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^{1}H NMR studies of human blood plasma

Assignment of resonances for lipoproteins

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Single-pulse and Hahn spin-echo 500 MHz ^{1}H NMR spectra of human blood plasma and isolated chylomicrons, VLDL, LDL and HDL are reported. The comparison has enabled specific assignments to be made for the resonances of individual lipoproteins in the CH_2 and CH_3 (fatty acid), and NMe_3^+ (phospholipid choline head group) regions of the spectra of plasma (0.8–1.3 and ~3.25 ppm, respectively). Fasting, and freeze-thawing of plasma samples led to marked changes in the intensities and linewidths of lipid resonances. Analysis of lipid resonances in the spectra of plasma in terms of individual lipoproteins may shed new light on many conditions of clinical and biochemical interest.

^{1}H-NMR; Lipoprotein; (Human plasma)

1. INTRODUCTION

^{1}H NMR spectra of blood plasma consist of relatively sharp resonances from low-M_r metabolites superimposed on a broad protein envelope arising mainly from albumin and immunoglobulins [1–3]. Strong signals in the 0.8–1.3 ppm region have been assigned to CH_3 and CH_2 groups of 'mobile' fatty acid components of chylomicrons and lipoproteins.

It appeared from our Hahn spin-echo NMR studies that these resonances [1,2] contained several overlapping components. We report here the assignment of specific ^{1}H NMR resonances in 500 MHz spectra of human blood plasma to chylomicrons, VLDL, LDL and HDL, using single-pulse and especially spin-echo NMR spectroscopy. This was achieved by studies of isolated lipoproteins, of plasma from fasting subjects, and the effects of freeze-thawing of samples.

The work should make possible the simulation of ^{1}H NMR spectra for lipids in intact plasma and thereby aid the diagnosis of a variety of conditions of clinical and biochemical interest.

Our findings are discussed in the light of a recent suggestion by Fossel et al. [4] that the linewidths of lipid CH_2 and CH_3 resonances can be correlated with the presence ($\Delta\nu_{1/2}$ 29.9 ± 2.5 Hz) or absence (39.5 ± 1.6 Hz) of malignant tumours in human subjects.

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2. EXPERIMENTAL

2.1. *Plasma samples and lipoprotein preparation*

Human plasma samples from healthy volunteers were collected as described [1,2]. 'Fresh' samples were studied by NMR within 5 min of separating from whole blood. 'Frozen' samples were stored at −20°C for various times (6 h, 2 days, 2 weeks, 3

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weeks), and then thawed at room temperature immediately prior to NMR study.

Lipoproteins were fractionated by sequential preparative ultracentrifugation at densities of <0.95 (chylomicrons), <1.006 (VLDL), 1.006–1.063 (LDL) and 1.063–1.21 (HDL) g/ml at $105\,000 \times g$ according to Cortese et al. [5].

Plasma and isolated HDL samples were treated with increasing concentrations of $Na_3Fe(CN)_6$ (BDH, 0.1–1 mM, added as μl aliquots of a 100 mM solution in D_2O).

2.2. *NMR spectroscopy*

All spectra were acquired using a Bruker AM500 spectrometer (MRC Biomedical NMR Centre, Mill Hill) operating at 500 MHz in quadrature detection mode and a probe temperature of 298 K. Each spectrum corresponds to 32–48 free induction decays (FIDs), using 16384 data points, 50–60° pulses and a 5 s pulse repetition rate. Hahn spin-echo spectra [6] were acquired using a t value of 60 ms in the sequence (90°-t-180°-t-collect). The large H_2O signal was suppressed either by continuous secondary irradiation or by a 3.5 s presaturation pulse at the H_2O resonance frequency using the decoupler coils.

Peaks were referenced to internal TSP (sodium 3-trimethyl[2,2,3,3-$^2H_4$]propionate) added in D_2O (50 μl to 450 μl of sample). When present, the methyl resonances of lactate (1.330 ppm), alanine (1.487 ppm) or valine (1.050 ppm) served as secondary internal references. For Gaussian resolution enhancement, the Bruker parameters were LB = -0.5 Hz and GB = 0.4. In other cases an exponential function equivalent to a line broadening of 0.8 Hz was applied.

3. RESULTS

The 500 MHz ^1H NMR spectrum of normal heparinized human blood plasma consists of a broad envelope of overlapping resonances from macromolecules such as glycoproteins, immunoglobulins, albumin and lipoproteins, and small molecules including glucose, alanine, lactate and valine. An expansion of the aliphatic region from 0.5–1.5 ppm is shown in fig.1a. The most prominent resonances near 0.9 and 1.3 ppm are assignable to lipid CH_3 and CH_2 groups, respectively, by comparison with similar spectra from isolated lipoproteins [7–10]; those for VLDL and HDL are shown in fig.1b and c. The CH_3 and CH_2 resonances of HDL and LDL are shifted slightly to lower frequency and broadened compared to those of chylomicrons and VLDL. The same appears to be true for the less intense C_{18}, C_{19} and C_{21} methyl resonances of cholesterol. These features appear to be additive in the plasma spectrum. This heterogeneity in the chemical shifts for cholesterol in different lipoproteins makes the peaks too broad to assign in the plasma spectrum. At 298 K only HDL and LDL give rise to relatively sharp NMe_3^+ resonances at 3.25 ppm assignable to phospholipids, that for LDL being slightly broader ($\Delta\nu_{1/2}$ 17 Hz) than that for HDL ($\Delta\nu_{1/2}$ 10 Hz).

A clearer differentiation between lipoproteins is obtained using Hahn spin-echo spectroscopy in which only resonances from the most mobile CH_3 and CH_2 groups remain and those with short T_2 values ($< \sim 40$ ms) are filtered out. This is shown in fig.2. The progressive shifts of the CH_2 and CH_3 resonances of chylomicrons, VLDL, LDL and HDL to low frequency can again be seen. With this t value (60 ms) HDL does not contribute a CH_2 resonance and that for LDL is only a small shoulder (peak 5). Similarly, only HDL (and not LDL)

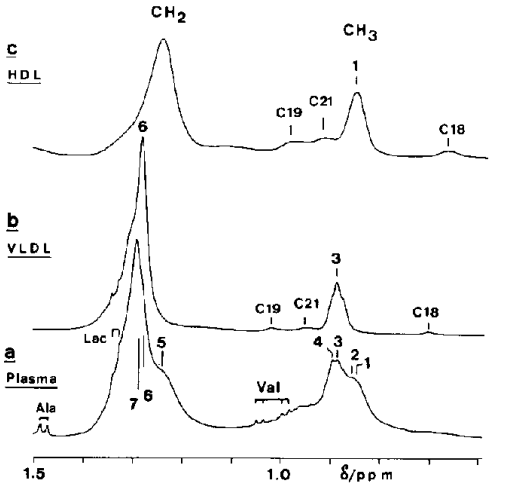

Fig.1. 500 MHz ^1H NMR spectra of (a) blood plasma, (b) VLDL, and (c) HDL. Assignments: Ala, alanine CH_3; Lac, lactate CH_3; Val, valine CH_3; C18, C19, C21, CH_3s of cholesterol (or ester); peaks 1–7, see fig.2. The broad CH_2 peak from HDL in (c) (not numbered) overlaps with the similar peak 5 from LDL.

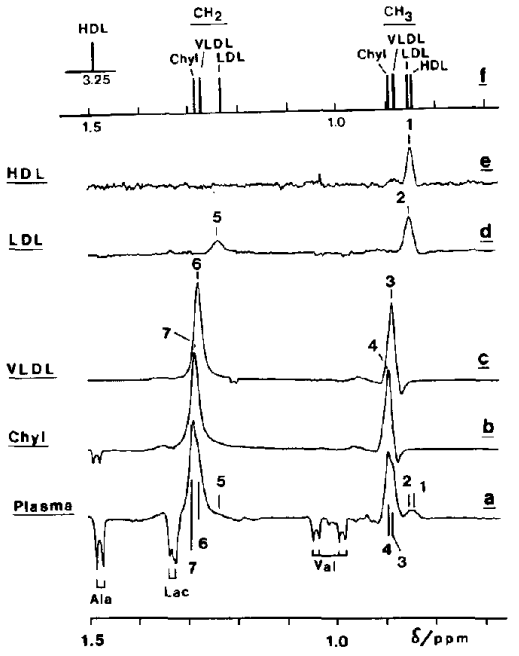

Fig.2. 500 MHz Hahn spin-echo ($t = 60$ ms) ^{1}H NMR spectra of (a) blood plasma (fresh) and (b–e) various lipoproteins. The assignments of spin-echo peaks obtained under these conditions are illustrated diagrammatically in (f).

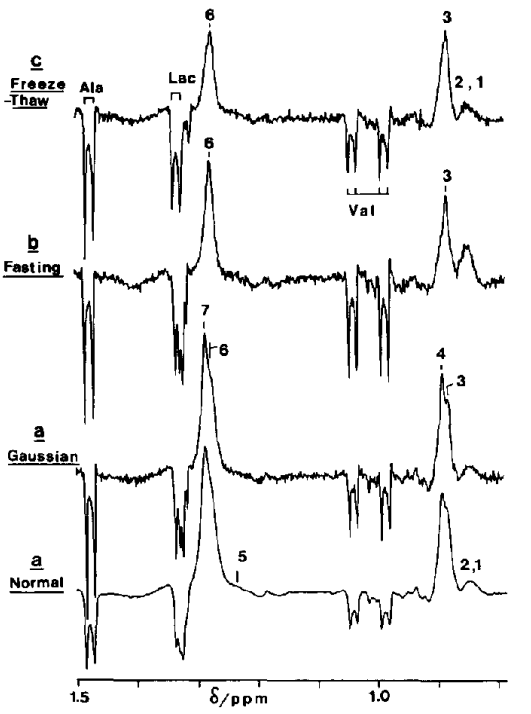

Fig.3. 500 MHz Hahn spin-echo ($t = 60$ ms) ^{1}H NMR spectra of (a) fresh blood plasma obtained using an exponential (normal) or Gaussian multiplication of the FID; (b) fresh plasma from the same subject after an overnight fast (Gaussian): and (c) the same plasma sample as for (b) after freeze-thawing (Gaussian).

contributes an $N(CH_3)_3^+$ resonance at 3.25 ppm (not shown). Thus, the assignments for spin-echo resonances can be deduced: overlapping peaks 1 (0.858 ppm) and 2 (0.863 ppm), to HDL and LDL, 3 (0.886 ppm) and 4 (0.894 ppm) to VLDL and chylomicron CH_3 resonances; peaks 5 (1.25 ppm), 6 (1.279 ppm) and 7 (1.285 ppm) to CH_2 resonances of LDL, VLDL and chylomicrons respectively, peak at 3.25 ppm to NMe_3^+ of HDL.

Gaussian resolution enhancement allows a clearer distinction to be made between peaks 3,4 and 6,7 in plasma (fig.3a). This spectrum was obtained from a fresh sample of plasma taken within 2 h of the subject eating a meal. Fresh plasma from the same subject after an overnight fast gave the spectrum shown in fig.3b. Resonances 4 and 7 attributable to chylomicrons have clearly decreased in intensity. We have observed similar changes to peaks 4 and 7 after freeze-thawing of plasma samples (fig.3c). The precipitates which appeared in samples after freeze-thawing were removed by centrifugation before spectral acquisition. No other changes in spin-echo peaks were observed, but in normal (single pulse) spectra both the CH_2 and CH_3 resonances broadened (from 28.1 to 38.4 Hz, and from 40.3 to 42.7 Hz, respectively). Prolonged storage at 4°C (3–4 days) also produced the same effects. Further cycles of freezing, storage at −20°C, and thawing led to progressive precipitation and further increases in the linewidths of the CH_2 and CH_3 resonances. Similar effects were also observed when EDTA rather than heparin was used as anticoagulant.

The addition of $Fe(CN)_6^{3-}$ to plasma produced low-frequency shifts (0.1 ppm at 5 mM) of the observable NMe_3^+ resonance in both normal and spin-echo spectra. No other lipoprotein resonances were affected. The effect on isolated HDL was similar.

4. DISCUSSION

^{1}H NMR spectra of lipoproteins at 60 MHz were first reported by Stein et al. [7] in 1968. Since then, spectra of chylomicrons, VLDL, LDL and HDL at higher frequencies (200, 220, 400 MHz) have been reported and resonances have been assigned [8–10]. As discussed recently by Hamilton and Morrisett [11], the spectra contain relatively few resolved resonances compared with the large number of magnetically distinct protons. The increase in resolution with observation frequency has appeared to be only modest.

The general appearances of our spectra of lipoproteins at 500 MHz are similar to those reported. However, by a careful comparison of chemical shifts and through the use of Hahn spin-echo spectroscopy we have been able to suggest new markers for individual lipoproteins that can be used to analyse the mixture found in blood plasma. There do not appear to be any previous reports of attempts to analyse mixtures of lipoproteins by ^{1}H NMR. ^{1}H NMR has distinct advantages (compared to e.g. ^{13}C NMR) for clinical purposes: small volumes, rapid spectrum acquisition.

Protein resonances are very broad and make little contribution to the spectra of LDL and HDL [9]. Both exhibit a resolved NMe_3^+ signal at 3.25 ppm due to mobile choline head groups of phospholipids. This resonance is broader for LDL than for HDL and disappears from the (t = 60 ms) Hahn spin-echo spectrum, and enables HDL to be distinguished from LDL. The CH_3 resonances from HDL and LDL (1,2) overlap, but only the CH_2 resonance LDL (5) appears in the spin-echo spectrum. It is not possible to say whether the NMe_3^+ protons and CH_3 protons seen in spectra of HDL belong to the same molecules (phosphatidylcholines).

The small low-frequency shifts of the LDL and HDL CH_3 and CH_2 resonances compared to chylomicrons and VLDL enable these to be distinguished. HDL and LDL are smaller particles than chylomicrons and VLDL (5–12 and 18–25 nm in diameter compared to 75–1200 and 30–80 nm, respectively) and contain higher proportions of protein (49 and 23% vs 2 and 8%, respectively), phospholipid (30 and 22% vs 4 and 12%, respectively) and cholesterol/cholesterol ester (17 and 46% compared to 9 and 21%, respectively). Chylomicrons (81%) and VLDL (53%) are richer in triacylglycerols than LDL (9%) and HDL (5%) [12]. Thus, the shift differences may be accounted for by the different type of fatty acids observed, packing of the particles, and possibly susceptibility effects.

All the mobile phospholipid choline head groups of HDL are accessible to the paramagnetic shift reagent $Fe(CN)_6^{3-}$ [13] whether isolated or in plasma, and appear to be on the surface of the particles.

It is difficult to make a clear distinction between fatty acid (largely triacylglycerols) resonances of chylomicrons and VLDL. Resonances 4 and 7 were assigned to CH_3 and CH_2 resonances, respectively, of chylomicrons since they decreased greatly in intensity in spectra of plasma from fasting subjects. Chylomicrons usually disappear from circulation after overnight fasting [14]. Clear decreases in intensities of these resonances also occur after freeze-thawing of plasma samples or prolonged storage of samples at 4°C. The overall linewidths of the lipid resonances also change.

Fossel et al. [4] have recently correlated the linewidths of these CH_3 and CH_2 resonances with the occurrence of malignant cancers. It is notable that the samples used in their study had been stored or freeze-thawed and that no dietary factors were considered. Our spectra of fresh plasma from normal healthy subjects fall closest to the 'cancer' category of Fossel et al. whereas the same samples after freeze-thawing lie in the 'normal' range. We have also detected linewidths for CH_3 and CH_2 resonances of <30 Hz, from plasma samples from subjects with types I and IV hyperlipidaemias (unpublished). These factors appear to complicate any attempt to obtain reliable correlation between linewidths and clinical conditions.

Our results suggest that a detailed analysis of the overlapping fatty acid CH_3 and CH_2 resonances of lipoproteins in ^{1}H NMR spectra of normal human plasma is possible. We have been able to simulate plasma spectra by addition of spectra of individual lipoproteins. This type of analysis may provide a valuable new approach to the study of conditions of clinical and biochemical interest.

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