthesized from cholesterol in the liver (1). These bile acids are, subsequently, conjugated to the amino acids glycine or taurine and secreted into the bile as salts of sodium or potassium (2). In addition, there are several other derivatives of cholic acid present in the bile, of which conjugated deoxycholic acid is in significant quantity (3). Total bile acid concentration in the bile varies from 3 to 45 mM, almost all of which are conjugated to glycine or taurine (1,3). Sulfonation and glucuronation of bile acids are minor metabolic pathways, except in cholestatic disorders (1,3–5). As a result of conjugation with the amino acids, the *p*Ka of the bile acids is drastically reduced and hence they become readily soluble in aqueous medium (4). The main function of the bile salts is to help in solubilization of dietary fats, fat-soluble vitamins, and cholesterol (6,7).

Abnormal and varied composition of the conjugated bile salts in bile is indicative of various hepatogastrointestinal diseases (3,8,9). Dietary fat malabsorption and gallstone formation are the common causes of abnormal bile composition in the bile (3,9). Hence, measurement of the conjugation pattern of bile acids is necessary for understanding the pathophysiology of these diseases. Although there are many reports that cite the percentage of various glycine and taurine conjugated bile salts in bile, the results are conflicting (1,10).

Commonly used methods for the quantification of conjugated bile acids usually involve tedious steps such as extraction, hydrolysis, derivatization, and/or purification before analysis (10–15). Subsequently, the assay of conjugated bile acids is performed by enzymatic method, gas–liquid chromatography, or high-performance liquid chromatography (HPLC) (16).

Nuclear magnetic resonance (NMR) spectroscopy offers a straightforward method for the quantification of bile acids. An attempt has been made to quantify total and taurine conjugated bile salts in rat bile (17) from the area of the H-18 methyl signals (around 0.7 ppm). However, since this peak partially or completely overlaps with other bile metabolites, accurate determination of total bile acids from this method is questionable (18). Thus, the major difficulty in quantification of conjugated bile acids from NMR spectra arises from the overlap of their signals with those from other biliary metabolites such as phospholipids and cholesterol. Due to similarity in the structures of various bile salts, the NMR signals of bile acids overlap, making quantitative estimation of individual bile acids further complicated. In this study, we report a simple and accurate method of quantifying glycine and taurine conjugated bile salts in human bile using ^{1}H NMR spectroscopy.

METHODS

Glycine and Taurine Conjugated Bile Salts

Sodium salts of glycocholic acid (GCA), glycodeoxycholic acid (GDCA), glychochenodeoxycholic acid (GCDA), taurocholic acid (TCA), taurodeoxycholic acid (TDCA), taurochenodeoxycholic acid (TCDA), cholesterol, deuterium oxide (D_2O), and trimethylsilylpropionic acid sodium salt-d_4 were purchased from Sigma–Aldrich and used without further purification.

Collection of human bile samples

Bile was collected from the gall bladder in patients undergoing cholecystectomy (laparoscopic or open) for symp-

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Grant sponsor: Department of Science and Technology, Government of India.

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Received 8 November 2004; revised 10 January 2005; accepted 20 January 2005.

DOI 10.1002/mrm.20513
Published online in Wiley InterScience (www.interscience.wiley.com).

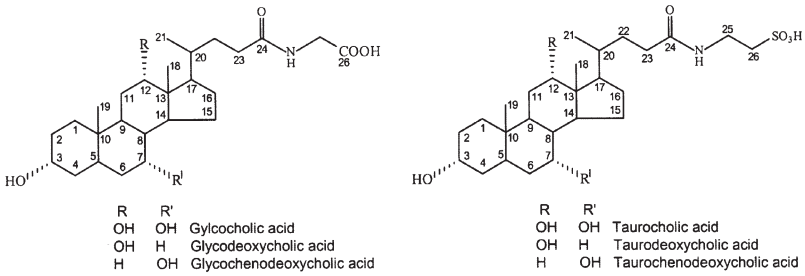

FIG. 1. Structures of glycine and taurine conjugated bile acids used in the current study.

tomatic gallstone disease. The samples were stored in sterile dark conditions at −80°C until the experiments were performed.

NMR Experiments

NMR experiments were performed on a Bruker Biospin Avance 400-MHz NMR spectrometer using a 5-mm broadband inverse probe. For quantitative estimation, a sealed reusable capillary tube consisting of a known quantity of sodium salt of trimethylsilylpropionic acid (TSP) dissolved in 35 μL of D_2O was inserted into the NMR tube while obtaining NMR spectra. While TSP served as a chemical shift reference as well as the quantitative standard for estimation of metabolites, deuterium oxide served as the "field-frequency-lock."

1D ^{1}H NMR Experiments

All 1D ^{1}H NMR experiments in aqueous media were performed at 25°C by suppression of water resonance by presaturation. The data were Fourier transformed after multiplying by a line broadening function and baseline correction was applied by making use of Bruker Biospin Xwinnmr software version 3.5. Parameters used were as follows: spectral width, 4800 Hz; time domain points, 32 K; relaxation delay, 6 s; pulse angle, 45°; number of scans, 32; spectrum size, 32 K; and line broadening, 0.3 Hz. For quantitative estimation of bile acids, a computer program was used that was developed for calculating the concentrations of metabolites, making use of the integral of signals relative to TSP.

2D Experiments

For assigning the amide (-NH) (Fig. 1) proton resonances in glycine and taurine conjugated bile salts, ^{1}H-^{1}H 2D double quantum filtered correlated spectroscopy (DQF-COSY) and ^{1}H-^{1}H 2D total correlated spectroscopy (TOCSY) experiments were performed on human bile and a mixture of standard bile acids in water (5 mg each of GCA, GDCA,

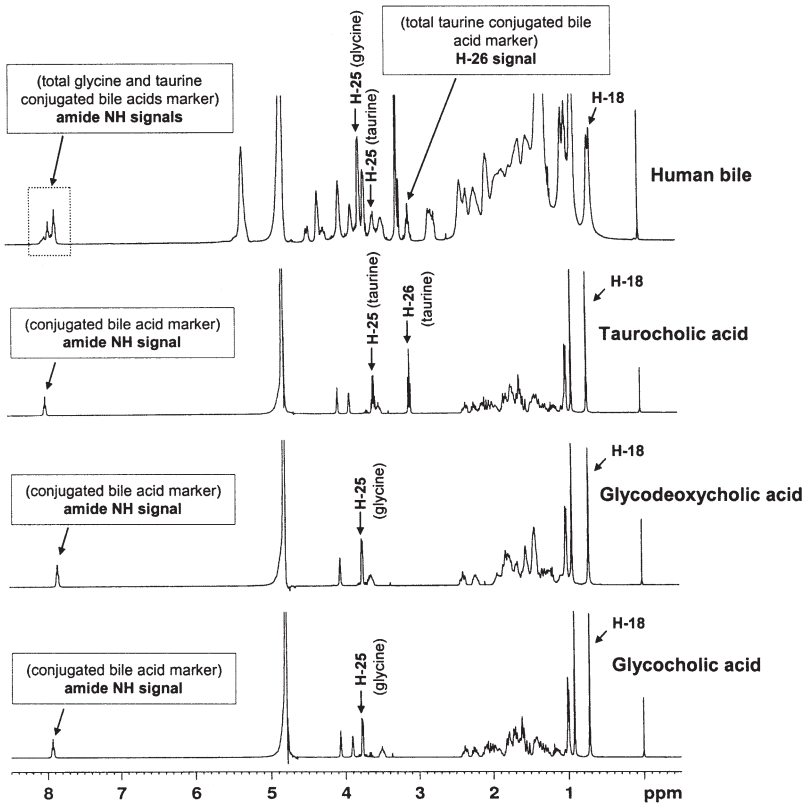

FIG. 2. Typical room temperature (25°C) ^{1}H NMR spectra of a human bile and standard glycine and taurine conjugated bile acids (TCA, taurocholic acid; GDCA, glycodeoxycholic acid; GCA, glycocholic acid) obtained on a Bruker 400-MHz spectrometer. The assignments of the relevant proton signals are marked.

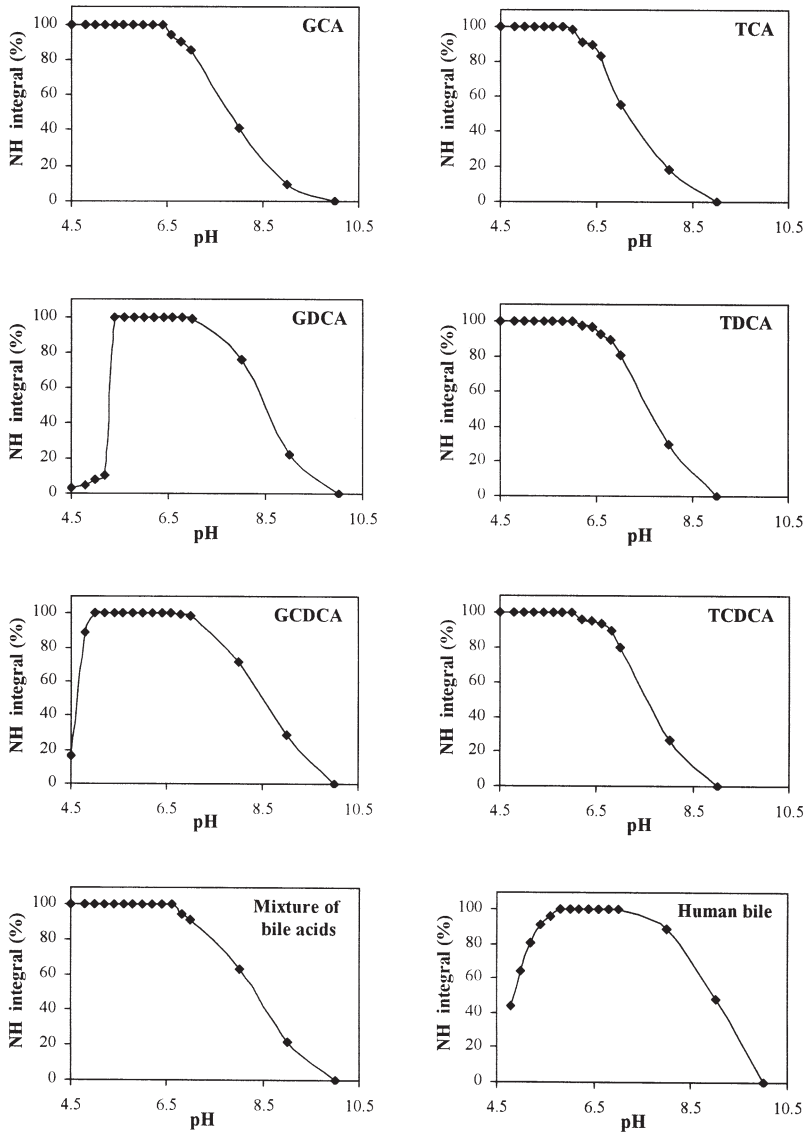

FIG. 3. Plots of percentage variation of amide signal(s) integral, obtained from the ^{1}H NMR spectra at 400 MHz, as a function of pH for the individual conjugated bile acid and mixture of standard bile acids (GCA, glycocholic acid; GDCA, glycodeoxycholic acid; GCDCA, glycochenodeoxycholic acid; TCA, taurocholic acid; TDCA, taurodeoxycholic acid; TCDCA, taurochenodeoxycholic acid) and human bile. Although there is some difference in the variation in the pH range in different bile acids (in standard solutions and human bile) at which the amide signal is maximum (100%), clearly, in all cases, the pH range of about 6.0 $\pm$ 0.5 shows maximum intensity.

TCA, and TDCA). Suppression of intense water signal in all the 2D experiments was done with presaturation. Parameters used were as follows: spectral width, 4800 Hz in both dimensions; time domain data points, 2048; number of FIDs with t_1 incrementation, 512; relaxation delay, 2.5 s; and number of transients, 24. A spin lock time of 80 ms was used for TOCSY experiment. Phase-sensitive data were obtained by the TPPI method. The resulting data were zero filled to 1024 points in t_1 dimension and Fourier transformed along both dimensions after multiplying the data by squared sine-bell window function shifted by $\pi/2$.

Experiments on Standard Bile Salts at Variable pH

Individual bile salts and mixtures of standard bile salts (GCA, GDCA, GCDCA, TCA, TDCA, and TCDCA) solutions were prepared in triple distilled water (5 mg/mL each). The pH of the solutions was measured using Thermo Orion model 520 A+ pH meter after the system was calibrated using standard pH solutions. A total of 500 μL of these bile acid solutions was taken separately in 5-mm NMR tubes and 1D ^{1}H NMR experiments were performed. The pH of these solutions was then increased to 10 in steps of about 0.2 units and at every step ^{1}H NMR spectra were obtained. Subsequently, the pH was decreased from 10 to about 4.5 again in steps of 0.2 units and, at every step, ^{1}H NMR spectra were obtained. For changing the pH, solutions of 6 N sodium hydroxide or hydrochloric acid (HCl) was used. Integrals of amide proton signals of individual bile acids and mixtures of bile acids at different pH were obtained relative to the integral of the reference, TSP, and H-18 methyl signal of bile acids. The quantity of the dissolved bile acids was also calculated at each pH.

Experiments on Human Bile at Variable pH

1D ^{1}H NMR experiments on five human bile samples were performed in duplicate to determine the effect of pH on signal intensity of amide resonances of conjugated bile acids. For this, 500 μL of bile was taken in a 5-mm NMR tube and ^{1}H NMR spectra were obtained before the pH was changed and every time after the pH was changed. The pH

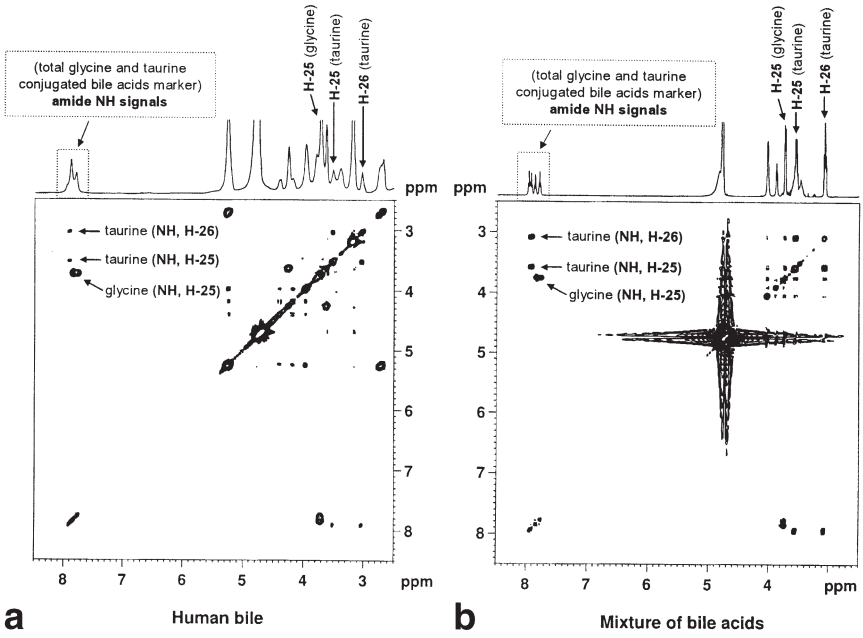

FIG. 4. Parts of 2D TOCSY spectra of (**a**) a typical human bile and (**b**) a mixture of standard aqueous solutions of GCA, glycocholic acid; GDCA, glycodeoxycholic acid; TCA, taurocholic acid; TDCA, taurodeoxycholic acid showing the connectivity of amide proton signals to H-25 and/or H-26 protons of glycine and/or taurine conjugated bile acids. The spectra were recorded at 25°C on a Bruker 400-MHz spectrometer.

was first increased to 10 in steps of about 0.2 units and then decreased to about 4.5, again in steps of about 0.2 units. Integrals of amide proton signals were obtained relative to the integral of the reference, TSP.

Recovery Experiments in Bile

A stock solution of bile was prepared by diluting 500 μL bile to 5 mL using triple distilled water. The pH was adjusted to 6.5 using 6 N HCl. From this stock solution of bile five samples were prepared, in duplicate (500 μL each); one set served as a control and to the other four sets, a linearly varying quantity (1.2, 2.4, 3.6, and 4.8 mg) of a standard bile acid (TCA) was added in separate tubes, all in duplicate. Subsequently, all solutions including controls were subjected to 1D ^{1}H NMR experiments. A sealed reusable capillary tube consisting of a known quantity of TSP in 35 μL D_2O was inserted into the NMR tube before the spectrum was obtained. The total integral of amide proton signals arising from conjugated bile acids was obtained relative to the integral of the reference, TSP. The contribution of the integral from the added TCA was determined from the difference of the integral after addition of TCA and the base value of the integral from the control spectrum. Using the integral of TCA thus determined, the quantity of TCA recovered was calculated. The correlation plot of added TCA versus recovered TCA was made and the regression coefficient was calculated using Excel software, Microsoft Office version 2000.

RESULTS

Standard Conjugated Bile Salts

Figure 1 shows the structure and numbering of the glycine and taurine conjugated bile acids used in this study. ^{1}H NMR spectra of conjugated bile acids are complex due to the large number of overlapping signals. Figure 2 shows typical ^{1}H NMR spectra of standard glycine and taurine conjugated bile acids (GCA, GDCA, and TCA). Integrals of the amide protons relative to H-18 protons and TSP protons were obtained at different pH for both individual and mixture of the conjugated bile acids. Figure 3 shows the plots of percentage integral of the amide proton signals for individual bile acids and mixtures of standard bile acids. In all standard bile acids, the intensity of amide proton signal varies as a function of pH. Above about pH 7, the amide proton signals diminish as the pH increases with the signal vanishing above pH 9 to10. On the other hand, the signal intensity is maximal and remains constant in the pH range of about 5 to 7. The variation in the intensity with the pH is erratic below pH 5 (Fig. 3). The chemical shifts of the amide protons were reproducible at any given pH with a maximum variation of less than 0.01 ppm.

Human Bile

A typical 1D ^{1}H NMR spectrum of human bile is shown in Fig. 2. The assignment of the amide signals in the spectra of bile was made from the analysis of 2D DQF-COSY and TOCSY spectra. Figure 4a shows part of a TOCSY spectrum of human bile highlighting the connectivity of amide protons with the H-25 and H-26 protons of glycine or taurine conjugated bile acids, respectively. The corresponding TOCSY spectrum of a mixture of standard bile acids (GCA, GDCA, TCA, and TDCA) is also shown for comparison (Fig. 4b). It is also clear from the spectra that all the amide signals in the bile arise only from glycine and taurine conjugated bile acids. Further, amide NH of taurine conjugated bile acids appear at slightly higher frequency than those of glycine conjugated bile acids, as in the case of individual bile acids and mixtures of standard bile acids (Figs. 2 and 4).

Similar to standard bile acids, the amide signals in bile decrease in intensity at alkaline pH with the signal intensity vanishing at pH 10. The intensity is maximal (100%) between about pH 5.2 and 6.8. Below about pH 5, the signal intensity again decreases (Fig. 3).

Recovery of TCA in Human Bile

The integral of amide NH signals in bile increases with the addition of known quantity of TCA. Figure 5a shows the amide signals of bile acids from human bile before and after the addition of different quantities of TCA. The quantities of TCA calculated from the increase in the integral of amide signals are compared with the actual quantity added. The plot of recovered versus added TCA is shown in Fig. 5b. As seen from Fig. 5b, very good precision and accuracy was obtained from the experiments performed in duplicate at pH 6.5 with the regression coefficient, R^2 = 0.997.

DISCUSSION

Quantitative estimation of bile acids from ^1H NMR spectroscopy requires that at least one of the proton signals is separated from the crowded regions of the spectra. Alternatively, by making use of the overlapping signals of several bile acids, it is possible to quantify the total bile acids provided (a) the number of protons of each bile acid in overlapping signals is known; (b) the number of protons is the same for all the bile acids; and (c) no signal overlaps in this region other than those of bile acids. The signals of H-18 methyl protons that appear around 0.7 ppm were thus proposed to estimate the total bile acids in rat bile with the assumption that no signals overlap other than those from the bile acids (17). However, cholesterol, which is the precursor of the primary bile acids and one of the major constituents of biliary fluids, contributes its signal (H-18 methyl), which overlaps with those of bile acids (18). Hence, use of this signal becomes erroneous in the quantitative estimation of total bile acids in bile.

Every conjugated bile acid has an amide NH proton arising from glycine or taurine conjugation to primary bile acids (Fig. 2). ^1H NMR spectroscopy may be used to quantify conjugated bile acids using signal from these amide protons. However, from the in vitro study on the standard bile acids, it is shown that amide integrals cannot be used for quantitative estimation of bile acids at all pH values due to the significant variation of the integrals at alkaline pH as well as at certain acidic pH range. The reduction of the amide integral in the alkaline pH is due to the attenuation of the signal arising from chemical exchange and the reduction at lower pH (below pH 5) is due to the precipitation of the bile acids. We estimated the attenuation due to exchange through the ratio of the integral of amide to H-18 signals in the individual bile salts as well as in mixtures of standard bile salt solutions; a ratio of 1:3 for amide NH to H-18 methyl protons indicates complete suppression of amide exchange.

At a substantial range of pH, the integral of amide signals of individual and mixture of standard bile acids is maximal (100%) and remains constant (Fig. 3). Although this range of maximum integral varies slightly from one bile acid to another, the amide integral is maximal within the pH range of about 6.0 ± 0.5 for all bile acids. Therefore, at any pH in this range the integral of amide signals represents the true concentration of the conjugated bile acids.

In human bile, ^1H NMR signals of the amide protons appear invariably in the region 7.8–8.1 ppm, similar to

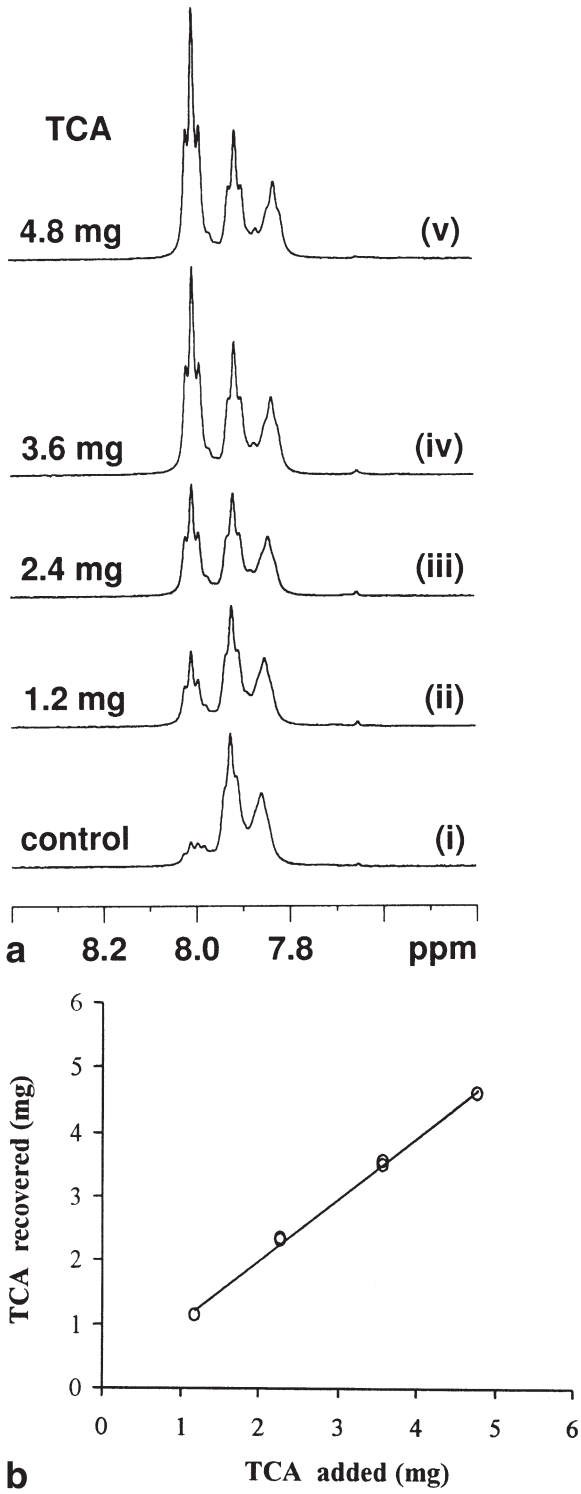

FIG. 5. (**a**) Parts of ^1H NMR spectra obtained on a 400-MHz spectrometer at room temperature (25°C) of amide proton regions of human bile before and after the addition of linearly incremental quantity of TCA (taurocholic acid) as mentioned. Note the proportional increase of integral of amide signals with the increasing quantity of TCA. The pH of the bile was adjusted to 6.5 before ^1H NMR spectra were obtained. (**b**) Plot of the quantity of TCA recovered from bile versus the quantity of TCA added (in duplicate). Note the excellent fit between the added and recovered quantity of TCA, which shows the regression coefficient R^2 = 0.997.

individual and mixture of standard glycine and taurine conjugated bile acids (Fig. 2). Typically, the bile specimens show two glycine conjugated bile acids in a large quantity and one taurine conjugated bile acid in a relatively small quantity (Fig. 4). Similar to the standard bile acids, the amide signals of bile acids in human bile showed reduction in the integral in alkaline pH and acidic pH (< 5) (Fig. 3). Since the physiologic pH of bile lies in the alkaline pH (7 to 7.7) (19), the amide integral under these conditions does not represent the true bile acid quantity. However, since there is no reduction in the amide signal either due to amide exchange or due to precipitation in the pH range of 6.0 ± 0.5, accurate quantification of conjugated bile acids can be made at any pH in this range. To further prove this, experiments of recovery of known quantities of standard bile acid (TCA) in neat bile were performed at pH 6.5. The quantity of TCA calculated from the integrals of the amide protons is in excellent agreement with the added quantity with a maximum error of 4.8%.

Taurine (H-26) signals that appear at 3.08 ppm usually do not overlap with other signals (Fig. 2). Hence, the integral of H-26 signal can be used to determine the total quantity of taurine conjugated bile acids (17). The quantity of total glycine conjugated bile acids can be subsequently determined by simply subtracting the quantity of taurine conjugated bile acids from total conjugated bile acids determined from the method described herein.

CONCLUSIONS

A simple method for the determination of glycine and taurine conjugated bile acids in human bile is presented. It is relatively easy to perform the analysis since it only requires the addition of a few microliters of an acid such as hydrochloric acid to bring down the pH anywhere in the region of 6.0 ± 0.5 before the NMR spectrum is obtained. Since the amide signals are very well separated from rest of the signals in the bile spectrum, the estimation can be performed even on routinely available spectrometers of moderate magnetic fields such as the one used in this study.

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