Quantification of components of bile acids in human bile and proton HR-MAS spectroscopy of gall bladder tissue: a route for diagnosis of gall bladder malignancy

B. S. Somashekar[1], O. B. Ijare[1], G. A. Naganagowda[1], A. Sharma[2], V. K. Kapoor[2], R. Roy[3], and C. L. Khetrapal[1]

[1]Centre of Biomedical Magnetic Resonance, Sanjay Gandhi Postgraduate Institute of Medical Sciences campus, LUCKNOW, UP, India, [2]Surgical Gastroenterology, Sanjay Gandhi Postgraduate Institute of Medical Sciences, LUCKNOW, UP, India, [3]NMR Laboratory, Division of Sophisticated Analytical Instrumentation Facility, Central Drug Reserach Institute, LUCKNOW, UP, India

SYNOPSIS: Human bile samples (n=81) from patients suffering from chronic cholecystitis (CC) (n=67), xanthogranulomatous cholecystitis (XGC) (n=6) and gallbladder cancer (GBC) (n=8) were subjected to NMR analysis and nine biliary components which included cholesterol, glycerophosphocholine, urea and six conjugated bile acids were quantified. Among all the bile samples, GBC bile samples contained more urea. The univariate analysis indicated urea to be statistically significant (P<0.01). The ^{1}H HR-MAS tissue spectra showed marked increase in the choline containing compound signals of GBC when compared to CC. We propose that the biochemical changes in bile acids are independent of Gall bladder tissues in GBC.

INTRODUCTION: Human bile is a complex mixture consisting of phospholipids and cholesterol, which exist as micelles or vesicles and several primary and secondary bile acids, which play a key role in the solubility of fats, fat soluble vitamins and cholesterol. The concentration and composition of these constituents vary in several hepatobiliary diseases such as gall stone disease and gall bladder cancer. Analysis of these various biliary components is important in understanding the pathophysiology and diagnosis of these diseases. With this aim, we have initiated the NMR studies on human bile and reported novel NMR methods for the quantitation of biliary constituents.[1-5] Detailed characterization of human bile using one- and two-dimensional NMR has been carried out. Using the distinct and characteristic amide proton signals, we have demonstrated that these bile acids can be quantified individually and accurately in a single step. With an objective to understand the biochemical information of human bile and gall bladder tissue in the case of malignant and nonmalignant gall bladder diseases, we have carried out the *in-vitro* ^{1}H NMR studies of human bile and ^{1}H HR-MAS of gall bladder tissue and the results are discussed.

MATERIALS AND METHODS: Human bile samples were collected from the gall bladder from the patients undergoing cholecystectomy (laparoscopic or open) for symptomatic gall stone diseases. Subsequently few gall bladder tissues were also stored under liquid nitrogen until HR-MAS NMR experiments were performed. Bile samples were stored in sterile dark conditions at -80°C until the NMR experiments were performed. Deuterated dimethyl sulfoxide (DMSO-d_6), deuterium oxide (D_2O) and trimethylsilylpropionic acid sodium salt-d_4 (TSP) were purchased from Sigma-Aldrich (USA). The pH of the neat bile samples were adjusted to 6.0 and NMR experiments were performed on a Bruker Biospin Avance 800 MHz NMR spectrometer using broadband inverse cryoprobehead. A reusable coaxial capillary tube containing known concentration of TSP in D_2O, which served as chemical shift as well as quantitative reference was inserted into the NMR tube before obtaining the NMR spectra. One-dimensional ^{1}H NMR experiments were performed with and without homonuclear decoupling (of glycine and taurine CH_2 protons of conjugated bile acids) by using single-pulse sequence with water presatuation during relaxation delay of 6s and from the characteristic marker signals, the concentrations of six individual conjugated bile acids were measured.[5] ^{1}H NMR spectra were also obtained on a Bruker Biospin Avance 400 MHz NMR spectrometer for all human bile samples (20µL) dissolved DMSO-d_6 (500µL). Using the distinct marker signals cholesterol, total bile acids, glycerophosphocholine and urea were quantified.[4] The HR-MAS experiments were carried out on a Bruker Biospin Avance 400 MHz NMR spectrometer using ^{1}H/^{13}C dual HR-MAS probehead with z-shielded gradient at magic angle. One-dimensional ^{1}H NMR on 25-35 mg of gall bladder tissue were performed using NOESY pulse sequence with water presaturation during relaxation delay of 2s in a 4-mm rotor at a spinning speed of 4.0 KHz.

RESULTS: From the characteristic marker signals in 400 MHz ^{1}H NMR spectra of human bile (20 µL) dissolved in DMSO-d_6, cholesterol, total bile acids, glycerophosphocholine and urea were quantified[4] and from the characteristic marker signals in the 800 MHz ^{1}H NMR spectra of neat bile, six conjugated bile acids viz., glycochenodeoxycholic acid, glycodeoxycholic acid, glycocholic acid, taurochenodeoxy cholic acid, taurodeoxycholic acid and taurocholic acid were quantified.[5] Among all the cases, GBC bile samples showed more urea (Mean±SD), (4.99±2.81 mmol) as compared to that of XGC (4.1±1.95 mmol) and CC (2.86 ± 1.02 mmol). The univariate analysis were carried out for all the quantified components, among all urea to be statistically significant (P<0.01). Figure 1 shows a part of 400 MHz ^{1}H NMR spectra of human bile (20 µL) dissolved in DMSO-d_6 of CC, XGC and GBC showing relative intensities of urea signal. ^{1}H HR-MAS experiments for some of the gall bladder tissues were carried out and observed a marked increase in the choline containing compounds in the cases of gall bladder malignant tissues as compared to that of benign. Figure 2 shows ^{1}H HR-MAS spectra of malignant and benign gall bladder tissue showing marked increase in the choline containing compounds in malignant tissue as compared to benign.

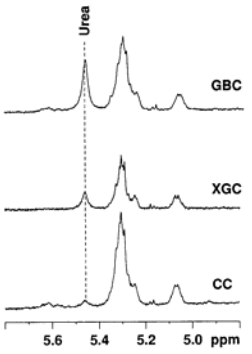

FIGURE 1: Parts of 400 MHz ^{1}H NMR spectra of bile (20µL) dissolved in 500 µL of CC, XGC and GBC showing relative concentration of urea.

DISCUSSION: Gall bladder cancer is a potentially lethal disease and accounts for 0.3 to 0.7% of all cancers. It is usually associated with untreated gall stone disease. The detailed characterization of bile components and quantitation of metabolites revealed that urea to be a marker for the differential diagnosis of GBC. It is for the first time, we are reporting HR-MAS of gall bladder tissues, which showed high concentration of choline containing compounds in GBC tissues when compared to CC. The detailed analysis of both human bile and gall bladder tissues suggested that the biochemical components of bile acids in bile are independent of tissue biochemistry. We propose that NMR analysis of both bile and tissue can be an additional diagnostic tool for the evaluation of GBC along with the histopathological examinations.

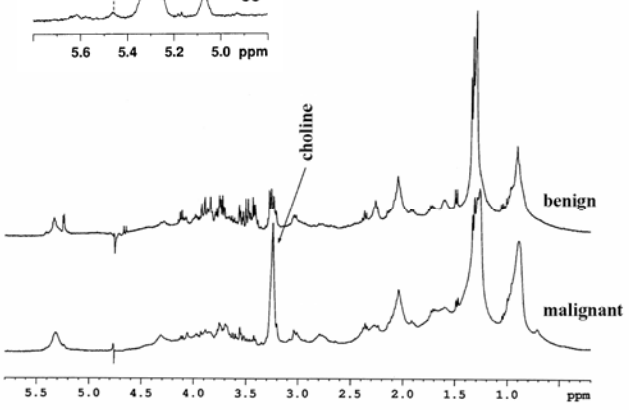

FIGURE 2: ^{1}H HR-MAS spectra of malignant and benign gall bladder tissues showing elevated levels of choline containing compounds in malignant tissue.

REFERENCES:

1. Ijare OB et al. *Magn. Reson. Med.* 2005, **53(6)**:1441-1446
2. Srivatsava M et al. *Anal. Lett*. 2005, **38(13):** 2135-41
3. Ijare OB et al. *Lipids* (2005), **40:** 1031-1041
4. Nagana Gowda GA *et al. Lipids* (2006), **41:** 577-589
5. Nagana Gowda GA *et al. Lipids* (2006), **41:** 591-603