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750 MHz ^{1}H-NMR spectroscopy of human blood plasma

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Abstract: The application of high-resolution 750 MHz ^{1}H-NMR spectroscopy to a biological fluid is demonstrated for the first time and its advantages over 600 MHz ^{1}H observation shown by reference to studies on human blood plasma. Improvements in signal dispersion were observed which facilitated improved signal assignments. Differences in lipid/lipoprotein signal line-widths between 600 and 750 MHz were noted indicating that ultrahigh field measurements may help to give insight into dynamic motional phenomena of lipids in whole plasma. The two-dimensional J-resolved (JRES) technique and spin-echo spectra measured at 750 MHz have enabled new signal assignments to be made in control plasma. The application of 750 MHz JRES to the clinical chemical problem of the detection of abnormal metabolites associated with chronic renal failure is also demonstrated.

Keywords: *750 MHz ^{1}H-NMR; spin-echo; J-resolved spectroscopy; blood plasma; uraemia; abnormal metabolites; aromatic amino acids; chronic renal failure.*

Introduction

High-resolution NMR spectroscopy has undergone many hardware developments in the last decade, such as the evolution of new high-field magnet technology, radio frequency electronics and novel probe designs, and these have resulted in ever increasing spectral sensitivity and dispersion. Parallel developments in software design and the evolution of new pulse-sequences to effect spectral editing and signal assignments have enabled a variety of complex biochemical problems to be addressed and solved [1, 2]. Perhaps the most effort in the production of new NMR spectrometers has been expended on the creation of new ultrahigh-field magnet technology; indeed, the superconducting magnet is the most expensive single item in an NMR spectrometer. The first 17.63 Tesla NMR magnets with high-field homogeneity were recently produced for NMR spectrometers operating at 750 MHz ^{1}H resonance frequency; such instruments would be expected to produce unparalleled performance with respect to sensitivity and signal dispersion. The sensitivity now attainable approaches *ca* 1 nM ml^{-1} for ^{1}H-NMR.

A diverse range of applications of high-field ^{1}H-NMR spectroscopy to biological fluids have been developed in the last 10 years, concerned with the investigation of toxicological processes, drug metabolism and clinical chemistry [3–10]. In previous studies the authors have shown that in order to obtain useful, clinically relevant biochemical information on blood plasma samples it is normally necessary to eliminate the broad resonances arising from macromolecules, by use of spin-echo pulse sequences or by physical pretreatment of the sample [3, 11–16]. Recently the authors have shown that two-dimensional J-Resolved (JRES) spectroscopy, when performed at very high field (14.1 T), can be a particularly efficient means of extracting data on low MW compounds in urine and blood plasma [9]. The authors now report the first application of 750 MHz single-pulse, spin-echo and JRES ^{1}H-NMR spectroscopy for assigning the metabolite resonances more completely and

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for studying the biochemical composition and interactions of compounds in normal and uraemic human blood plasma.

Methods and Materials

Samples and sample-preparation

Blood was taken from a healthy male volunteer with normal renal function and from a uraemic patient with chronic renal failure (CRF). The patient had a plasma creatinine level of 1043 $\mu M\ l^{-1}$ and a blood urea level of 33.7 $mM\ l^{-1}$, but was not undergoing treatment by dialysis and drug therapy was limited to calcium carbonate supplementation. Whole blood was collected by venepuncture into lithium heparinized vacutainers and the plasma separated by centrifugation and frozen prior to use. The samples (0.7 ml) were diluted (10% v/v) with D_2O to establish a field frequency lock. Proton signals were referenced internally to the α-glucose H_1-resonance at δ 5.233 already present in each sample.

^{1}H-NMR spectroscopy

Single-pulse and Carr Purcell Meiboom Gill (CPMG) spin-echo ^{1}H-NMR spectra were obtained on blood plasma samples using either a Bruker AMX600 spectrometer (Bruker Analytische Meßtechnik GmbH, Silberstreifen, Germany) operating at the ^{1}H resonance frequency 599.945 MHz (14.1 T), or a Bruker AMX750 spectrometer operating at the ^{1}H resonance frequency 750.143 MHz (17.63 T). All spectra were measured at ambient probe temperature. At 750 MHz, 128 free induction decays (FIDs) were collected as 64 K data points with a spectral width of 10,000 Hz. In single-pulse experiments 90° pulses were used with an acquisition time of 3.28 s and a total pulse recycle time of 5.28 s. The water signal was suppressed by using the one-dimensional NOESY (nuclear Overhauser effect spectroscopy) presaturation pulse sequence applied at the water resonance frequency for 2 s in the delay between 90° pulses. This type of water-suppression pulse sequence results in attenuation factors of 10^5 or more for water signals in biofluid samples, thus avoiding the potential problem with limited dynamic range. No further physical or chemical treatment of the sample is necessary to ensure water suppression. Prior to Fourier transformation (FT), an exponential apodization function was applied to the FID, corresponding to a line broadening of 0.5 Hz. The CPMG pulse sequence is as follows:

$$D\ [-90° - (\tau - 180° - \tau)_n - \text{acquire FID}],$$

where: D = 1 s (to allow T_1 relaxation); τ = 343 μs [a fixed delay to allow spectral editing via T_2 relaxation (attenuation of broad signals) and refocusing of spin-coupled multiplets]; n = a fixed loop of 128 cycles, giving a total spin–spin relaxation delay $2n\tau$ of 87.8 ms. The CPMG spin-echo experiment allows spectral editing according to molecular mobility and permits the attenuation of signals from macromolecules [1, 10].

Homonuclear two-dimensional J-resolved ^{1}H-NMR spectroscopy (JRES) [17]

750 MHz ^{1}H-JRES spectra of blood plasma were obtained by sequential repetition of the pulse sequence:

$$[D - 90° - t_1 - 180° - t_1 - \text{collect FID for time } t_2],$$

where D = 2 s; t_2 was 0.507 s; and t_1 was an incremented variable delay to allow modulation of the spin–spin couplings. A secondary irradiation field was applied at the water resonance frequency during delay D, which was gated-off during the application of the pulse sequence and acquisition. The F2 (chemical shift) domain was collected as 8192 data points with a spectral width of 8064 Hz, while the F1 (J-coupling) domain spectral width covered 63 Hz with 64 increments of t_1. Typically eight transients were collected for each t_1 increment in the JRES experiments. Prior to the double FT, the F1 data were zero-filled to 1024 data points and the data were apodised by means of a sine-bell function in t_2 and a sine-bell squared function in t_1. The spectra were tilted by 45° to ensure orthogonality of the chemical shift and coupling-constant axes, and symmetrized about the F1 axis. Following a magnitude calculation, spectra were displayed both in the form of contour plots of the F1 and F2 domains and as skyline F2 projections.

Results and Discussion

600 and 750 MHz single-pulse and CPMG ^{1}H-NMR spectra of blood plasma

Even at 750 MHz single-pulse ^{1}H-NMR

spectra of human blood plasma do not give helpful data with respect to most of the low MW components, due to the extensive overlap with signals from broad resonances from macromolecules such as proteins (particularly albumin and immunoglobulins) and lipids [1] [see Fig. 1(a)]. Application of the CPMG spin-echo pulse sequence to plasma yields spectra in which protein ^{1}H resonances are mostly absent, except those from the acetyl methyl signals from the *N*-acetylated sugar moieties of glycoproteins such as α_1-acid glycoprotein [12] [Fig. 1(b)], which are relatively motionally unconstrained. Some of the stronger lipid signals from lipoproteins and chylomicrons are also detected in the CPMG spectrum, but these are heavily attenuated [Fig. 1(b)].

Many signals from low-MW species that are readily detected in the CPMG experiment cannot be resolved easily in the single-pulse experiment, e.g. lactate, glucose, acetate, valine, and other amino acids. The authors have previously found the related Hahn spin-echo (HSE) approach to be a useful method

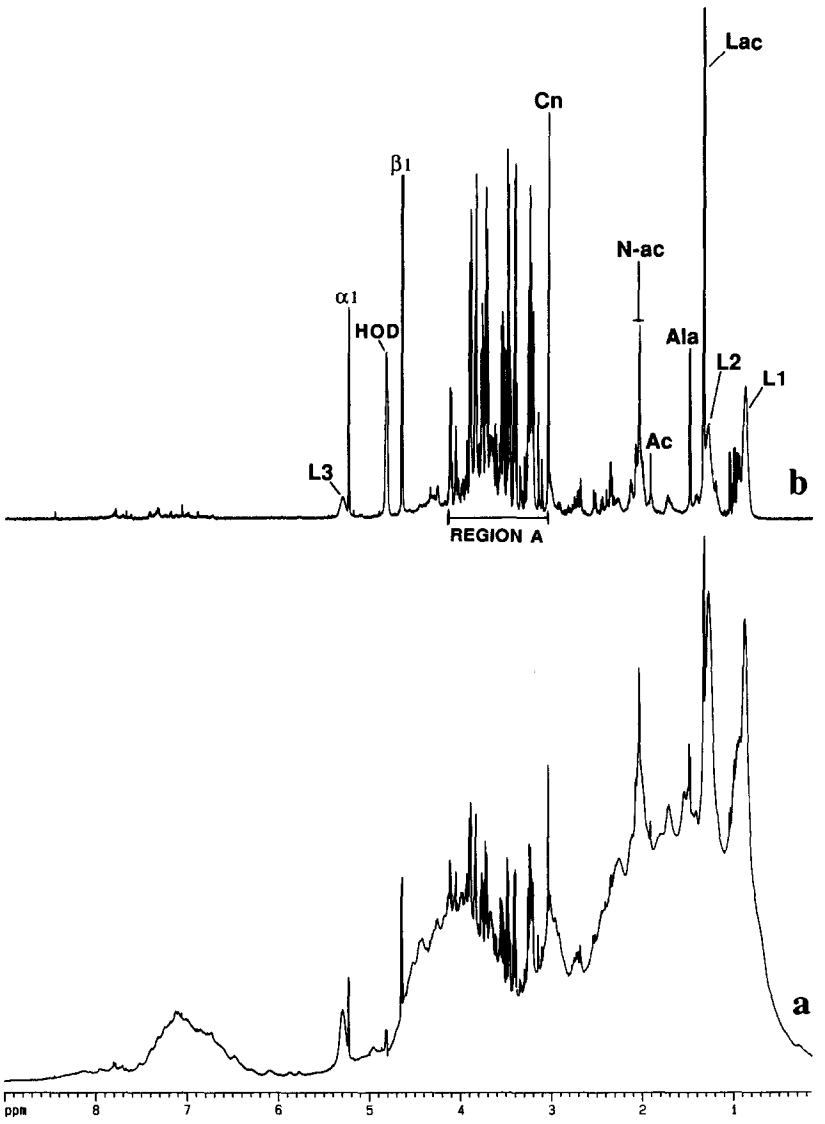

Figure 1
Comparison of (a) the 750 MHz single-pulse ^{1}H-NMR spectra of human blood plasma from a uraemic patient; and (b) the CPMG spin-echo spectrum of the same (128 scans in each case). Key to assignments: L1, L2, L3, terminal CH_3, $(CH_2)_n$ and CH=CH groups of mobile lipids and lipoproteins; Lac, lactate; Ala, alanine; Ac, acetate; N-ac, acetyl groups of N-acetylated sugars on glycoproteins and sialic acids; Cn, creatinine; HOD, residual water signal; α_1 and β_1, H_1 proton signals of α and β anomers of glucose. Region A contains many overlapped resonances from glucose, other sugars and polyols, *N*-methylated compounds and amino acids.

for eliminating protein resonances and for increasing the biochemical information content of the spectra of low-MW components of blood plasma [11–13]. These methods have proved to be qualitatively reliable when using the internal calibration method based on standard additions. The HSE experiment, unlike the CPMG spin-echo methods results in phase-modulation of spin–spin coupled multiplets. Although this can be useful as a signal assignment aid, it may also considerably complicate signal quantitation, particularly with complex or second-order spin–spin coupling patterns [12]. A serious problem in HSE spectra also arises for certain metabolite signals (such as glutamine and glucose) which may be cancelled out due to partial overlap of signals that are opposite in phase.

In earlier ^{1}H-NMR studies [15] performed at lower field strengths (9.4 T), the authors did not readily observe resolved signals from aromatic amino acids, either in single-pulse or in HSE spectra (with $2n\tau$ = 136 ms) of normal human blood plasma, unless the pH was lowered to <3 by the addition of dilute HCl. Signals from aromatic amino acids and histidine were only clearly detected and resolved in 400 MHz HSE spectra of pathological plasma samples, such as those from patients with significant liver damage, as in Wilson's disease [15] or paracetamol overdose [16]. In such patients aromatic amino acid concentrations tend to be higher, and plasma protein levels lower, than in controls. It was concluded that in normal plasma at near neutral pH, the major fraction of the 'free' phenylalanine, tyrosine and a significant proportion of the histidine was in fact bound to plasma proteins such as albumin [15]. Such binding results in restricted molecular motion (slow tumbling) in solution and/or chemical exchange between bound and free fractions. In consequence, the T_2 relaxation times of protons from these aromatic amino acids, histidine and their derivatives are shorter than those observed in solutions containing no protein.

At the higher frequencies used here (i.e. 600 or 750 MHz) signals for free phenylalanine, tyrosine and histidine, 1-methylhistidine, 3-methylhistidine, *p*-hydroxyphenyl-lactic acid and certain other unassigned metabolites are clearly detected in the CPMG spectra of plasma at neutral pH from the uraemic patient (Fig. 2). Histidine signals are also well resolved in the CPMG spectra of normal plasma, whereas tyrosine signals are weak and phenyl-

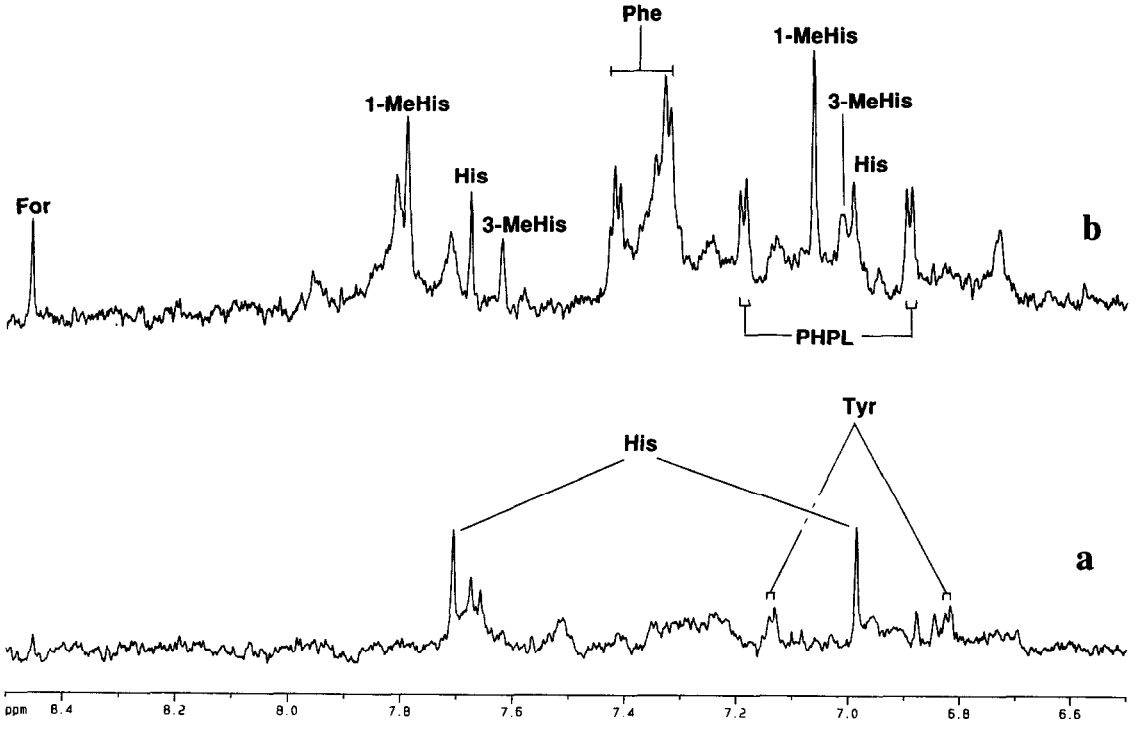

Figure 2
Aromatic region of the 750 MHz CPMG spin-echo spectrum of (a) normal human blood plasma and (b) uraemic plasma. Key to assignments: Tyr, tyrosine; His, histidine; Phe, phenylalanine; For, formate; 1-MeHis, 1-methyl histidine; 3-MeHis, 3-methyl histidine; PHPL, *p*-hydroxyphenyl-lactate.

alanine was not detected under the solution and spectrometer conditions used here (Fig. 2). The much stronger contributions of the metabolites to the aromatic region of the CPMG spectra of uraemic plasma and the presence of abnormal metabolites such as *N*-methyl histidine indicates an accumulation of motionally unconstrained (i.e. non-protein bound) substances due to the incipient renal failure. The observation of well-resolved signals for histidine and tyrosine in the normal plasma may be consistent with a different observational time-scale of the chemical exchange processes on going from 400 to 600 or 750 MHz, i.e. the exchange rate appears to have moved from intermediate to slow on the NMR time-scale. The signal-to-noise ratio is still low in the aromatic region of the control spectrum because the protein-bound fraction is still largely non-observable due to the shortening of the T_2 relaxation times imposed by the motional constraint of macromolecular binding. The advent of very high field NMR spectrometers will do much to benefit this area of the dynamic study of small to large molecule interactions in biological fluids and opens up the possibility of observing intermolecular nuclear Overhauser effects and the use of saturation transfer experiments to probe protein binding sites of small molecules.

Partial 600 and 750 MHz CPMG spectra of normal human blood plasma collected with the same number of scans and under identical processing conditions (i.e. exponential line-broadening of 0.5 Hz) are compared in Fig. 3. Both dispersive and dynamic changes are detectable on going to higher field. For instance, at 600 MHz the two leucine methyl signals are overlapped at δ0.96 giving the appearance of a triplet, whereas at 750 MHz they are dispersed to give a pair of doublets (resolution enhancement shows this more clearly). The resolution of a multiplet at δ1.2 from a long-chain fatty acid moiety from a lipoprotein (probably very low density lipoprotein, VLDL and low density lipoprotein, LDL) is also superior at 750 MHz. There is also a significant improvement in the dispersion and hence resolution of the many spectral lines in the region of the spectrum from δ1.95 to 2.1 corresponding to signals from N-acetylated sugars of glycoproteins and sialic

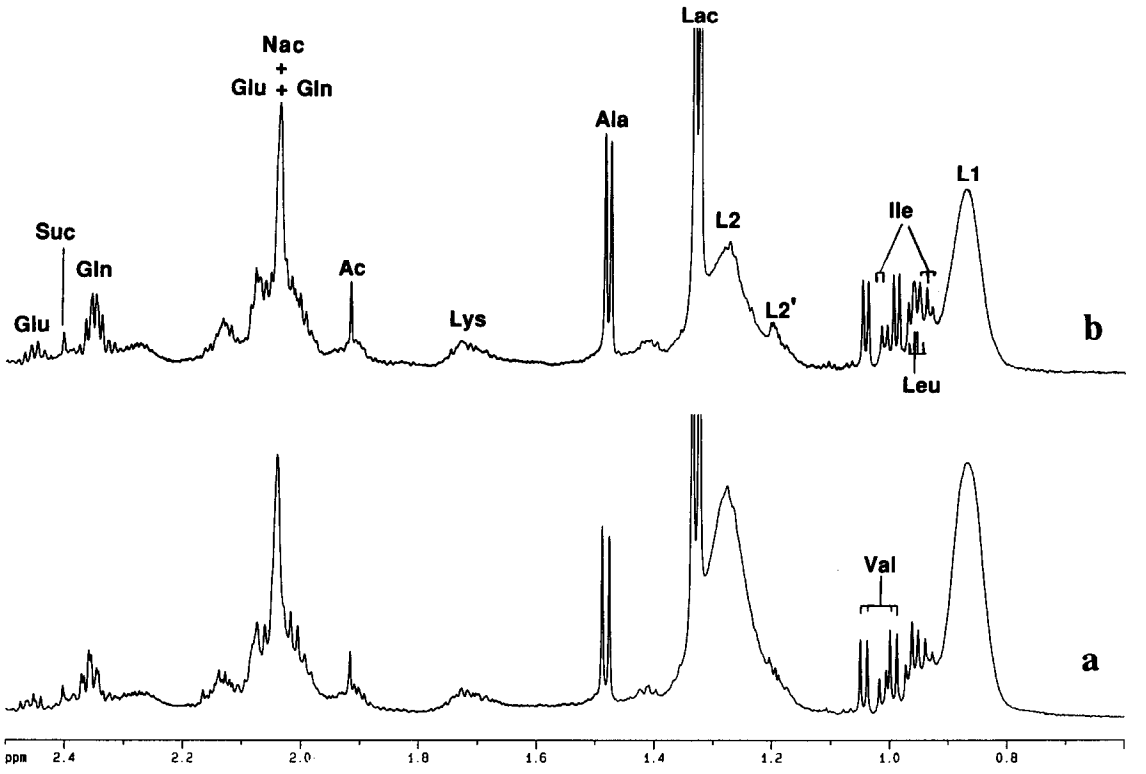

Figure 3
Comparison of the partial (a) 600 and (b) 750 MHz ^{1}H-NMR CPMG spin-echo spectra of uraemic human blood plasma. Key to assignments: as in Fig. 1, plus: L2′, extra resolved $(CH_2)_n$ group of lipid component; L2′ and L2 are signals from LDL and VLDL. Leu, leucine; Ile, isoleucine, Gln, glutamine; Glu, glutamate; Suc, succinate, Lys, lysine; Val, valine.

acids and the β-CH_2 signals of glutamine and glutamate. There appear to be a maximum of eight resolved signals at 600 MHz and 14 resolved signals at 750 MHz even without the use of resolution enhancement methods. At 600 MHz the γ-CH_2 signal from glutamine gives a doublet of triplets at δ2.36 whereas at 750 MHz this has been simplified because of the larger frequency difference between the two protons of the methylene group.

In addition to the added dispersion of the low-MW metabolite signals at 750 MHz, there is also a substantial reduction in the intensity and line-broadening of the lipid signals at *ca* δ 0.84 and 1.25 in the 750 MHz spectra. In the single-pulse spectrum, signals in these regions arise from the overlapped terminal CH_3 and long-chain $(CH_2)_n$ groups of fatty acids integral to the various lipoprotein particles, triglycerides and chylomicrons. In the CPMG spectrum from a plasma sample collected after an overnight fast, as in this case, the signals probably arise mainly from VLDL and LDL.

The line-widths of these and other lipid signals contributing to this region of the single-pulse NMR spectrum have been measured in a number of previous studies and it has been suggested that the arithmetic mean line-width of the two composite signals may in some way relate to the presence of metastatic tumours in the donor [18]. This work has been extensively reviewed elsewhere [19, 20] and the subject remains controversial, as the peaks are composites of several lipoproteins and lipids, together with a variety of low-MW species. Moreover, the line-widths are subject to many influences including temperature, the mode of sample preparation and spectrometer field strength. Clearly 750 MHz ^{1}H-NMR spectroscopy of plasma can be expected to do much to further the study of these interesting phenomena because of the significant improvement in dispersion and extension of the observed motional time-scale on which dynamic events can be studied.

750 MHz two-dimensional J-resolved spectroscopy of blood plasma

The authors have previously reported that 600 MHz JRES ^{1}H-NMR spectroscopy results in a dramatic simplification of biological fluid spectra and enables complex overlapped resonances in certain crowded chemical shift ranges (e.g. from δ3 to 4) to be resolved more completely. As the JRES experiment is a two-dimensional application of a spin-echo pulse sequence, there is also nearly complete attenuation of any broad macromolecular signals. Two regions of the 750 MHz ^{1}H-NMR JRES spectrum of normal human blood plasma are shown in Fig. 4 with signal assignments. The protein and lipid resonances are attenuated even more effectively than in the CPMG spin-echo experiment (Figs 1 and 2) and the skyline projection through the JRES map results in a greatly simplified spectral profile of the effectively fully ^{1}H-decoupled ^{1}H-NMR spectrum of the motionally-unconstrained low-MW metabolites in plasma (Fig. 4). The skyline projection could offer an attractive method for quantitating minor metabolites in plasma where attenuation due to T_2 relaxation can be accounted for or calibrated. The relative intensity pattern of the metabolite signals in the JRES spectra are likely to be of more diagnostic value than absolute intensities, as practical quantitation of JRES spectra requires internal calibration by standard additions of metabolites. It should be noted that signals from any small molecules that are extensively protein-bound are also severely attenuated due to constrained moelcular tumbling and a shortening of the T_2 relaxation time [15].

The authors have previously stated [15] that the use of JRES for spectral simplification of plasma would also be indicated in cases where abnormal sugar or polyol components are being examined, as resonances from these compounds largely occur in the complex spectral region from δ3 to 4 and this is very effectively simplified by this technique (Fig. 4). This region of the ^{1}H-NMR spectrum of any biofluid is perhaps the most complex (i.e. the richest in biochemical information) and has hitherto been difficult to study because of spectral interference due to glucose.

Detailed examination of the expanded and most complex region of the JRES spectrum of plasma from δ3 to 4.5 reveals a wealth of previously hidden signals amongst the complex glucose resonances. The authors have previously assigned [9] the signals from many other biochemically-important metabolites, including amino acids, glycerol, trimethylamine-*N*-oxide (TMAO), and choline in 600 MHz JRES spectra, their signals being undetectable in the single-pulse spectrum due to overlap with glucose; this list has now been augmented as seen in Fig. 4. For instance, it was possible to resolve and assign all of the

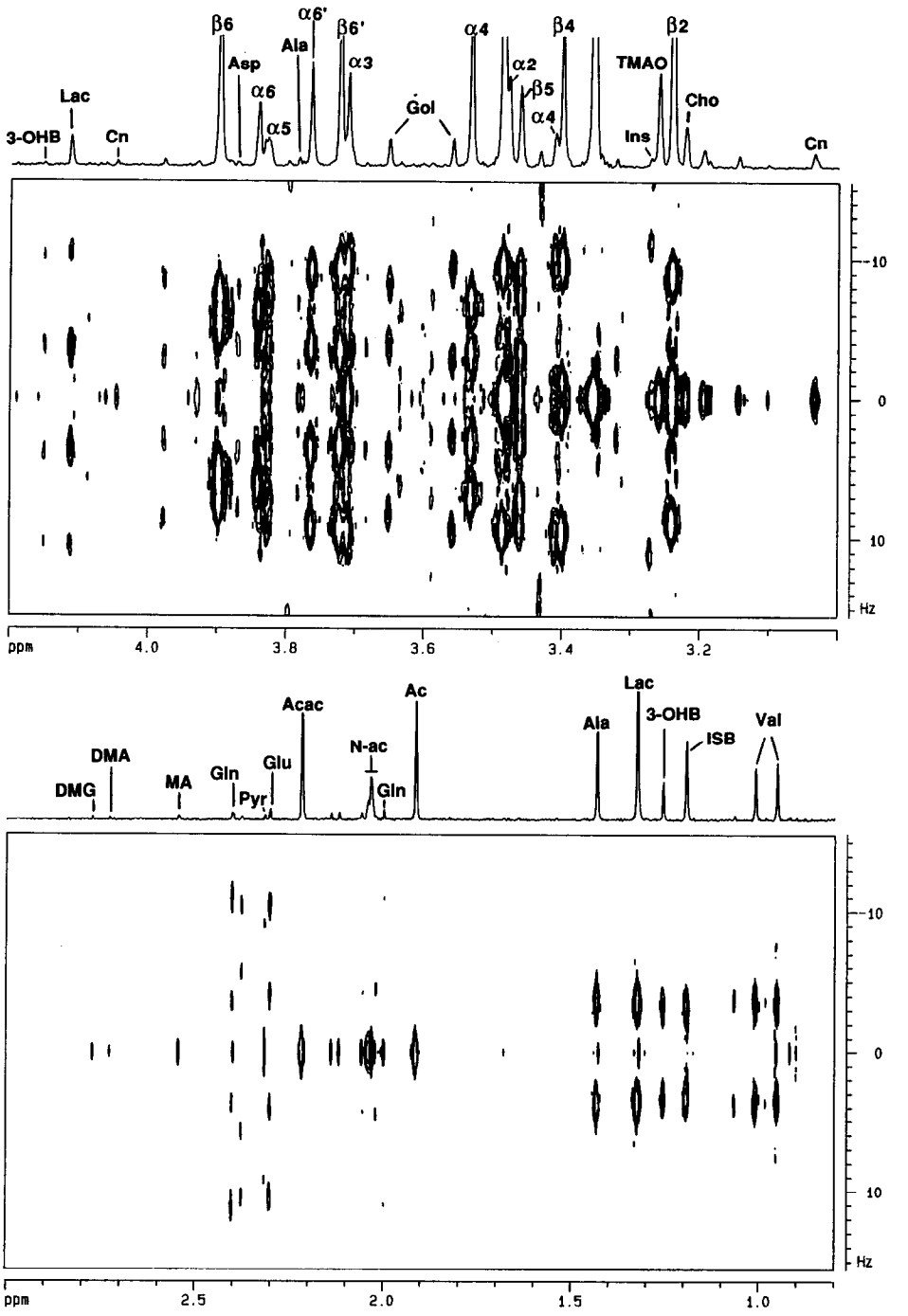

Figure 4
Contour plots of 750 MHz JRES ^{1}H-NMR spectrum of normal human blood plasma with skyline F2 projections above. Key to assignments: as in Figs 1 and 3, plus: ISB, isobutyric acid; 3-OHB, 3-D-hydroxybutyric acid; Acac, acetoacetate; Pyr, pyruvate; MA, methylamine; DMA, dimethylamine; DMG, dimethylglycine; Cho, choline (N-CH_3 group); TMAO, trimethylamine *N*-oxide; Ins, myo-inositol (H_5); Gol, glycerol; Asp, aspartate; signals enumerated α and β refer to the various proton signals of the two glucose anomers.

signals of α- and β-glucose in plasma using 750 MHz JRES spectroscopy. In a recent report [21] on the assignment of 400 MHz ^{1}H-NMR spectra of blood plasma, four of the signals (βH_6, αH_3, αH_5 and $\alpha H_6'$) were not assigned at all, and the βH_3 βH_4 and βH_5 signals were wrongly assigned, probably due to the insuperable problem of peak overlap at that field strength (9.4T) even when using JRES spectroscopy.

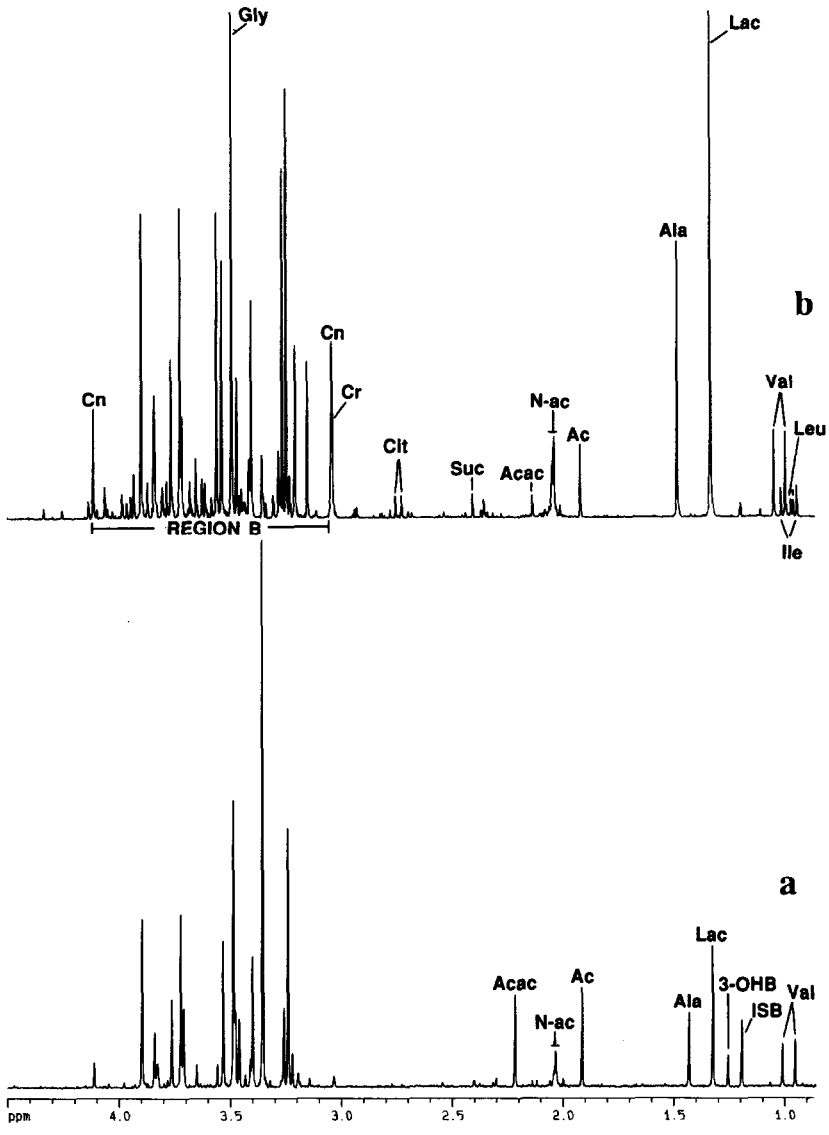

Figure 5
Comparison of the skyline F2 projections of 750 MHz JRES ^{1}H-NMR spectra of the aliphatic regions of: (a) normal; and (b) uraemic blood plasma. Key to assignments: as in Figs 1, 3 and 4 plus: Cit, citrate; Cr, creatine; Gly, glycine.

The partial skyline F2 projections of the 750 MHz ^{1}H-JRES spectra of plasma collected from both the normal, healthy volunteer and the CRF patient are shown in Figs 5 and 6. The uraemic plasma sample contains many more resolved lines, corresponding with a greater number of motionally unconstrained and/or more abundant low-MW metabolite species, than in the control. It has long been known that the clinical syndrome of uraemia is associated with the accumulation of a variety of molecules over a large MW range. However, the so-called "uraemic toxins", i.e. those substances which cause the diverse clinical symptoms (e.g. nausea, itching, headaches and malaise) associated with CRF, have not been identified or characterized [22]. In previous NMR studies abnormal levels of compounds such as TMAO, methylamine and dimethylamine have been detected in the blood plasma of CRF patients by use of 500 MHz HSE ^{1}H-NMR studies and by single-pulse 500 MHz ^{1}H-NMR spectroscopy of ultrafiltered and solid-phase extracted plasma [23]. Here it has been shown that a variety of other substances with possible significance to uraemic renal failure can be identified with the aid of 750 MHz JRES spectroscopy. The biological significance of the increased levels of a number of metabolites, e.g. 1- and 3-methylhistidine,

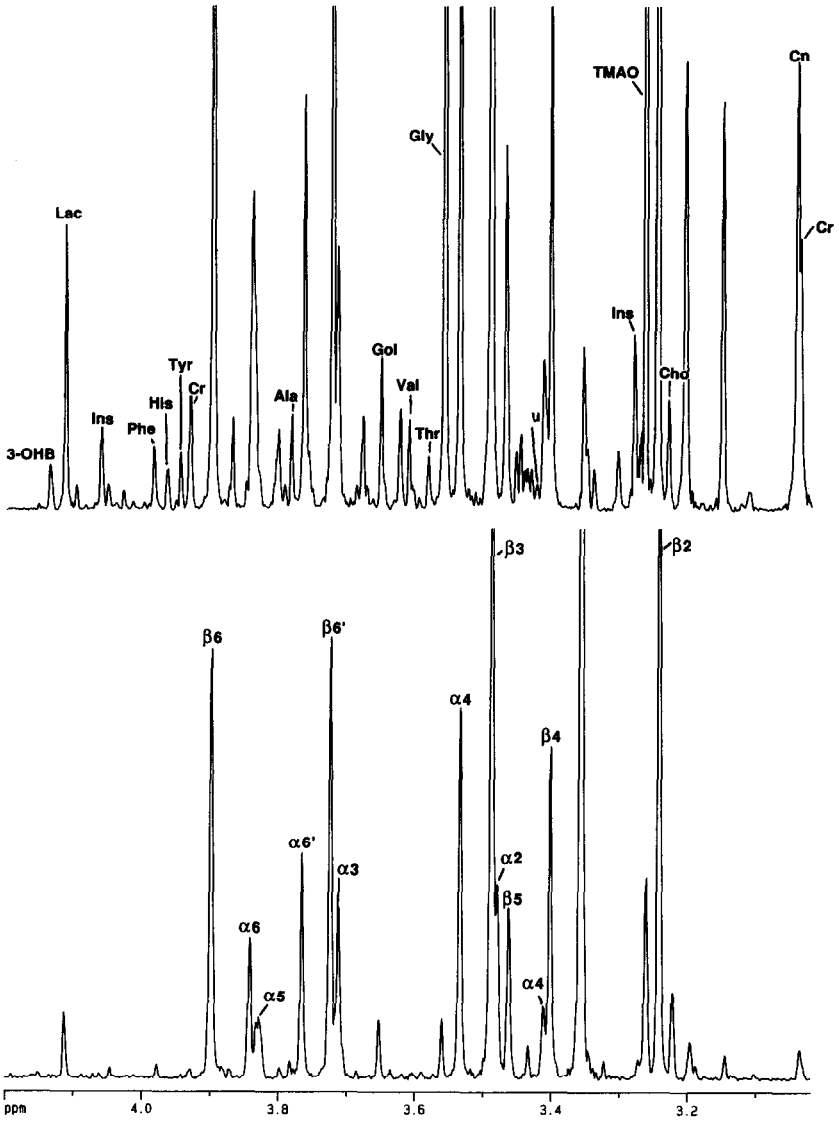

Figure 6
Comparison of the expanded skyline F2 projections (labelled region B in Fig. 5) of 750 MHz JRES ^{1}H-NMR spectra of the aliphatic regions of (a) normal and (b) uraemic blood plasma. Key to assignments: as in previous figures, plus: Thr, threonine.

TMAO, dimethylamine and methylamine, in plasma from patients with CRF is curently being explored. This first report of the use of JRES at very high fields appears to be much superior, in terms of the useful information generated, to the HSE or CPMG spin-echo experiment and promises to be particuliarly useful in the identification of potential uraemic toxins and for the study of abnormal low-MW components in blood plasma.

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References

[1] J.K. Nicholson and I.D. Wilson, *Prog. NMR Spectrosc.* **21**, 449–501 (1989).
[2] J.K. Nicholson and I.D. Wilson, In: *Drug Metabolism from Molecules to Man* (D. Benford and G. Gibson, Eds), pp. 189–207. Taylor Francis, London (1987).
[3] E. Holmes, P.J.D. Foxall and J.K. Nicholson, *J. Pharm. Biomed. Anal.* **8**, 955–958 (1990).
[4] P.J.D. Foxall, G. Mellotte, M. Bending, J.C. Lindon and J.K. Nicholson, *Kidney Internat.* **43**, 234–245 (1993).
[5] P.J.D. Foxall, K. Jones, R.G. Price, G. Neild, R. Thompson and J.K. Nicholson, *Biochem. Biophys. Act.* **1138**, 305–314 (1992).
[6] K.P.R. Gartland, J.C. Lindon, C. Beddell and J.K. Nicholson, *Mol. Pharmacol.* **39**, 629–642 (1991).

[7] E. Holmes, F.W. Bonner, B.C. Sweatman, J.C. Lindon, C.R. Beddell, E. Rahr and J.K. Nicholson, *Mol. Pharmacol.* **42**, 922–930 (1992).
[8] M.C. Malet Martino and R. Martino, *Biochemie* **74**, 785 (1992).
[9] P.J. Foxall, J. Parkinson, I.H. Sadler, J.C. Lindon and J.K. Nicholson, *J. Pharm. Biomed. Anal.* **11**, 21–31 (1993).
[10] S.C. Connor, J.K. Nicholson and J.E. Everett, *Anal. Chem.* **59**, 2885–2891 (1987).
[11] J.K. Nicholson, M.J. Buckingham and P.J. Sadler, *Biochem. J.* **211**, 605–615 (1983).
[12] J.D. Bell, J.C.C. Brown, J.K. Nicholson and P.J. Sadler, *FEBS Lett.*, **215**, 311–315 (1987).
[13] J.K. Nicholson, M. O'Flynn, P.J. Sadler, A. Macleod, S.M. Juul and P.H. Sonksen, *Biochem. J.* **217**, 365–375 (1984).
[14] J.D. Bell, J.C.C. Brown, R.E. Norman, P.J. Sadler and D.R. Newell, *NMR Biomed.* **1**, 90–94 (1988).
[15] J.K. Nicholson and K.P.R. Gartland, *NMR Biomed.* **2**, 63–70 (1989).
[16] J.R. Bales, J.D. Bell, J.K. Nicholson, P.J. Sadler, J.A. Timbrell, R.D. Hughes, P.N. Bennett and R. Williams, *Magn. Reson. Med.* **6**, 301–309 (1988).
[17] W.P. Aue, J. Karham and R.R. Ernst, *J. Chem. Phys.* **64**, 4226–4227 (1976).
[18] E.T. Fossel, J.M. Carr and J. McDonagh, *New Engl. J. Med.* **315**, 1369–1376 (1986).
[19] P. Okvnieff, A. Zietman, J. Kahn, S. Singer, L.J. Neuringer, R.A. Levine and F.E. Evans, *New Engl. J. Med.* **322**, 953–958 (1990).
[20] T. Engan, J. Kranz and S. Kvinnsland, *NMR Biomed.* **4**, 142–149 (1991).
[21] M. Kriat, S. Confort-Gouny, J. Vion-Dury, P. Viout and P.J. Cozzone, *Biochemie* **74**, 913–918 (1992).
[22] R. Vanholder, A. Schoots and S. Ringoir, *Replacement of Renal Function by Dialysis* (J.F. Maher, Ed.), 3rd edn, pp. 4–9. Kluwer (1989).
[23] J.D. Bell, J.A. Lee, H.A. Lee, P.J. Sadler, D.R. Wilkie and R.H. Woodham, *Biochim. Biophys. Acta* **1096**, 101–107 (1991).

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