methods

Proton magnetic resonance assay of total and taurine-conjugated bile acids in bile

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Abstract **Biliary bile acids, coexisting with phospholipid and cholesterol, are partly conjugated with taurine. In the present report we show that total and taurine-conjugated bile acids in bile can be simultaneously and quantitatively measured by high-resolution ^{1}H-nuclear magnetic resonance (^{1}H-NMR) spectroscopy. We used a 7.05-Tesla NMR spectrometer to obtain the ^{1}H-NMR spectra of model and biological biles. Only addition of trimethylsilyl-3-propionic acid sodium salt-D_4 (TSP) to each sample as an internal standard was required in preparation for ^{1}H-NMR measurement. In ^{1}H-NMR spectra of rat bile, peaks of C-18 methyl protons of bile acids and of C-25 methylene protons on the taurine moiety of taurine-conjugated bile acids were detected at 0.7 ppm and 3.1 ppm, respectively. Peak areas, of C-18 and C-25 peaks, increased in proportion to the concentrations of bile acids or taurine-conjugated bile acids, even in the presence of phospholipid and cholesterol. The accuracy of NMR measurement of total and taurine-conjugated bile acids was confirmed by comparing the results of NMR with those of enzyme-fluorimetry. The results clearly demonstrate that ^{1}H-NMR spectroscopy can be applied to the quantitative determination of total and taurine-conjugated bile acids in bile without troublesome preparative steps.**—Ishikawa, H., T. Nakashima, K. Inaba, H. Mitsuyoshi, Y. Nakajima, Y. Sakamoto, T. Okanoue, K. Kashima, and Y. Seo. **Proton magnetic resonance assay of total and taurine-conjugated bile acids in bile.** ***J. Lipid Res.*** **1999.** 40: **1920–1924.**

Supplementary key words ^{1}H-NMR • cholic acid • phospholipid • cholesterol

Bile acids are the major solutes in bile and are amphiphilic detergent-like molecules that solubilize other biliary components such as phospholipid and cholesterol (1). Bile acids, phospholipid, and cholesterol co-exist in bile in the form of mixed micelles or vesicles. Perturbation of these biliary components might result in the formation of gallstones (2, 3). Bile acids in bile exist mostly in the form of conjugates with taurine or glycine, which increases their water solubility (4). Human bile acids mostly consist of 3α-hydroxycholanic acids: cholic acid (CA), deoxycholic acid (DCA), chenodeoxycholic acid (CDCA), and ursodeoxycholic acid (UDCA). Taurine-conjugated bile acids account for about 25% of total bile acids in normal human bile. Rat bile acids are also 3α-hydroxycholanic acids: mainly CA and β-muricholic acid, and more than 90% of biliary bile acids are usually conjugated with taurine (5). As the ratio of glycine-conjugates to taurine-conjugates, or the ratio of conjugates to unconjugates, varies in various hepato-gastrointestinal diseases (6), measurement of the conjugation pattern of biliary bile acids is necessary to understand the pathophysiology of these diseases. Although it has become possible to quantitatively determine total bile acid levels by enzyme-fluorimetry with 3α-hydroxysteroid dehydrogenase (7), measuring the conjugation pattern of bile acids requires either the use of a column such as Piperidinohydroxypropyl-Sephadex LH-20 (8) to separate unconjugated and conjugated bile acids, or high performance liquid chromatography (HPLC) (9, 10). The column method includes many tedious and time-consuming preparatory steps. Although the analytical method using HPLC offers high sensitivity and selectivity (11), the procedure requires both extraction and purification of the sample before assay, and the enzyme-fixed column must be carefully maintained.

Recent advances in nuclear magnetic resonance (NMR) instruments have provided a useful tool for studying the structural biochemistry and metabolism of biomolecules. Several NMR studies on the structure and dynamics of simple bile acid micelles and mixed micelles have been reported (12–14). Groen et al. (15) have measured micellar phosphatidylcholine by NMR techniques. In this study, we investigated whether it is possible to quantify bile acids in bile by using high-resolution ^{1}H-NMR.

Abbreviations: NMR, nuclear magnetic resonance; TSP, trimethylsilyl-3-propionic acid; CA, cholic acid; DCA, deoxycholic acid; CDCA, chenodeoxycholic acid; UDCA, ursodeoxycholic acid; HPLC, high-performance liquid chromatography; TCA, taurocholic acid; TUDCA, tauroursodeoxycholic acid.

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MATERIALS AND METHODS

Materials

Sodium salts of CA, taurocholic acid (TCA), glycocholic acid (GCA), DCA, CDCA, UDCA, tauroursodeoxycholic acid (TUDCA), phosphatidylcholine (lecithin) from egg yolk, cholesterol, β-alanine, and taurine were obtained from Nacalai Tesque Ltd. (Kyoto, Japan). Deuterium oxide (D_2O, 99.9%-D) and trimethylsilyl-3-propionic acid sodium salt-D_4 (TSP) were purchased from Eurisotop (Saint-Aubin, France). All lipids were used without further purification as they were of guaranteed grade, and their purity was confirmed by ^{1}H-NMR analysis of their individual solutions (16, 17).

^{1}H-NMR spectroscopy

^{1}H-NMR spectra were obtained on a Bruker AMX-300wb (7.05 Tesla) spectrometer operating at 300.15 MHz at the Laboratory for Nuclear Magnetic Resonance, Physiological Division of Central Laboratories, Kyoto Prefectural University of Medicine (Kyoto, Japan). The spectra were recorded at 25°C using a spectral width of 5,000 Hz and digitized into 16,384 computer points to yield a digital resolution of 0.30 Hz/point. Standard one-pulse ^{1}H-NMR experiments used a 90° flip angle, a 5 μ sec pulse, an acquisition time of 0.82 sec, a recycle time of 3.82 sec, and 16 as the number of accumulations. Large water resonance was suppressed by homogated decoupling.

Aliquots of 0.45 ml of the sample were transferred to 5-mm diameter NMR glass tubes, and 0.05 ml of 50 mm TSP in D_2O was added to each sample to provide an internal reference standard and a field-frequency lock (at 0.00 ppm). Peak areas of each peak obtained were calculated automatically using analytical software (UXNMR) installed in the instrument.

Preparation of model bile

Model bile was prepared according to Kibe et al. (18) with some modification. Briefly, sodium salts of bile acids in methanol were mixed with phosphatidylcholine in the presence or absence of cholesterol in chloroform, and the mixture was shaken at 37°C for 2 h. After flushing with nitrogen to remove organic solvents, the mixture was placed under high vacuum for 2 h. The dry lipid film was solubilized in a Tris-buffered saline solution (140 mm NaCl, 10 mm Tris-HCl, pH 7.4) and incubated at 55°C for 12 h.

Preparation of rat bile

Rat bile samples were obtained from male Wistar rats weighing 200–250 g. Some rats were given β-alanine (3% in drinking water) for 7 days to deplete taurine in the liver (19). Rats were anesthetized with sodium pentobarbital (50 mg/kg b.w., i.p.), and the bile ducts were cannulated with a PE-10 polyethylene tube (Nippon Becton Dickinson Co. Ltd. Tokyo, Japan) to collect bile. At the start of bile collection, an intravenous infusion of 0.9% NaCl (1.0 ml/h/100 g) in the tail vein was begun and continued during bile collection. The bile samples obtained were stored at −20°C and were analyzed within 2 weeks.

Enzymatic analysis

Total bile acid levels of the rat bile were measured by enzymefluorimetry using 3α-hydroxysteroid dehydrogenase (Enzabile2, Dai-ichi Chemicals, Tokyo, Japan) (7).

RESULTS AND DISCUSSION

Assignments of ^{1}H-NMR spectra of rat bile

A typical ^{1}H-NMR spectrum of rat bile is presented in **Fig. 1**. Each peak obtained was assigned according to chemical shift (δ, ppm) based on previously published data (12, 20, 21) and the present data as obtained from the solutions containing bile acids, phosphatidylcholine, and cholesterol, alone or in combination. Peaks derived from bile acids were assigned as follows: C-18 and C-19 methyl proton peaks were at 0.7 ppm and 0.9 ppm, respectively, and C-25 and C-26 methylene proton peaks on the taurine moiety of taurine-conjugated bile acids were at 3.1 ppm and 3.5 ppm, respectively. The downfield side of the C-18 peak

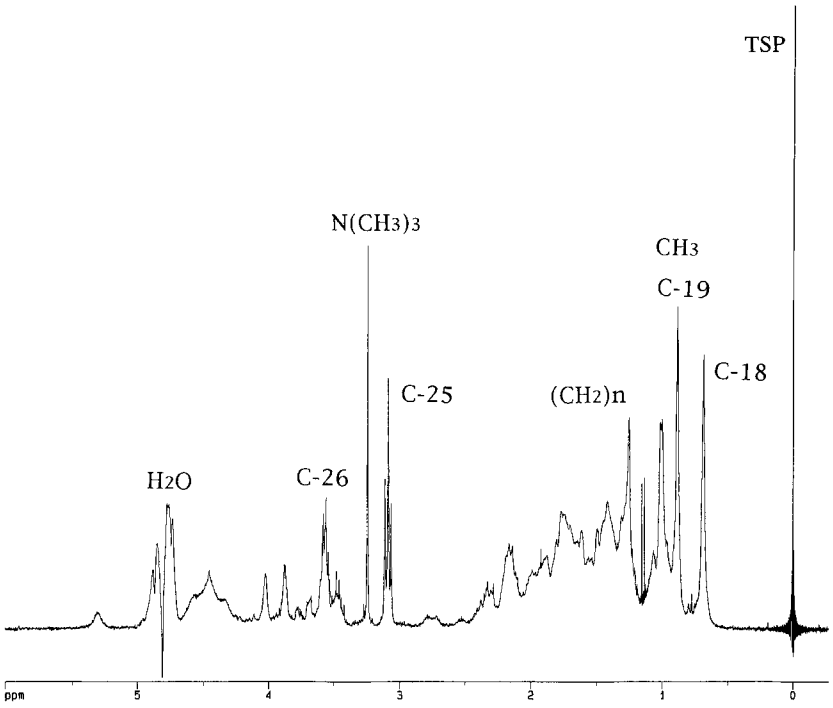

Fig. 1. A typical ^{1}H-NMR spectrum of bile collected during a 1-h period through a biliary cannula from a normal rat. TSP was added to the bile sample in order to provide an internal reference standard at 0.00 ppm. Assigned peaks are as follows: C-18, C-18 methyl protons of bile acids; C-19, C-19 methyl protons of bile acids; C-25 and C-26, C-25 and C-26 methylene protons, respectively, of taurine moieties of taurine-conjugated bile acids; CH_3 and $(CH_2)n$, methyl and methylene protons, respectively, of fatty acid moieties of phospholipids; $N(CH_3)_3$, choline head group of phospholipids.

partly overlapped the neighboring peaks, but not so with the C-25 peak. C-25 methylene proton peaks on the glycine moiety of glycine-conjugated bile acids should have been at 3.8 ppm, but were difficult to detect because of overlapping with neighboring peaks.

Peaks derived from phospholipid were assigned as follows: the choline head group ($N(CH_3)_3$) peak was at 3.2 ppm, and peaks of $(CH_2)n$ and CH_3 in the fatty acid moiety were at 1.3 ppm and 0.9 ppm, respectively. The CH_3 peak overlapped with the C-19 peak of the bile acids.

A resolved signal peak could not be assigned to cholesterol in the present study. Ellul et al. (16) claimed that peaks of micellar cholesterol were observed in human bile, but Graaf, Groen, and Bovee (22) in a later report stated that no significant cholesterol resonance could be detected in bile.

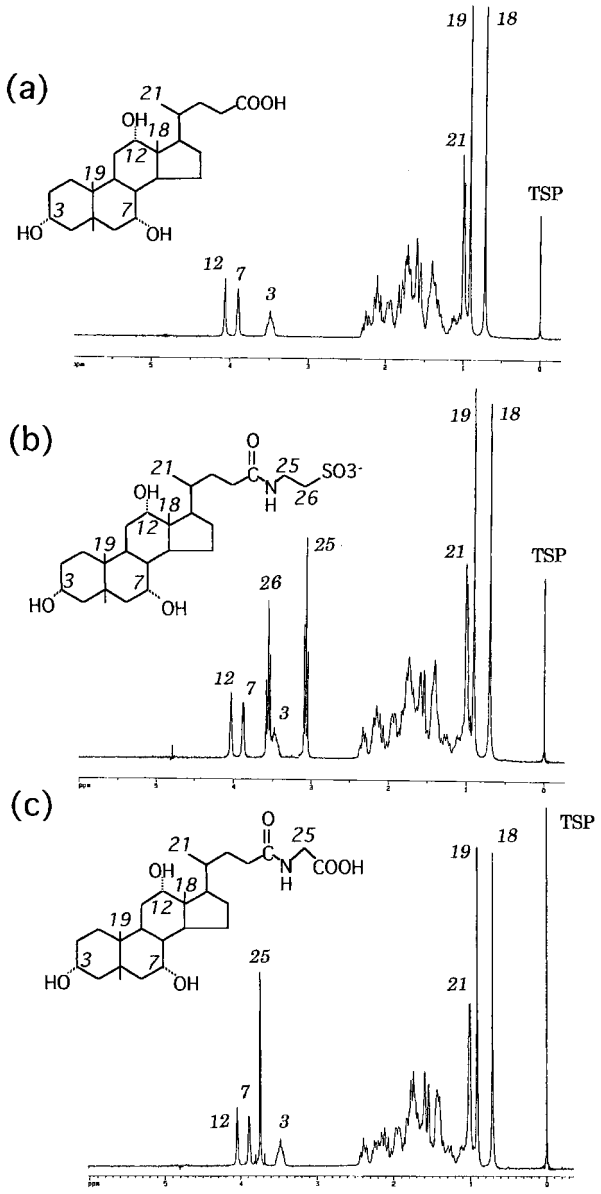

Fig. 2. ^{1}H-NMR spectra of the solutions of (a) cholic acid (b) taurocholic acid, and (c) glycocholic acid. The sodium salts of each bile acid (50 mm) were dissolved in D_2O. The assignment of each peak is described in the text.

^{1}H-NMR spectra of authentic bile acids

The ^{1}H-NMR spectra of unconjugated, taurine-conjugated, and glycine-conjugated CAs prepared in D_2O are presented in **Fig. 2**. The sharp C-18 methyl proton peak at 0.7 ppm was common to these bile acids, and its chemical shift was quite constant as previously reported (23). The triplet C-25 methylene proton peaks at 3.1 ppm were specific to TCA. The spectra of taurine itself showed two triplet peaks, at 3.5 and 3.6 ppm (data not shown), which did not overlap with the C-25 peak of TCA. The C-25 peak at 3.8 ppm, of the glycine moiety of GCA, overlapped with some hydroxy group proton signals.

In view of the above data for the ^{1}H-NMR spectra of rat bile and authentic bile acids, we decided to use C-18 and C-25 bile acid peaks to measure total and taurine-conjugated bile acids in bile. As a result, model studies showed a linearity between peak area and bile acid concentration below 50 mm (data not shown).

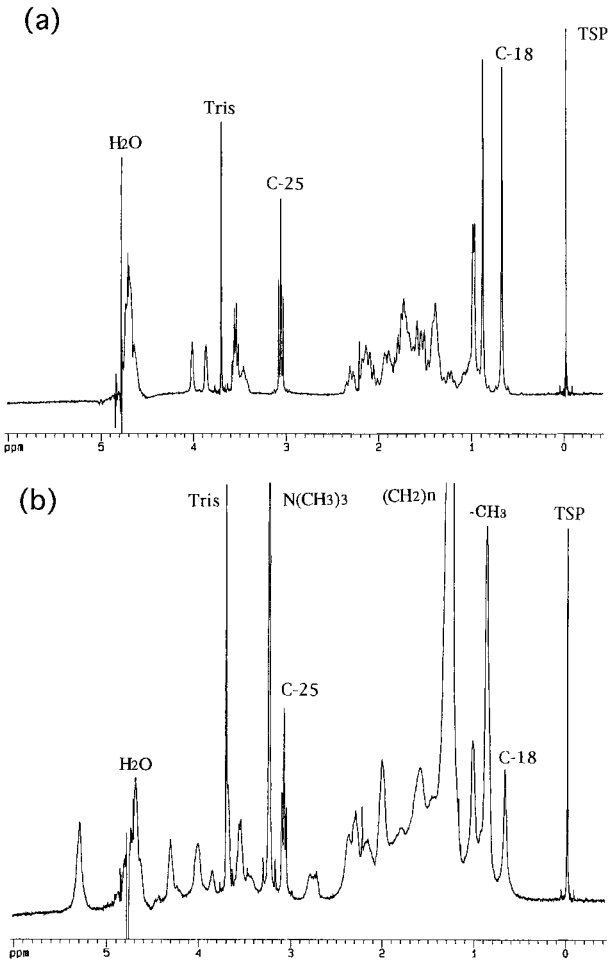

Fig. 3. ^{1}H-NMR spectra of (a) 50 mm taurocholic acid and (b) 50 mm taurocholic acid plus 20 mm phosphatidylcholine dissolved in 10 mm Tris-buffered saline (pH 7.4).

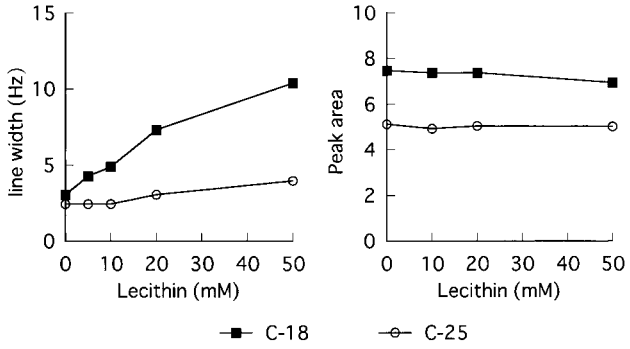

Fig. 4. Effects of varying amounts of phosphatidylcholine on line width and peak areas of C-18 and C-25 of 50 mm taurocholic acid in 10 mm Tris-buffered saline (pH 7.4).

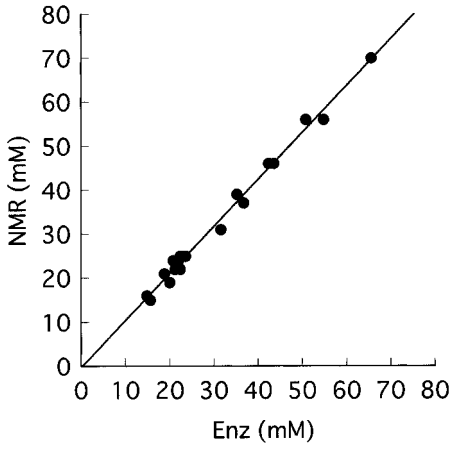

Fig. 5. A linear relationship between total bile acid levels determined by enzyme-fluorimetry and estimated by the peak area of the C-18 (NMR) in 17 separate samples of rat bile. The line obtained passed through the origin.

Effects of phosphatidylcholine and cholesterol on ^{1}H-NMR spectra of bile acids

Because phospholipids and cholesterol are insoluble in water, they usually exist in mixed micelles with bile acids or in phospholipid–cholesterol vesicles in bile. The structure of micellar particles in bile is still controversial: disc-shaped bimolecular leaflets of phospholipids whose hydrophobic parts are surrounded by bile acids (1), cylindical worm-like particles (24, 25), and so on, depending on the concentration of the components, have been proposed. When more phospholipids are solubilized in the micelles, the micelles should swell and the motion of bile acids might be restricted to some extent (12). Therefore, the influence of phospholipids and cholesterol on the bile acid spectra was investigated next.

As shown in **Fig. 3** and **Fig. 4**, the line width of C-18 and C-25 bile acid peaks increased with increasing phosphatidylcholine concentrations, but peak areas were unaffected. Neither line width nor peak areas of these peaks were altered by the addition of cholesterol (data not shown).

Application to measurement of total and taurine-conjugated bile acids in rat bile

The total bile acid levels obtained by NMR measurement (C-18) were compared to those obtained by enzyme-fluorimetry using 3α-hydroxysteroid dehydrogenase in rat bile samples (7). The results showed an excellent linear relationship between the total bile acid levels obtained by the two methods (**Fig. 5**). Furthermore, the change in the peak area of C-25 after addition of known amounts of TCA to the sample matched the amount of TCA added (data not shown), ascertaining the accuracy of the NMR method for quantifying taurine-conjugated bile acids.

On the other hand, the line widths of C-18 and C-25 from real rat bile were found to be slightly broader than those of model bile, suggesting that real bile contains some paramagnetic metal ions, such as Fe^{2+} and Mn^{2+} (26, 27). Even so, chemical shifts and peak areas of C-18 and C-25 did not differ between real and model bile.

We also applied the present NMR method to the measurement of sequential changes in total and taurine-conjugated bile of a taurine-depleted rat bile. Because the conjugation of bile acids depends on the taurine concentration in the liver, the ratio of taurine-conjugated bile acids increases after administration of taurine (28). The effects of infusions of CA and taurine on the composition of bile acids are clearly shown in **Fig. 6**. In this experiment, aliquots of 0.45 ml of each sample were measured as obtained. If the samples were adequately diluted with aqueous solution, lipid complexes in the sample would become

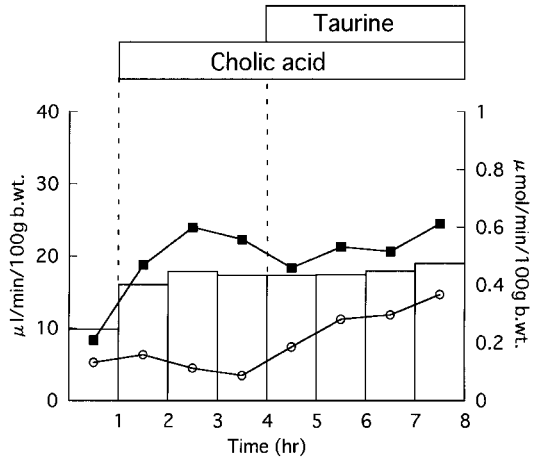

Fig. 6. Changes in the levels of total bile acids (■), taurine-conjugated bile acids (○), and bile flow volume (bars) in a β-alanine-treated rat after administration of cholic acid and taurine. Saline (1.0 ml/h/100 g body wt) was continuously infused through the tail vein during the experiment, and cholic acid (0.7 μmol/min/kg body wt) and taurine (0.7 μmol/min/kg body wt) were added to the perfusate during the period indicated. A bile sample was collected through a biliary cannula every hour. The concentrations of total bile acids and taurine-conjugated bile acids were determined by the peak areas of C-18 and C-25, respectively. Taurine-conjugated bile acids accounted for about half of total bile acids at the start of infusion. Bile flow volume and total bile acid levels increased during infusion of cholic acid. Taurine-conjugated bile acid levels increased markedly after infusion of taurine in addition.

smaller and the quantification of bile acids would become more precise. Indeed, we have confirmed that the linearity of the peak areas of C-18 and C-25 are preserved after dilution.

In conclusion, we confirm that the present NMR method can be applied to quantitative assay of total and taurine-conjugated bile acids in rat bile. As glycine-conjugated bile acids can generally be determined as the difference between total bile acids and taurine-conjugated bile acids (28), conjugation patterns (taurine/glycine ratio) of bile acids can be measured at once. The present technique should be extendable to human bile as these mostly consist of 3α-hydroxycholanic acid, as in rat.

Manuscript received 21 September 1998, in revised form 25 February 1999, and in re-revised form 16 June 1999.

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