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Nuclear magnetic resonance spectroscopy to determine the micellar cholesterol in human bile

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The cholesterol of gallstones comes from the vesicular rather than the micellar phase of bile. Progress in this field has been limited because conventional analytical methods disturb the distribution of cholesterol between the two phases. The resonance of the cholesterol C_6 proton occurs at a chemical shift of 5.4 ppm, to be shown by 2D NMR to be specific for biliary cholesterol, and arises only from the micellar mode. Thus integration of the C_6 proton resonance peak area provides a direct non-invasive determination of the cholesterol distribution in human bile.

Biliary cholesterol; Cholesterol NMR; Micelle; Vesicle

1 INTRODUCTION

Cholesterol is virtually insoluble in water and consequently its transport in an aqueous medium such as human bile is dependent on aggregation with other biliary lipids. The major aggregates in bile are vesicles of phospholipid and cholesterol and mixed micelles of phospholipid, bile acid and cholesterol. Recently much evidence has accumulated to show that cholesterol from the vesicular aggregates may be deposited to form stones in the gallbladder [1,2]. However, studies of cholesterol transport modes in human bile have been hampered by the lack of an appropriate and accurate analytical procedure. In particular, physical separation techniques such as ultracentrifugation and gel chromatography inevitably disturb the equilibrium distribution of cholesterol between micelles and vesicles, whilst quasielastic light scattering techniques cannot readily be applied to concentrated native gallbladder bile. Nuclear magnetic resonance spectroscopy may be used to analyse biological fluids without physical processing. Here we report the identification, characterisation and quantitation of cholesterol in native human gallbladder bile by proton nuclear magnetic resonance spectroscopy (^{1}H NMR). We also show that cholesterol is detectable by ^{1}H NMR only from micelles. Thus, we propose a method to measure directly the contribution of the micellar (and indirectly the vesicular) cholesterol in human bile.

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2. MATERIALS AND METHODS

We used a JEOL GSX500 NMR spectrometer (11.74 Tesla) operating at 500 MHz to obtain proton NMR spectra. All spectra were recorded at 37°C. The sample was injected into a 4 mm diameter NMR sample tube and this was placed concentrically inside a 5 mm diameter tube which contained the external reference standard sodium trimethyl silyl-[$^2H_4$]-propionate (TSP, set at 0.0 ppm) in ^2H_2O to provide a field frequency lock. The large water resonance of aqueous solutions, particularly whole bile, was suppressed by the application of a gated secondary irradiation at the water frequency. These techniques avoided the need to add any reagents to the bile sample itself.

Standard solutions of phospholipid (25 mM phosphatidylcholine from egg yolk; Sigma) and cholesterol (10 mM; BDH) were prepared in deuterated methanol; 20 mM sodium taurocholate solution (20 mM; BDH) was prepared in ^2H_2O. Unilamellar vesicles were prepared by the co-evaporation technique [3]. After negative staining of the vesicle preparations with phosphotingstic acid transmission electron microscopy showed small vesicles measuring 40–100 nm diameter.

Gallbladder bile was obtained by direct peroperative needle aspiration of the gallbladder before clamping of the cystic duct. It was stored in the dark under sterile conditions and spectra were recorded within 24 hours after collection. Total cholesterol was measured in bile using a modified enzymatic method [5].

3. RESULTS

The spectra of cholesterol (Fig. 1), phospholipid and sodium taurocholate were assigned and the characteristic resonances for cholesterol confirmed using 2D homonuclear correlation spectroscopy (COSY). Cholesterol peaks were not detectable in any of the vesicular preparations. The phospholipid choline head group resonance (at 3.3 ppm) and fatty acid tail peak (at 1.4 ppm) were broad, indicating restricted molecular motions but became progresively sharper and more intense as the vesicular phospholipid /cholesterol ratio increased (Fig. 2). In contrast, pure phospholipid vesicles gave sharp intense peaks at all concentrations studied.

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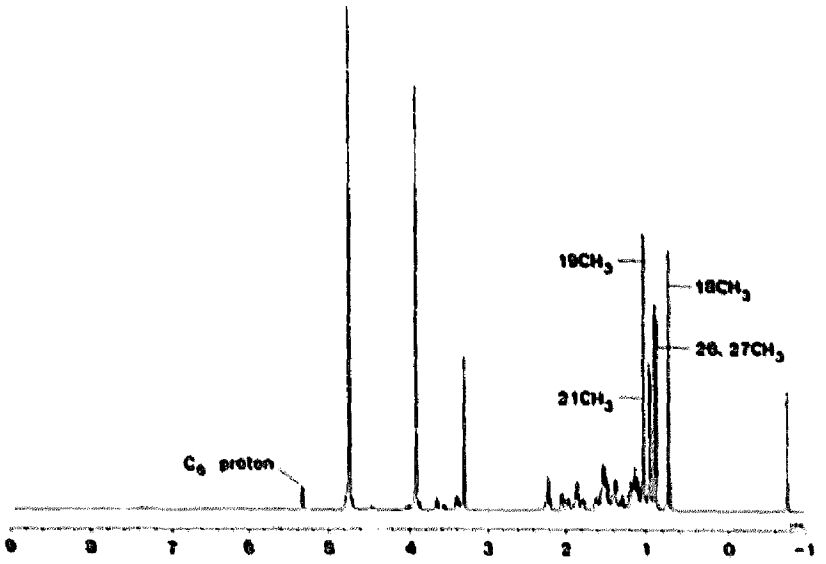

Fig. 1. ^{1}H NMR spectrum of cholesterol (15 mM) in CD_3OD with D_2O/TSP in external tube.

Integration of the resonances from the protons at positions of cholesterol C_{26} and C_{27} and that of the proton at cholesterol C_6, in alcoholic standard solutions of cholesterol, gave areas corresponding to the cholesterol concentrations. The doublet from the methyl groups at C_{26} and C_{27}, although identifiable, was obscured by several other peaks in all bile samples by several other peaks. The resonance of the cholesterol C_6 proton occured at a chemical shift of 5.4 ppm, a region of the bile spectrum relatively devoid of signals (Fig. 3). 2D spectra showed that minor peaks from phospholipid could be differentiated from the peak of the cholesterol C_6 proton (Fig. 4). Quantitation of micellar cholesterol was thus accomplished by measurement of the area under the C_6 peak by computer integration and reference to the external standard (TSP). After dilution of native biles with sodium taurocholate solutions, an increase was detected in the C_6 proton peak presumably due to increased solubilisation of cholesterol in the micellar phase.

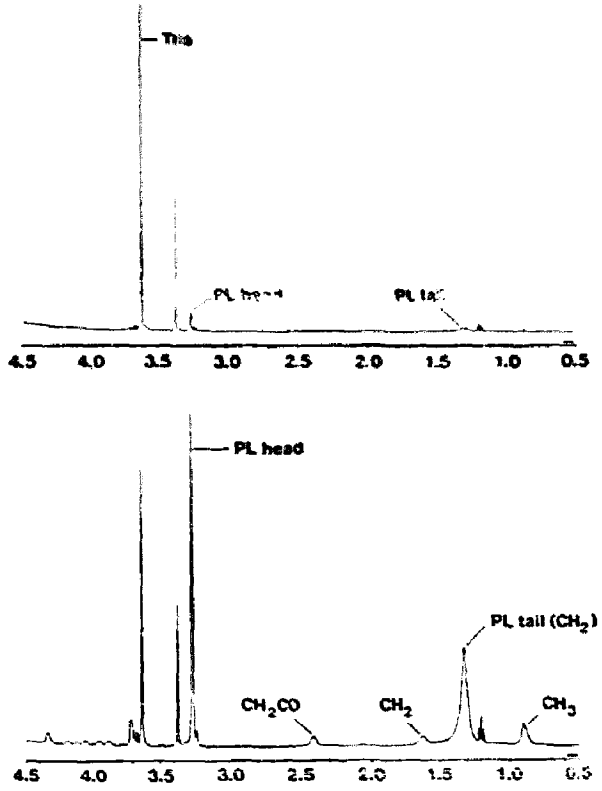

Fig. 2. ^{1}H NMR spectra of 5 mM PL/5 mM Ch vesicles (upper) and 20 mM PL/5 mM Ch vesicles (lower) in Tris buffer.

4. DISCUSSION

This work demonstrates that cholesterol may be identified in human bile and differentiated from other lipids present by resonances arising from the protons at C_6 and C_{26} and C_{27}. Cholesterol imparts rigidity to vesicle bilayers and leads to a reduction in molecular

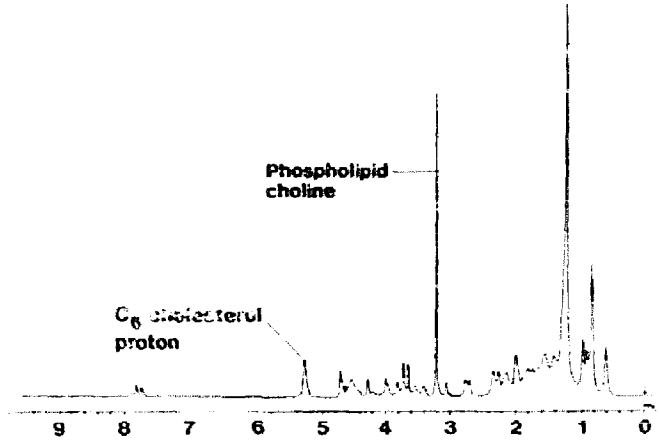

Fig. 3. ^{1}H NMR spectrum of human gallbladder bile.

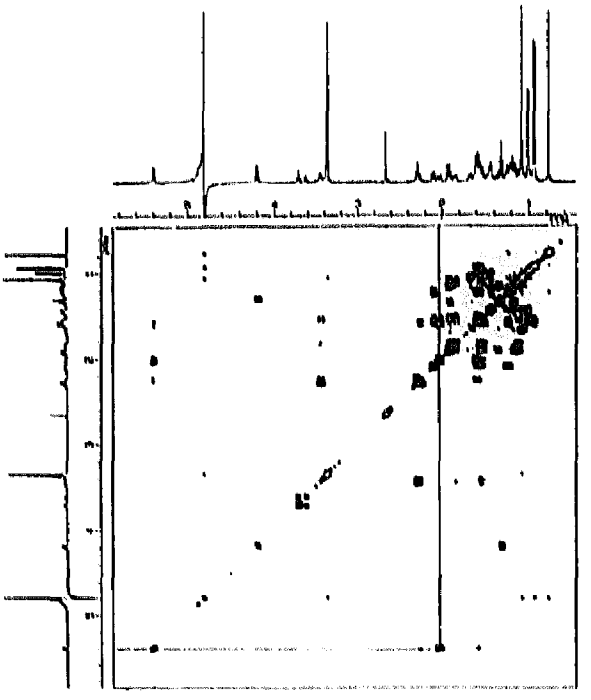

Fig. 4. 2D COSY spectrum of cholesterol in CD_3OD.

motion. This reduced motion gives rise to a greatly increased line width via T_2 relaxation and as a result, the signals are not observed in the NMR spectrum. We have reasoned, therefore, that the detectable cholesterol peaks in native bile arise from the micellar phase. Although the doublet arising from the methyl groups at positions 26 and 27 is often identifiable in human bile spectra, overlap with other lipid resonances makes measurement of the peak areas very inaccurate. In native bile spectra, the peak at 5.4 ppm has been shown to arise from the proton at cholesterol C_6, and may be integrated. The increase in intensity of this peak which results from the addition of bile acid to the sample indicates that the peak being measured indeed arises from the micellar portion of the cholesterol in bile. Although comparisons with currently used techniques such as density gradient ultracentrifugation and gel filtration may be made, they are of little value as these methods are widely acknowledged to be crude and irreproducible, because they change the very equilibrium they seek to measure [4]. The study of the distribution of cholesterol in bile by proton NMR provides a less invasive technique which may prove more accurate than older methods.

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REFERENCES

[1] Somjen, G.J. and Gilat, T. (1985) J. Lipid Res. 26, 699–704.
[2] Halpern, Z., Dudley, M.A. and Lynn, M.P. et al. (1986) J. Lipid Res. 27, 295–306.
[3] Groen, A.K., Goldhorn, B.G., Egbers, P.H.M., Chamuleau, R.A.F.M., Tytgat, G.N.J. and Bovee, W.M.M.J. (1990) J. Lipid Res. 31, 1315–1321.
[4] Donovan and Carey (1990) Hepatology 12, 94S–105S.
[5] Bolton, C.H., Nicholls, J.S. and Heaton, K.W. (1980) Clin. Chim. Acta 105, 225–230.