Rapid and simultaneous analysis of all major constituents in human bile using 1H NMR spectroscopy

O. B. Ijare[1], B. S. Somashekar[1], G. A. Nagana Gowda[1], A. Sharma[2], V. K. Kapoor[2], C. L. Khetrapal[1]

[1]Centre of Biomedical Magnetic Resonance, LUCKNOW, UTTAR PRADESH, India, [2]Department of Surgical Gastroenterology, Sanjay Gandhi Postgraduate Institute of Medical Sciences, LUCKNOW, UTTAR PRADESH, India

SYNOPSIS: One step analysis of major bile components in intact bile is difficult due to broad and overlapping NMR signals. This problem is overcome by using dimethylsulfoxide which results in resolved NMR signals. Using rigorous 1D and 2D NMR studies at 400 and 700 MHz, distinct markers for cholesterol, bile acids and glycerophospholipids were identified and used for quantitative analyses of major constituents of bile in a single step. Accurate, rapid and simultaneous analyses of major bile components shown here for the first time may have immense implications for pathophysiology, diagnosis and prognosis of biliary and intestinal diseases.

INTRODUCTION: Major constituents of human bile are glycerophospholipids, bile acids and cholesterol. Variation in its composition causes various hepato-gastrointestinal diseases. Hence measurement of bile composition is important for the study of pathophysiology, diagnosis and prognosis of the diseases associated with abnormal bile composition. Currently available methods invariably involve tedious steps such as extraction, hydrolysis, derivatization and/or purification before analyses. Further, separate procedures are required for the analysis of glycerophospholipids, cholesterol and bile acids. Efforts were made to quantitatively estimate major bile constituents using ^{1}H NMR spectroscopy. However, in intact bile, ^{1}H NMR signals severely overlap due to the presence of several molecular species and, further, this overlap problem becomes compounded due to the broadening of ^{1}H NMR signals since all major biochemicals in bile exist in micellar form. This has been the major impediment in the spectral assignment and accurate quantitative analysis of major bile constituents in a single step using NMR. In this study we show that, by disrupting the micelles in the bile, well resolved NMR signals could be obtained in dimethylsulfoxide (DMSO) and all the major bile constituents could be identified and accurately quantified in a single step.

MATERIALS AND METHODS: Human bile was collected from the gall bladder from 25 patients undergoing cholecystectomy (laparoscopic or open) for symptomatic gall stone disease. Cholesterol, phospholipid, sodium salts of glycocholic acid, taurocholic acid, deuterated acetone, deuterated acetonitrile and deuterated dimethylsulfoxide were used in this study were obtained from Sigma-Aldrich (USA). ^{1}H spectra of human bile were obtained in all the three deuterated polar organic solvents, acetone, acetonitrile and dimethylsulfoxde. In dimethylsulfoxide, which showed well resolved signals for all the major biliary constituents such as cholesterol, lipids, and bile acids, complete spectral assignment was made using rigorous NMR study using two dimensional experiments such as ^{1}H-^{1}H DQF-COSY, ^{1}H-^{1}H TOCSY, ^{1}H-^{13}C multiplicity edited HSQC and ^{1}H-^{13}C HMBC at 400 and 700 MHz spectrometers. ^{1}H spectra in DMSO were also studied as a function of concentration of water and pH, separately, and monitored the variation of signal intensity for conjugated glycine and taurine amide protons. Major bile components were quantitatively analyzed using integrals of characteristic signals with reference to the signal intensity of trimethylsilylpropionate (TSP) taken in a reusable co-axial capillary. Precision and accuracy of quantitation was determined for total conjugated bile acids using one dimensional single pulse ^{1}H NMR experiments performed before and after addition of known quantities of standard bile acids. Precision and accuracy for total cholesterol and glycerophospholipids were determined by comparing the quantities estimated by one pulse ^{1}H NMR in the bile in DMSO with that determined after extraction following Folch's method.

RESULTS: Among the polar organic solvents used in the study, only DMSO solvent gives well resolved signals for all major bile constituents (Figure). From the assignment made using ^{1}H and ^{13}C 1D and 2D experiments, it is found that glyceryl 2-CH signal of glycerophospholipids (5.07 ppm), H-18 methyl signal of cholesterol (0.63 ppm) and H-18 methyl signals of bile acids (conjugated and unconjugated) (0.570-0.585 ppm), and amide signals of taurine and glycine conjugated bile acids (7.70 and 7.12 ppm, respectively) are the distinct marker signals for quantitative estimation of lipids, cholesterol, total bile acids (conjugated and unconjugated), respectively (see Figure). Excellent linearity, precision and accuracy were obtained from the quantitation of the metabolites obtained in one step using the integrals of the characteristic signals ($R^2 > 0.99$ for all). Further, to distinguish between total conjugated and total unconjugated bile acids, amide signals integrals were used. The amide signal intensity of total taurine conjugated bile acids represented the true concentration in pure DMSO while total glycine conjugated bile acids showed attenuated intensity. However, this attenuated signal gains its full intensity in presence of water anywhere in the range 10-60 %.

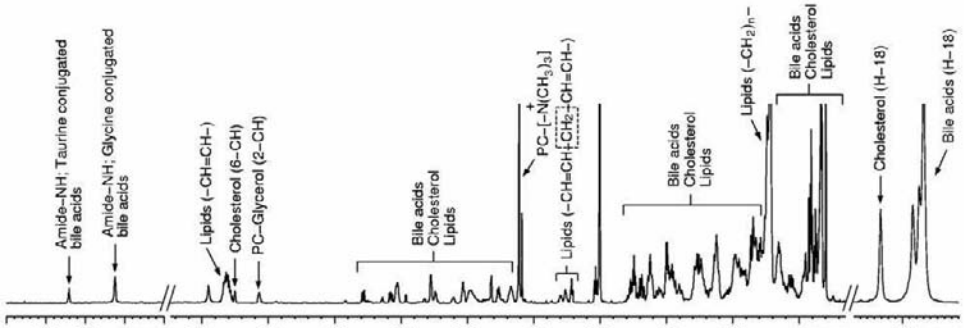

Figure: ^{1}H NMR spectrum of typical human bile in DMSO solvent; well resolved signals for major biliary constituents are marked.

DISCUSSION: Major difficulty in quantification of all bile constituents simultaneously, from NMR spectra, arises from the overlap of a large number of signals of biliary metabolites. Existence of glycerophospholipids, bile acids and cholesterol in the form of micelles in intact bile results in broad signals in ^{1}H NMR spectra thus making quantitative analysis further complicated. In this work, we have studied NMR spectra of bile in different polar organic solvents to get well resolved signals. Our study shows that, among the various solvents employed, dimethylsulfoxide gives the best results. Distinct marker signals were clearly observed for all major bile metabolites thus allowing their accurate quantitation in a single step. Thus, from a single measurement using single pulse 1D ^{1}H NMR sequence glycerophospholipids, cholesterol and total bile acids could be determined. Additional spectrum obtained in presence of about 10% water further enabled distinction between conjugated and unconjugated bile acids and, between glycine and taurine conjugated bile acids. The demonstration that all major bile metabolites can be quantified accurately, rapidly and simultaneously using ^{1}H NMR would have immense implications for the study of pathophysiology of hepatobiliary diseases.

ACKNOWLEDGMENT: This work was supported by Research Grants from Department of Science and Technology, Government of India. Use of 700 MHz NMR facility at NMR Research Centre, Indian Institute of Science, Bangalore, India, is gratefully acknowledged.