Accurate and simultaneous analysis of six glycine/taurine conjugated bile acids in human bile using 1H NMR spectroscopy

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SYNOPSIS: Glycocholicacid, glycodeoxycholicacid, glycochenodeoxycholicacid, taurocholicacid, taurodeoxycholicacid and taurochenodeoxycholicacid are identified for the first time in intact human bile using several 1D and 2D [^{1}H-^{1}H and ^{1}H-^{13}C] experiments at 400, 700 and 800 MHz and a method is proposed for their accurate analyses. ^{1}H and ^{13}C chemical shifts for ten standard bile acids were determined under physiological conditions and used for unambiguous identification of bile acids in the complex mixture of human bile. Simple one step method for accurate analyses of individual bile acids in bile presented herein may have implications in the study of bile acid metabolism associated with biliary diseases.

INTRODUCTION: Bile acids are implicated in several important physiological functions, including cholesterol homeostasis, lipid absorption, enabling bile flow that help in the excretion and recirculation of drugs, vitamins, and endogenous and exogenous toxins. Abnormal and varied composition of the conjugated bile acids results in various hepato-gastrointestinal diseases. Defects in bile acids synthesis/excretion cause cholestasis, or impaired bile flow. Bile acids are considered to be involved in the development of biliary tract carcinoma and colorectal cancer through DNA damage. However, the underlying molecular mechanisms of the role of involvement of individual bile acids are unclear. Convenient and rapid measurement of bile acids becomes an important component in understanding their role in the pathophysiology of the diseases. Commonly used methods for the quantification of conjugated bile acids usually involve tedious steps such as extraction, hydrolysis, derivatization and/or purification before analyses. NMR spectroscopy offers a straightforward method for the quantification of metabolites in bio-fluids. However, the major difficulty in quantification of conjugated bile acids in human bile arises from the severe overlap of signals among the bile acids and also with other biliary metabolites. This makes identification and quantitative estimation of individual bile acids difficult. Recently we have reported a simple and accurate method of quantifying total glycine- and total taurine conjugated bile acids in human bile (1). In continuation of this study, we present herein for the first time identification of six major conjugated bile acids using several 1D and 2D NMR experiments at 400, 700 and 800 MHz and their accurate and simultaneous quantification individually using simple one pulse ^{1}H NMR spectra.

MATERIALS AND METHODS: Human bile was collected from the gall bladder from 30 patients undergoing cholecystectomy (laparoscopic or open) for symptomatic gall stone disease. Sodium salts of glycocholic acid (GCA), glycodeoxycholic acid (GDCA), glycochenodeoxycholic acid (GCDCA), taurocholic acid (TCA), taurodeoxycholic acid (TDCA) and taurochenodeoxycholic acid (TCDCA), tauroursodeoxycholic acid (TUDCA), cholic acid (CA), deoxycholic acid (DCA), and chenodeoxycholic acid (CDCA), cholesterol, deuterium oxide (D_2O), and trimethylsilylpropionic acid sodium salt-d_4 were purchased from Sigma-Aldrich, USA. ^{1}H, ^{13}C 1D spectra, DQF-COSY, TOCSY, ^{1}H-^{13}C multiplicity edited HSQC and ^{1}H-^{13}C HMBC spectra were obtained for all the standard bile acids in aqueous media at 400 MHz spectrometer under the physiological pH region of human bile (7.4 ± 0.1). From the analysis of these spectra a library of ^{1}H and ^{13}C chemical shifts for all the standard bile acids was established. Subsequently, 1D and 2D NMR experiments for human bile were obtained at 400, 700 and 800 MHz spectrometers. From the rigorous analyses of all the NMR spectra and in comparison with the library of ^{1}H and ^{13}C chemical shifts for several bile acids, individual bile acids in human bile were identified. To further confirm all the individual bile acids identified in the human bile, 1D ^{1}H spectra were obtained before and after addition of known bile acids. After the unambiguous identification, the conjugated bile acids in intact human bile were quantitatively analyzed using the integrals of the amide signals obtained by deconvolution and following the simple procedure established recently (1). Accuracy and precision of analyses of six major bile acids were determined from the spectra obtained before and after addition of known quantities of standard bile acids.

RESULTS: Standard bile acids: ^{1}H and ^{13}C spectra of all individual bile acids were similar due to the close similarities in their molecular structures. However, ^{1}H and ^{13}C chemical shifts at and near hydroxyl substitution or epimerization were significantly different and, based on these differences, the ring moieties of four groups of bile acids [(a) CA, GCA, TCA; (b) DCA, GDCA, TDCA; (c) CDCA, GCDCA, TCDCA; and (d) TUDCA] could be distinguished. Further, distinction within each group of bile acids could be made based on the signals arising from those in the vicinity of glycine or taurine conjugation of the conjugated bile acids. **Human bile:** Rigorous analysis of human bile using 1D and 2D NMR experiments at 400, 700 and 800 MHz NMR instruments and in comparison with the data from standard bile acids resulted in identification of six major bile acids (GCA, GDCA, GCDCA, TCA, TDCA and TCDCA). All these six conjugated bile acids were invariably seen in all the bile specimens. Although, there was overlap of ^{1}H and ^{13}C signals of most of the bile acids, amide NH signals which invariably appeared in the region 7.8-8.1 ppm in ^{1}H NMR spectra distinctly differentiated them individually (Figure). The assignments were further confirmed from ^{1}H 1D spectra obtained before and after the addition of standard bile acids. Quantitative estimation of the bile acids determined from the peak integrals of individual amide signals from the spectra before and after addition of a known quantity of standard bile acids into human bile showed excellent precision and accuracy with the recovery ranging from 93 - 99 %.

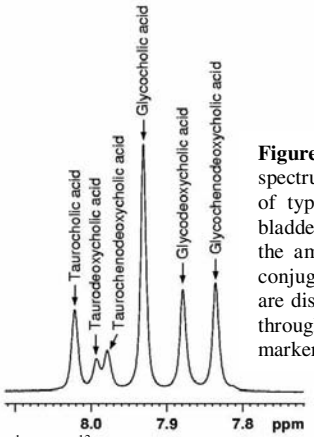

Figure: ^{1}H NMR spectrum at 800 MHz of typical human gall bladder bile showing the amide signals. Six conjugated bile acids are distinctly identified through their amide marker signals.

DISCUSSION: In order to identify the individual bile acids in human bile unambiguously, we first made *in vitro* ^{1}H and ^{13}C characterization of ten standard conjugated and unconjugated bile acids in aqueous media under physiological pH conditions. Subsequently, we have identified six conjugated bile acids in human bile from the rigorous ^{1}H and ^{13}C NMR study of bile at 400, 700 and 800 MHz spectrometers, and in comparison with the library of ^{1}H and ^{13}C chemical shifts data established for this purpose. Distinct amide signals of the six conjugated bile acids are shown here as the markers of the bile acids (Figure). After their identification, a simple method for quantitative estimation of all the bile acids, in human bile, accurately and simultaneously is presented which makes use of one dimensional single pulse ^{1}H NMR spectra obtained after addition of a few microlitre of 6N hydrochloric acid to bring down the pH anywhere in the range 6 ± 0.5 so as to avoid attenuation of the amide signals due to exchange of amide protons with water (1). It is for the first time that we have identified six conjugated bile acids and shown a simple and rapid method of accurately quantifying all these bile acids, individually. The outcome of this study may have immense potential in the *in vivo* NMR assessment of human bile to examine bile acid metabolism non-invasively that is currently inaccessible.

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REFERENCE

Quantification of glycine and taurine conjugated bile acids in human bile using ^{1}H NMR spectroscopy
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