IDENTIFICATION OF BILE ACIDS AS THEIR DERIVATIVES BY 100 MHZ PMR SPECTROSCOPY*

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1. Introduction

Bile acids have been characterized and identified by mass spectrometry (ms) [1,2], gas liquid chromatography (glc) [3–5] and thin layer chromatography (tlc) [3,6]. Recently, Small, Penkett and Chapman [7] reported on the identification of bile acids by proton magnetic resonance (pmr) spectroscopy with a 60 MHz instrument, particularly with regard to the composition of micelles containing lecithin and salts of the bile acids. With concentrations of bile acids of 5 g per 100 ml of D_2O or $CDCl_3$ their studies were somewhat limited because of solubility. Since bile acids may occur in biological specimens at much lower concentrations, we have investigated the feasibility of analysis of microgram quantities with a 100 MHz NMR spectrometer. Results reported here demonstrate the ability to identify small quantities of four common bile acids as their methyl esters, as the trimethylsilyl (TMSi) ethers of these esters (derivatives commonly used in glc or glc- ms analysis), or as their combined TMSi ester-ethers.

2. Materials and methods

Purified samples of methyl lithocholate (L), chenodeoxycholate (CD), deoxycholate (DC), and cholate (C) were prepared from the free acids with diazomethane [5]. TMSi derivatives were prepared from these methyl esters, or from the free bile acids with a mixture of hexamethyldisilazane (HMDS), trimethylsilyl chloride, and pyridine [5]. Excess solvent and reagents were removed in vacuo prior to pmr studies. Samples of approximately 2 mg of the esters were dissolved in 0.5 ml of $CDCl_3$ containing 25% $CHCl_3$ and 1–2 μl of tetramethylsilane as internal references. The Varian Model HA-100 NMR spectrometer was locked on the chloroform signal and scanned upfield towards tetramethylsilane. For samples of lower concentration (~ 0.25 mM) repetitive scans were cumulated with a Model C-1024 time-averaging computer (CAT).

3. Results and discussion

The chemical shifts for the C-21, C-19 and C-18 methyl groups of the methyl esters of lithocholate (L), chenodeoxycholate (CD), deoxycholate (DC), and cholate (C) are given in Hz in table 1. The proton resonance of the methyl ester appeared in all cases at 366 Hz. The C-21 methyl group appeared as a doublet [7,8] with a coupling constant of about 6 Hz [8]. The deshielding of the 12α-hydroxyl group on this doublet is clearly shown by the values of 97 and 99 Hz for compounds DC and C in comparison with 92 Hz for the 12-deoxy derivatives (L and CD). The chemical shifts for the C-19 methyl group for the four compounds showed little variation (92–90 Hz), whereas the shifts for the C-18 methyl groups varied stepwise by 2 Hz between compounds CD, DC, and

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Table 1
Proton resonance of methyl groups of bile acid derivatives

Compound[a]	Methyl esters					
	Free alcohols			TMSi ethers[b]		
	C-18	C-19	C-21	C-18	C-19	C-21
L	65	92	91	64	92	92
CD	66	90	92	63	88	93
DC	68	91	97	65	89	94
C	70	90	99	64	88	94

[a] L = lithocholate; CD = chenodeoxycholate, DC = deoxycholate; C = cholate. All values are Hz relative to tetramethylsilane.
[b] Proton resonance for the TMSi reagents are: TMSiCl, 42 Hz; HMDS, 5–6 Hz.

C. This difference suggested that these three derivatives could be identified in a mixture, particularly at a sweep width of 50 Hz. The lower curve of fig. 1 is the summation of 140 scans of a mixture of approximately 50 μg of each of these three compounds (CD, DC and C) in 0.5 ml of $CDCl_3$–$CHCl_3$ (3:1). The upper curve (resolved scan) shows clearly the C-18 methyl groups of cholate (70 Hz), deoxycholate (68 Hz) and chenodeoxycholate (66 Hz). The C-19 methyl group of deoxycholate (91 Hz) was separated from those of cholate and chenodeoxycholate (90 Hz), and the doublet of the C-21 methyl group of each of the components could be distinguished. These data indicate that as little as 2.5 μg of each of these components could be detected in this manner in a 25 μl micro tube.

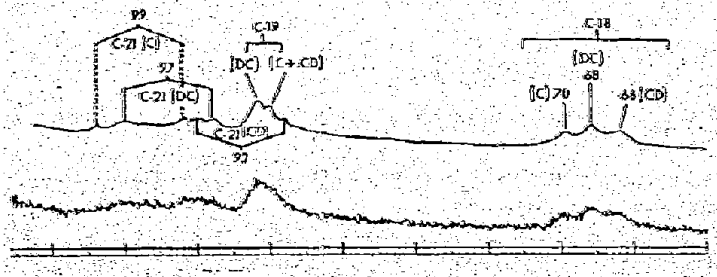

Fig. 1. 100 MHz PMR spectrum of a mixture of 50 μg each of methyl cholate (C), methyl deoxycholate (DC) and methyl chenodeoxycholate (CD) in 500 μl of $CDCl_3$–$CHCl_3$ (3:1). The lower tracing is the summation of 140 scans accumulated with a Model C-1024 CAT. The upper curve is the resolved scan; the proton resonances of the C-18, C-19 and C-21 methyl groups are expressed in Hz.

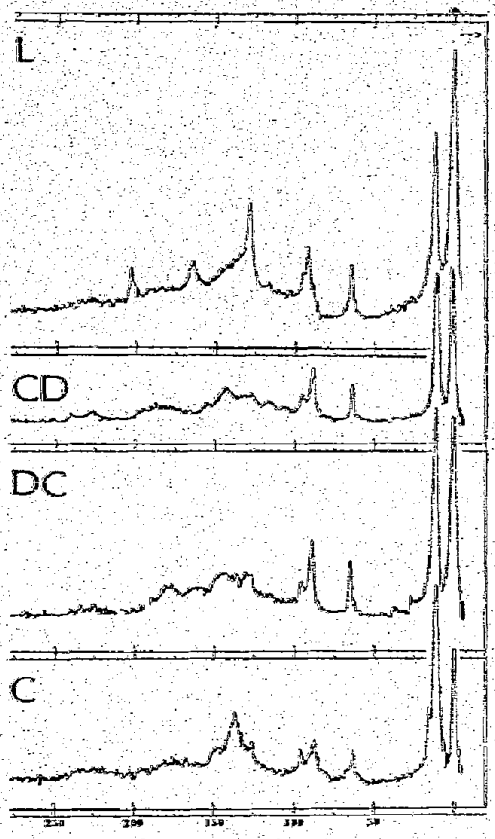

Fig. 2. 100 MHz PMR spectra of trimethylsilyl ethers of methyl hydroxy cholanates. From top to bottom: methyl lithocholate (L) (1.87 mg); methyl chenodeoxycholate (CD) (2.53 mg); methyl deoxycholate (DC) 2.32 mg); methyl cholate (C) (2.11 mg) in a total volume of 0.5 ml of $CDCl_3$–$CHCl_3$ (3:1).

A similar investigation was undertaken of the chemical shifts of the TMSi ethers of the methyl esters of these bile acids (table 1). The methyl ester persists at 366 Hz (fig. 3). The chemical shifts for the doublet of the C-21 methyl group and the proton resonance of the C-19 methyl group for these four derivatives now show little variation (92–94 Hz and 88–92 Hz, respectively) (fig. 2), while the shift for the C-18 methyl varies by 1–2 Hz (63–65 Hz). Since lithocholate and cholate are conveniently separated by several systems of tlc [3,6,9], and the dihydroxy acids (CD and DC) are difficult to separate, a mixture

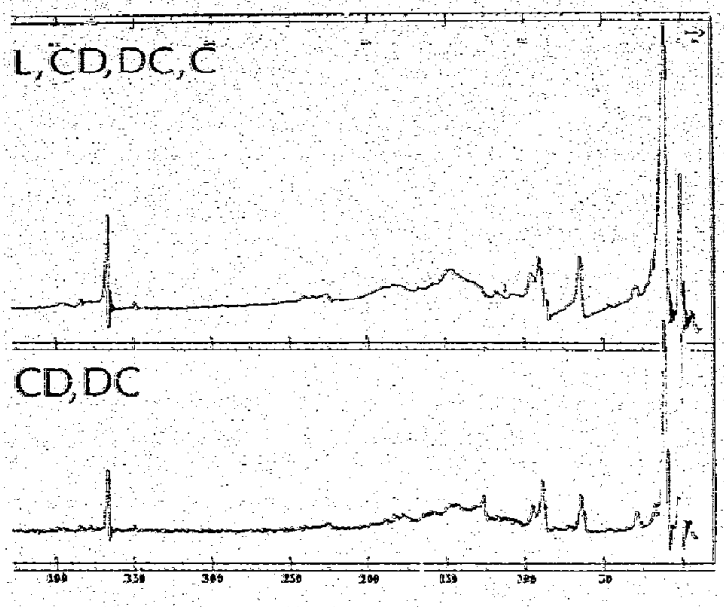

Fig. 3. 100 MHz PMR spectra of mixtures of trimethylsilyl ethers of methyl hydroxycholanates. Upper panel: Mixture of the four compounds; L (2.91 mg), CD (2.41 mg); DC (2.40 mg); C (2.09 mg). Lower panel: Mixture of compound CD (2.22 mg) and compound DC (2.67 mg).

of the TMSi ethers of these compounds (CD and DC) was prepared for pmr analysis (fig. 3). The single peak due to the C-18 methyl groups from the mixture of the four derivatives was not resolved at this concentration (~ 10 mM). However, the two peaks at 65 and 63 Hz, respectively, shown for the mixture of the two compounds (DC and CD) demonstrates the ability to distinguish one of these components in the presence of approximately an equal quantity of the other. The sharp peaks at 10–14 Hz are those of the TMSi ethers at C-3, C-7 and C-12. Similar peaks were observed for the TMSi derivatives of the free bile acids; the chemical shifts for the C-21, C-19 and C-18 methyl groups are the same (within 1 Hz) as those observed for the TMSi ethers of the methyl esters. The TMSi ester showed a strong sharp peak at 27 Hz. A subsequent manuscript will deal with these and other derivatives in greater detail. The TMSi derivatives are remarkably stable in $CDCl_3$; the pmr spectrum of a sample of the Tris TMSi ether of methyl cholate taken 38 days after its preparation was identical with that of the freshly prepared material.

Since completion of these studies, Leibfritz and Roberts [10] published cmr spectra of several bile acids and their esters in CH_2Cl_2, CH_2Cl_2—CD_2Cl_2 or CH_2Cl_2—dioxane at concentrations of 0.1–1 M. Hase and Hase [11] have reported the quantitative determination of organic hydroxyl groups by means of trimethylsilyl derivatives, particularly with methyl esters of hydroxy fatty acids and certain tertiary alcohols and phenols. Samples of 20–1000 mg were studied with a Varian A-60 NMR spectrometer; the height of the integrals of the TMSi signal was compared with that of an internal standard (*p*-nitroacetophenone). Dutton et al. [12] have undertaken similar studies on hydroxy acids of lower molecular weight, and suggest that measurements can be made directly on fractions isolated by gas–liquid chromatography. The procedures described here indicate that samples of methyl ester or methyl ester TMSi ethers (2–5 μg) prepared for use in analysis for glc or glc–ms are utilizable also for PMR analysis, by replacement of the solvent with $CDCl_3$—$CHCl_3$ (3:1) in concentrations of ~0.25 mM.

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