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Analysis of biological fluids using 600 MHz proton NMR spectroscopy: application of homonuclear two-dimensional J-resolved spectroscopy to urine and blood plasma for spectral simplification and assignment

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Abstract: The application of 600 MHz two-dimensional J-resolved ^{1}H NMR spectroscopy (JRES) to the analysis of human urine and blood plasma is demonstrated. This method when applied at very high field gives a rapid means of simplifying and aiding the assignment of highly overlapped resonances of minor metabolites in biofluids. Using this approach, mixtures of drug and endogenous metabolites were identified in untreated urine samples, the signals of which were extensively overlapped in single pulse 600 MHz spectra. For untreated blood plasma samples the JRES experiment was also effective for the selective attenuation of signals from the plasma proteins thus revealing strong well-resolved signals from the low molecular weight components. For the first time it was shown to be possible to assign in detail the spectral region from 3 to 4 ppm in blood plasma, including the complete assignment of the signals from α- and β-glucose. JRES spectra of plasma were much easier to interpret and had a much higher information content than equivalent one-dimensional Hahn spin-echo spectra, thus aiding the identification of non protein-bound low molecular weight metabolites in plasma.

Keywords: *Proton NMR spectroscopy; 600 MHz; urine; plasma; J-resolved spectroscopy; metabolites; signal assignment.*

Introduction

High resolution ^{1}H NMR spectroscopy can be used for the analysis of a wide range of endogenous and drug metabolites present in untreated biological fluids such as urine and plasma and hence can be applied to the solution of problems in clinical biochemistry, toxicology and drug metabolism [1–11]. NMR spectrometers operating at high magnetic field strengths (e.g. 9.4 or 11.57*T*, corresponding to ^{1}H resonance frequencies of 400 and 500 MHz) have generally been used in order to obtain maximum sensitivity and dispersion of signals for complex biofluids. The authors have argued that use of the highest field strength available is probably mandatory if the most accurate quantitative measurements on metabolites are to be obtained [11, 12].

Currently, the highest field NMR spectrometers that are commercially available operate at 14.1 Tesla (i.e. 600 MHz ^{1}H frequency). However, even at this operating frequency, single-pulse spectra of biological fluids such as urine still show considerable peak overlap in certain chemical shift ranges (especially the 'aliphatic' region of the spectrum from δ 0.7 to 4.7), that have previously been described in terms of chemical noise [12]. Such noise occurs where there is multiple overlap and superposition of peaks arising from low concentrations of metabolites that are within the NMR detection range (i.e. that result in digitized signals in the free induction decay),

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but cannot be identified using currently available multiple pulse sequences. This is because their resonances overlap so extensively that only a broad envelope results in the single pulse spectrum and individually the levels of individual metabolites may approach the lower limits of NMR detection (currently <10 nM ml^{-1}), rendering them difficult to observe in less sensitive two-dimensional experiments. This presents a problem both as regards signal assignment for many endogenous biomolecules and as regards a possible source of error in quantitation of single-pulse spectra in which the chemical noise is greater in certain chemical shift ranges than in others [12]. Indeed the higher the field strength used, the greater is the spectral dispersion and hence more resonances are resolved. Also, increasing the sensitivity of the experiment improves the detection limit. Thus, paradoxically, moving to higher field increases the apparent complexity of the spectra. However, the biochemical information content of a 600 MHz biofluid NMR spectrum is intrinsically much higher than that of a spectrum obtained at lower field and efficiency. Therefore solutions to problems raised by chemical noise and spectral assignments for minor metabolites become worth pursuing.

In the past, several possible solutions have been presented by this research team to problems of peak overlap, in order to aid assignment and subsequent quantitation of peaks. For instance we applied two-dimensional proton–proton shift correlation (2D-COSY) spectroscopy at 500 MHz for the identification of five paracetamol metabolites in human urine samples, where single-pulse 500 MHz spectroscopy was incapable of allowing resolution of all the aromatic and acetyl resonances, even in simple aqueous solutions [13, 14]. It has also been shown that the application of solid-phase extraction chromatography with off-line NMR detection (SPEC–NMR [15, 16]) can provide a simple and efficient means of separating and detecting complex mixtures of drugs and endogenous metabolites. Although both of these approaches (2D-COSY and SPEC–NMR) are of considerable value within a general NMR strategy for studying the composition of biofluids, they still have some practical drawbacks, specifically the requirement for the destruction of the biofluid matrix by SPEC–NMR procedures, and the relatively long spectral accumulation times required for 2D-COSY analysis if good signal-to-noise ratios and adequate spectral digitization are to be realised. The 2D-COSY experiments also require relatively large experimental data arrays and consequently high usage of disk storage space and more time-consuming data processing.

The authors have now applied 600 MHz two-dimensional J-resolved ^1H NMR spectroscopy (JRES [17]) to the analysis of human urine and plasma. The JRES experiment was one of the first two-dimensional FT–NMR pulse sequences to be published [17] but it has now been largely abandoned in protein and nucleic acid biochemistry because of the problems of short T_2 relaxation times posed by macromolecular structures and the apparently insurmountable problems associated with the complexity of JRES patterns for second-order or moderately strongly-coupled signals, which give rise to extra interfering peaks in the two-dimensional spectrum. In biological fluids such as urine, the NMR-detectable molecules are largely in 'free solution' conditions, i.e. they do not have significant constraints placed on their molecular motions (and hence possess relatively long T_2 relaxation times) and the vast majority of the endogenous metabolites of interest have first-order spin systems with simple coupling patterns. For these reasons the 600 MHz JRES technique has been examined, as this combines a relatively low total spectral acquisition time (<1 h) with high sensitivity and dispersion, together with good conditions for rapid digitization because of the relatively small second-dimension array in which coupling constant information is collected. In the case of plasma ^1H NMR spectra are dominated by signals from plasma proteins which have short T_2 relaxation times. Another useful feature of the JRES experiment is that projection of the spectrum through F2 gives a high resolution proton-decoupled proton spectrum in which all the peaks are presented as singlets.

In the past spin-echo pulse sequences have been used by the authors to effect selective attenuation of protein resonances and thereby enable visualization of the low molecular weight components. As the JRES experiment consists of an array of spin-echo experiments it should also result in attenuation of protein signals at the same time as aiding signal assignment. The efficiency of the JRES experiment and its ease of implementation could

allow its use on a routine basis for the analysis of biofluid samples at ultra high field.

Materials and Methods

Sample preparation

Urine was collected on four separate occasions from a healthy adult male volunteer and from a patient who had received a renal allograft and was undergoing antirejection therapy (cyclosporin A 440 mg day^{-1}, prednisolone 20 mg day^{-1}, cimetidine 600 mg day^{-1}, thyroxine, 0.1 mg day^{-1} and cefotaxime 2 g day^{-1}) together with paracetamol analgesia (1–2 g day^{-1}). For control urine samples D_2O was added to each sample at a final concentration of 7.7% v/v, to provide an internal field-frequency lock. Chemical shifts were referenced internally to the singlet methyl resonance of sodium 3-(trimethylsilyl-2,2,3,3-$^2H_4$)-1-propionate (TSP, δ 0 ppm) present in the D_2O to give a final concentration of 1 mM. Urine samples from the renal allograft patient were freeze-dried and reconstituted in D_2O containing TSP prior to NMR analysis. Blood was collected by venepuncture from a healthy male volunteer and was placed in a vial containing lithium heparin as an anticoagulant. The blood plasma was separated immediately by centrifugation (5000*g* for 5 min) and 0.7 ml was diluted with 10% D_2O and placed in a 5 mm NMR tube prior to measurement.

Proton NMR spectroscopy

Single-pulse and Hahn spin-echo ^1H NMR spectra were measured on urine samples using either Varian VXR 600 or Bruker AMX 600 spectrometers operating at 600 MHz proton resonance frequency. The Hahn spin-echo pulse sequence is as follows:

$$D\text{–}90°x\text{–}\tau\text{–}180°y\text{–}\tau\text{–acquire},$$

where $D = 3$ s (to allow T_1 relaxation), and τ = 68 ms (a fixed delay to allow T_2 relaxation).

All spectra were measured at ambient probe temperature (298 ± 1 K). For each sample, 64 free induction decays (FIDs) were collected into 32,768 computer data points with a spectral width of 8000 Hz. In single-pulse experiments 30° pulses were used with an acquisition time of 2 s and a delay between pulses of 2.7 s. The water signal was suppressed by applying a gated secondary irradiation field at the water resonance frequency during the delay between pulses (gated off during acquisition). Prior to Fourier transformation, an exponential apodization function was applied to the FID corresponding to a line broadening of 0.2 Hz. Spectral assignments were made by consideration of literature values of chemical shifts in biological fluids [2–4], the spin–spin coupling patterns and constants of the resonances, by standard additions of candidate compounds and in some cases by measurement of two-dimensional COSY spectra.

On selected samples Carr Purcell Melboom Gill (CPMG) spin-echo spectra were recorded using the pulse sequence:

$$D - 90°x\text{–}\tau\text{–}(180° \pm y\text{–}\tau)_n\text{–acquire},$$

where $D = 3$ s, $\tau = 1$ ms, and n is an incremented variable delay to allow T_2 relaxation. The total relaxation delays ($2n\tau$) used were 8, 16, 32, 64, 128, 256, 512 and 1024 ms. The CPMG experiment does not result in J-modulated signal phases, and losses of signal intensity during the relaxation delay period due to diffusion through field gradients are minimized by virtue of the short τ delay between the 180° pulses [18].

Two-dimensional JRES spectra of urine were obtained at 600 MHz by sequential repetition of the pulse sequence:

$$[D\ (90°x\text{–}t_1\text{–}180°x\text{–}t_1)\ \text{collect FID}],$$

where $D = 2.7$ 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 which was gated-off during the application of the pulse sequence and acquisition. The F2 (chemical shift) domain was collected into 8192 computer points with a spectral width of 6000 Hz, and the F1 domain covered 30 Hz with 64 increments of t_1. Typically, 16 transients were collected for each t_1 increment in the JRES experiments. Prior to the double Fourier transform, the data were apodized by means of a sine-bell function in t_1 and t_2. The spectra were tilted by 45° to provide orthogonality of the chemical shift and coupling constant axes. Spectra were displayed both in the form of contour plots of the F1 and F2 domains and as skyline F2 projections following a magnitude calculation.

Results and Discussion

^{1}H NMR spectroscopy of human urine

The single-pulse 600 MHz ^{1}H NMR spectrum of normal human urine is shown in Fig. 1 with assignments for many of the major metabolite resonances. This provides a graphic illustration of the enormous biochemical information content of such spectra (there are >3000 resolved lines in a typical spectrum) and the considerable practical problems posed by urine samples with respect to ^{1}H NMR signal assignment. In low vertical expansions a relatively simple spectral profile is presented in which the resonances from the most abundant urinary metabolites are readily assigned (Fig. 1). Higher vertical expansions reveal the underlying complexity of the resonance patterns from a large number of partially overlapping resonances from less abundant, but almost certainly equally interesting and biochemically-important organic molecules. Many of these resonances cannot be assigned in the single pulse spectrum due to extensive signal overlap, even when measured at 600 MHz on the highest field spectrometer that is currently commercially available.

Assuming a detection limit of about 20 nM ml^{-1} for 600 MHz ^{1}H NMR spectra collected over relatively short total accumulation times

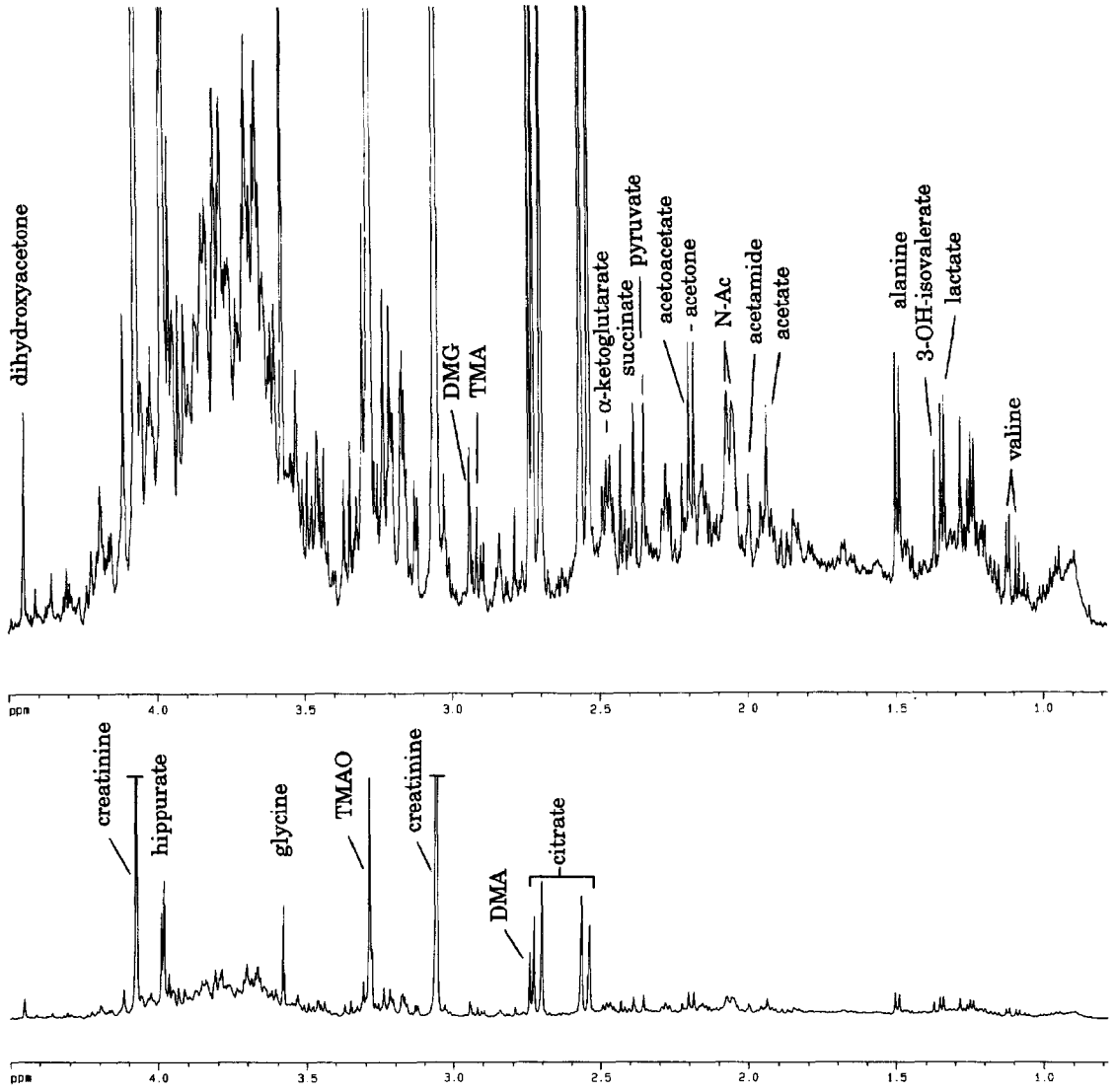

Figure 1
Single-pulse 600 MHz ^{1}H NMR spectrum of control human urine and a ×8 vertical expansion (above). TMAO, trimethylamine-*N*-oxide; DMG, dimethylglycine; DMA, dimethylamine; TMA, trimethylamine.

of about 15 min, it can be calculated from metabolite reference ranges [9] that at least 160 organic compounds present in normal human urine are NMR-detectable. This number will be greatly exceeded where abnormal biochemical, physiological or disease conditions are present and may be further complicated by the presence of drugs and their metabolites. It is the very richness of the ^{1}H NMR spectra of biofluids that makes them attractive to the biochemist as a source of diagnostic information, given that a range of widely different compound classes representing many biochemical pathways contribute to the NMR spectra of biofluids. Furthermore, information on these compounds can be gained from standard NMR experiments without extensive sample preparation or preselection of analytical conditions (which is essential for chromatographic procedures). The authors have previously described the influence of field strength on the reliability of quantitative NMR measurements on drug metabolites in urine and discussed the influence of chemical noise on quantitation [12]. Many compounds are present in sufficient amounts to give an envelope of resonances which places limits on the reliability of measurements on metabolites present at much higher concentrations, but close to, or within these chemical noise envelopes. It is chemical noise which effectively limits the detection of metabolites in biofluids, rather than the electronic noise generated within the spectrometer.

Figure 1 shows that these chemical noise envelopes extend from 0.8 to 4.8 ppm even at 600 MHz observation frequency where high vertical expansions are used, but these are less severe in chemical shift ranges >6 ppm (not shown). The most severe and extensive peak overlap occurs in the proton shift range 3–4 ppm, which is consistent with the presence of numerous sugars and amino acids (α-CH resonances). At least 30 such compounds are present in sufficient quantities in urine or plasma to be NMR-detectable [19], although

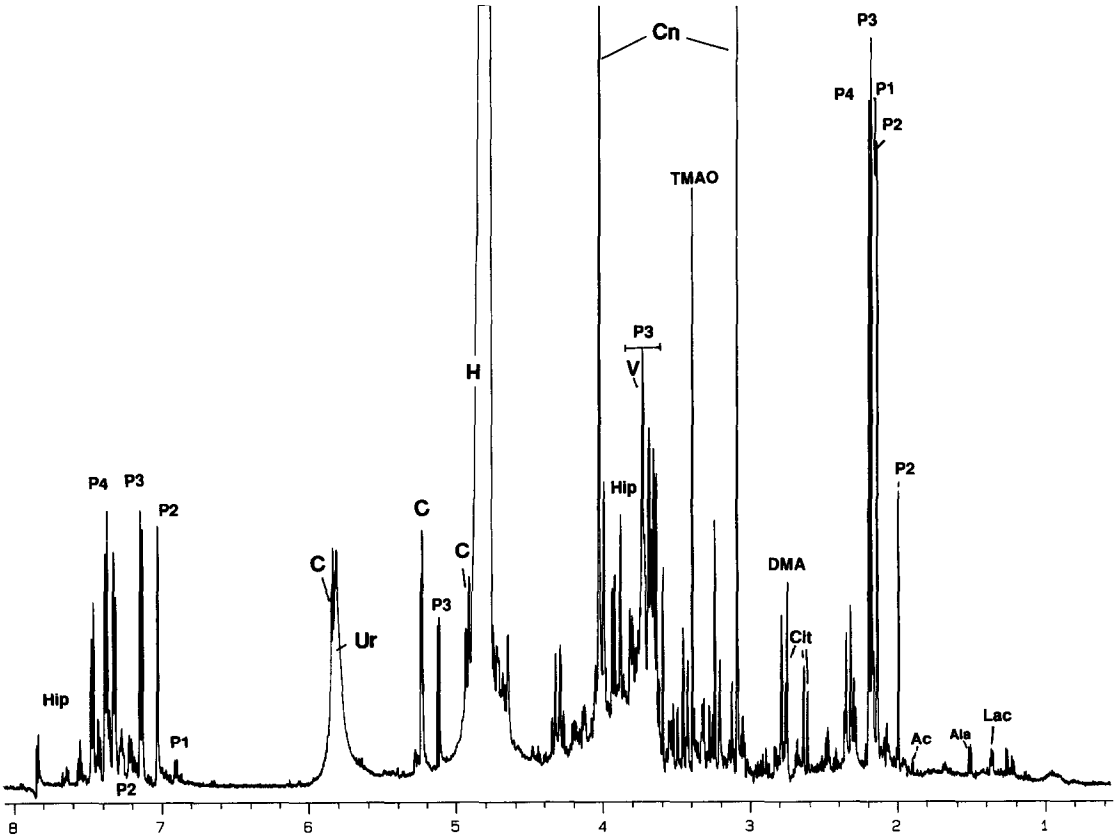

Figure 2
Single-pulse 600 MHz ^{1}H NMR spectrum of urine from a patient following renal transplantation. Abbreviations as Fig. 1 plus: Lac, lactate; Ala, alanine; Ac, acetate; DMA, dimethylamine; V, Cremaphore (polyethoxylated castor oil); P1, paracetamol; P2, *N*-acetylcysteine conjugate of paracetamol; P3, paracetamol glucuronide; P4, paracetamol sulphate (detailed assignments are to be found in ref. 13); C, cefotaxime signals; Ur, urea; H, residual water signal.

glucose is by far the most abundant, and should contribute to this narrow region of the spectrum. Some of the ring-closed sugars (e.g. glucose) also have α- and β-anomeric forms which gives a further degree of spectral complexity.

As a typical example of the type of spectral complexity that is frequently encountered when biofluids from diseased patients on drug therapy are studied, the single pulse 600 MHz ^{1}H NMR spectrum is shown of urine from a renal transplant patient undergoing a standard post-graft therapeutic régime (Fig. 2). All the endogenous components normally present in urine probably contribute to such a spectrum, although these will be in abnormal proportions as a consequence of the mechanisms and effects of the disease process. Numerous drug metabolites, including those from paracetamol, cefotaxime and from Cremaphore (a polyethoxylated castor oil used as the vehicle in the cyclosporin A dosing solution) also contribute to the spectrum (Fig. 2). Application of the JRES ^{1}H NMR experiment at 600 MHz to this sample allows a much greater proportion of the biochemical information latent in the single pulse spectrum to be accessed and further signals to be assigned in both the contour plot and the skyline F2 projection (Fig. 3). The effective dispersion is improved in the F2 projection allowing improved resolution of many peaks, e.g. the Cremaphore vehicle (V) resonance are resolved from those of the paracetamol glucuronide ring protons (at 3.7 ppm), whereas these were extensively overlapped in the single pulse spectrum (Fig. 2). To illustrate this further an expansion of the low frequency methyl region of the spectrum is shown in Fig. 4, where more than 30 separate methyl groups arising from endogenous metabolites can be resolved. Although not all of these signals have yet been assigned, many more resonances are detected in the JRES plots that could not be identified in the single-pulse spectra due to peak overlap.

The extra dispersion of data into the coupling constant domain considerably aids signal assignment, especially as most of the small molecules present have first-order NMR spectra. The authors have established that a JRES ^{1}H NMR experiment on human urine that is required to give good signal-to-noise ratios on minor metabolites (concentrations <100 nM ml^{-1}) typically takes less than 70 min

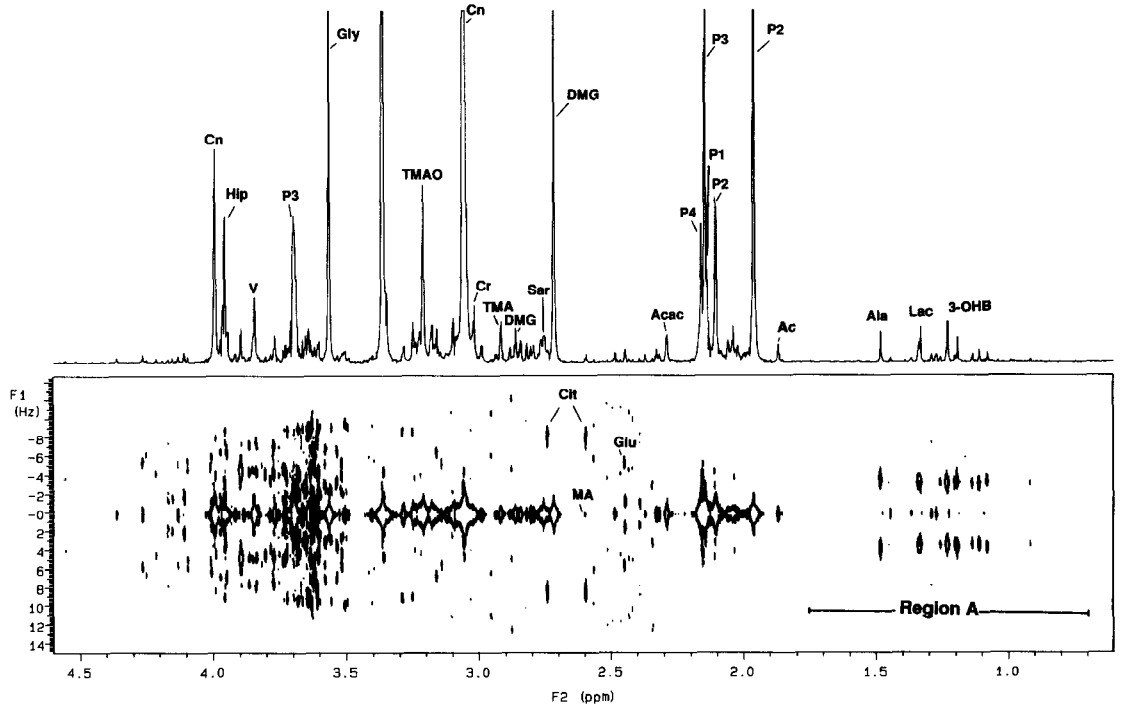

Figure 3
A 500 MHz JRES ^{1}H NMR spectrum of the aliphatic region of the urine sample shown in Fig. 2 showing contour plot and F2 projection. Abbreviations as in Figs 1 and 2 plus: 3-OHB, 3-D-hydroxybutyrate; sar, sarcosine; glu, glutamate; Cit, citrate; Cn, creatine; Hip, hippurate; Gly, glycine; Acac, acetoacetate.

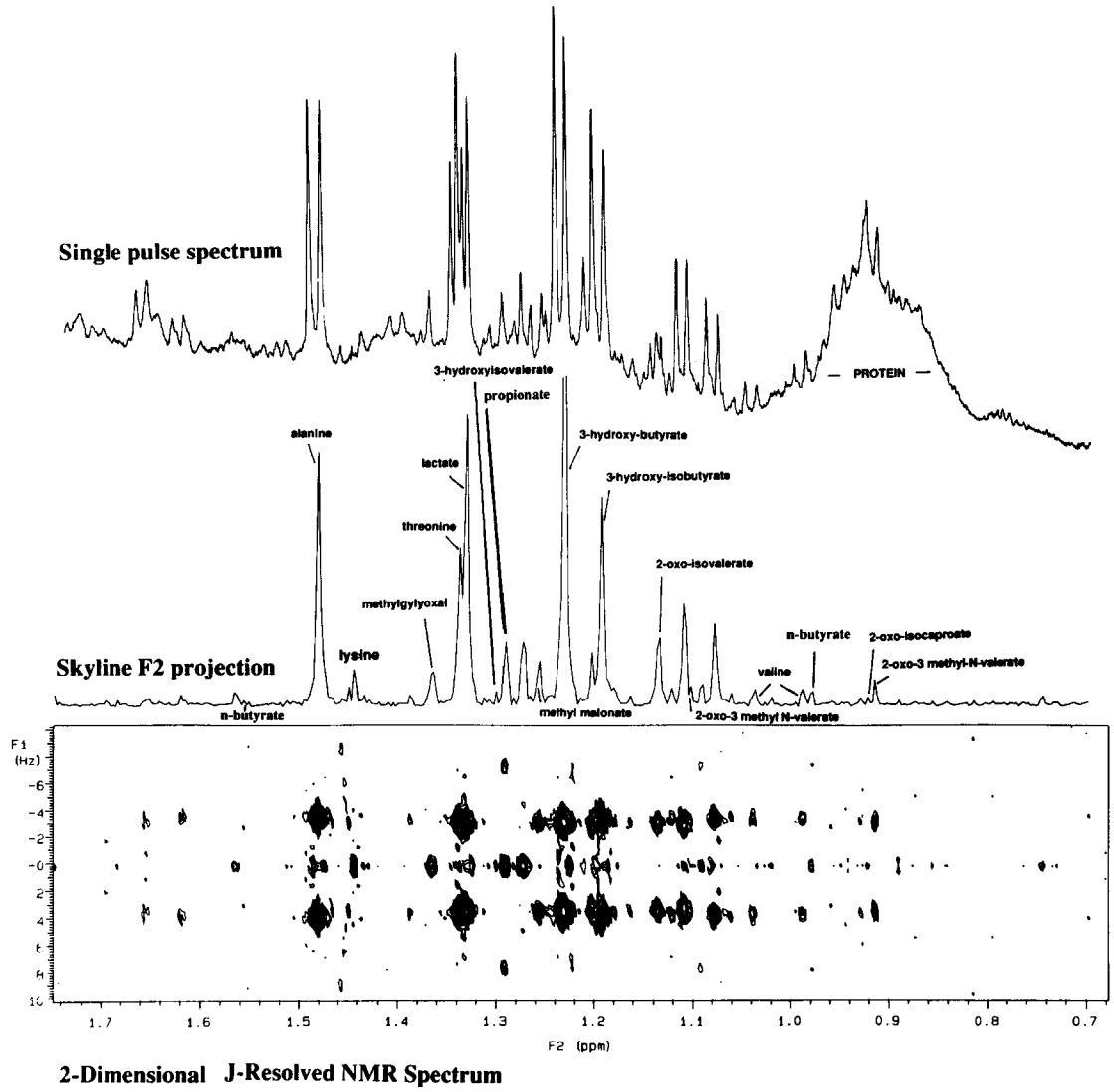

Figure 4
A 600 MHz JRES ^{1}H NMR spectrum of urine (expansion of region marked A in Fig. 3) in relation to F2 projection and single-pulse spectrum expansion showing resolution of the methyl signals in the 2DJ-RES contour map.

to complete (including probe turning, field shimming, data acquisition and processing). This compares favourably with the standard single-pulse experiment which requires a total time of about 20 min (of which <6 min is the actual acquisition time). Furthermore, in the JRES experiment as virtually all the ^{3}J coupling constants for small molecules found in urine are <15 Hz and most of the protons from low MW metabolites (with the exception of some sugar resonances) have first-order spin systems, the F1 domain can be kept small, e.g. 30 Hz which allows good digitization in F1 (0.47 Hz point^{-1} in Fig. 3) with a relatively small array of experiments (in this case 64) in which t_1 is varied. This also allows a reasonably good estimation of coupling constants to be made by reading the F1 axis. This obviates two of the main drawbacks associated with the application of the two-dimensional ^{1}H-^{1}H shift correlation experiment (^{1}H COSY), i.e. long spectral acquisition time (normally several hours) and relatively poor digital resolution of signals in F1 (a typical value might be 10–20 Hz point^{-1} for a full chemical shift range spectrum of a biofluid taking 8–12 h).

Examination of the skyline F1 projection of the JRES spectrum in Figs 3 and 4 shows that the relative intensities of the methyl signals appear to be largely preserved, whereas certain other resonances may be enhanced (e.g. acetate and dimethylamine) or attenuated (e.g.

citrate and hippurate). The relative changes in intensities of the acetyl methyl signals of the paracetamol metabolites in the single-pulse (Fig. 2) and JRES projection (Fig. 3) are also notable. These changes are all consequences of differential T_2 relaxation of the various metabolite protons, and this is highlighted in the JRES experiment which is based on a spin-echo pulse sequence designed to attenuate signals with short T_2 relaxation times.

The T_2 relaxation rates of biofluid metabolites are also modulated by chemical interactions in the intact biochemical matrix. For instance, metabolites such as citrate and hippurate both undergo chemical exchange reactions due to complexation with metal ions such as Ca^{2+} and Mg^{2+} in biological fluids which contributes to and therefore enhances the T_2 relaxation mechanism. Additionally, hippurate can also form an exchanging carbamic acid species in the presence of the bicarbonate ions abundant in urine. The intensity of signals in JRES spectra should therefore be interpreted with caution, unless the spectra have been individually calibrated by means of standard additions of compounds (i.e. before and after measurement).

Nevertheless, exact calibration of complete or even partial JRES spectra still poses problems because the Fourier transformed JRES data have to be presented in the absolute value mode due to their unfavourable phase-twist lineshape in phase sensitive mode. The intensities are also perturbed in situations when signals overlap. Although sine-bell or Gaussian-echo weighting [20] may alleviate this problem, the intensities in the projected spectrum are still unreliable and should be interpreted with caution.

The main analytical advantage of using JRES is as an aid to signal assignment and to detect peak overlap, which might result in inadvertent quantitation errors in one-dimensional spectra. The detailed assignment of the methyl region of the JRES spectrum shown in Fig. 4 would not have been possible from inspection of the single-pulse experiment alone and many of the minor metabolites give very weak signals in 2D-COSY experiments (not shown). Given the relatively short total accumulation time for acquisition of a JRES experiment at high field, it should now perhaps be considered seriously as a routine procedure where ^{1}H NMR spectra of biofluids are being assigned and to help eliminate quantitative overlap errors in the corresponding single-pulse spectra.

^{1}H NMR spectroscopy of human blood plasma

Single-pulse 600 MHz ^{1}H NMR spectra of human blood plasma are unrevealing with respect to most metabolites, due to the extensive overlap of, and resulting spectral complication from, broad resonances from macromolecules such as proteins (particularly albumin and immunoglobulins) and lipids (Fig. 5). The application of a Hahn spin-echo pulse experiment to the same plasma sample results in spectra that are largely devoid of protein–proton resonances [2], except for those from N-acetyl signals from glycoproteins [6] which are less motionally-constrained [Fig. 5(B)]. Many signals from low molecular weight species are readily detected in the HSE experiment that cannot be resolved in the single-pulse experiment (e.g. 3-D-hydroxybutyrate and 3-D-hydroxyisobutyrate). The authors have previously used the HSE approach as a useful method for eliminating protein resonances and increasing the biochemical information content of the spectra of low MW components of blood plasma [2, 3, 6]. Indeed, it has been shown that HSE experiments may be valuable in monitoring the changing patterns of low molecular weight metabolites in plasma in metabolic disease states such as diabetes mellitus, and for monitoring the changes in plasma biochemistry that accompany therapy [3].

The JRES experiment results in a dramatic simplification of the spectrum, does not suffer from phase modulation of multiplet peaks in the FT spectrum and enables the complex overlapped resonance in the chemical shift range from δ 3 to 4 to be more completely resolved (Fig. 5). Furthermore, the protein resonances are attenuated as effectively as was seen in the application of the simple spin-echo experiment (Fig. 5). The skyline projection through the JRES map results in a greatly simplified spectral profile of the effectively proton-decoupled proton spectrum of the motionally unconstrained low molecular weight metabolites in plasma. The skyline projection might therefore offer an attractive method for quantitating minor metabolites in plasma where attenuation due to T_2 relaxation can be accounted for or calibrated. It should be noted that signals from any small molecules that are extensively protein-bound will also be

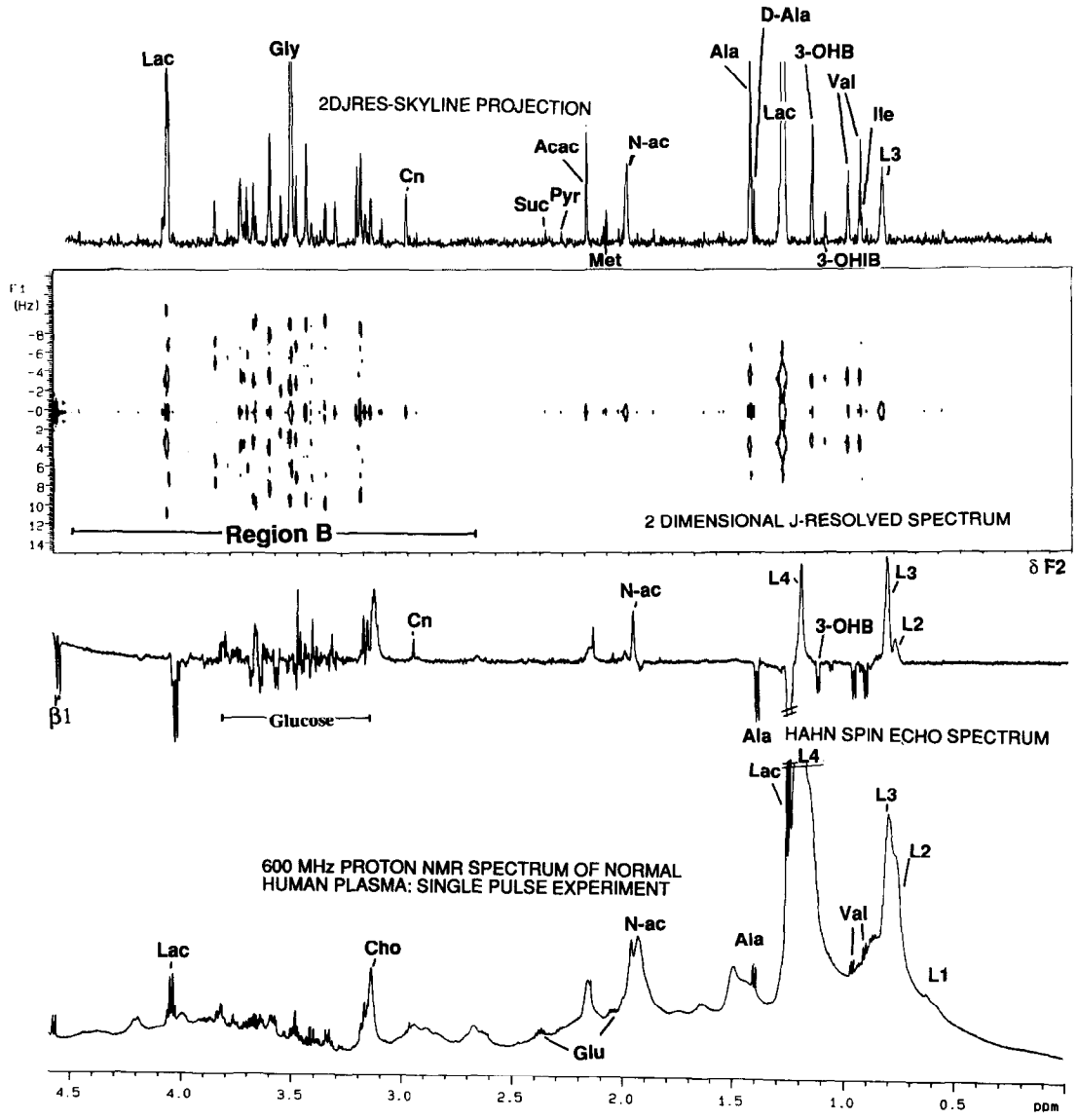

Figure 5
600 MHz ^{1}H NMR spectra of plasma. The lowest trace is a normal single-pulse spectrum with a spin-echo spectrum above showing J-modulation of multiplet peaks. The contour plot of the JRES spectrum is shown above this with its F2 projection above giving a proton-decoupled proton spectrum (all lines are singlets). Abbreviations as for urine, plus: Nac, N-acetyl signals of acetylated sugars on glycoproteins; Cho, choline; glu, glutamate; met, methionine; pyr, pyruvate; L1, terminal methyl groups of fatty acids in high-density lipoprotein; L2, terminal methyl groups of fatty acids in low-density lipoprotein; L3, terminal methyl groups of fatty acids in very low density lipoprotein; L4, aliphatic CH_2 groups of long-chain fatty acid in lipoproteins and chylomicrons (see ref. 21 for detailed signal assignments).

severely attenuated due to constrained molecular tumbling and a shortening of the T_2 relaxation time [3].

The use of JRES experiments 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 δ 3–4 that is so effectively simplified by this technique. Detailed examination of the expanded and most complex region of the JRES spectrum of plasma from δ 2.8 to 4.5 reveals a wealth of previously hidden signals amongst the complex glucose resonances (Fig. 6).

Virtually all of the heavily spin–spin coupled signals of both α- and β-glucose can be separated and have now been fully assigned for the first time in an intact biological fluid. Furthermore, the signals from many other

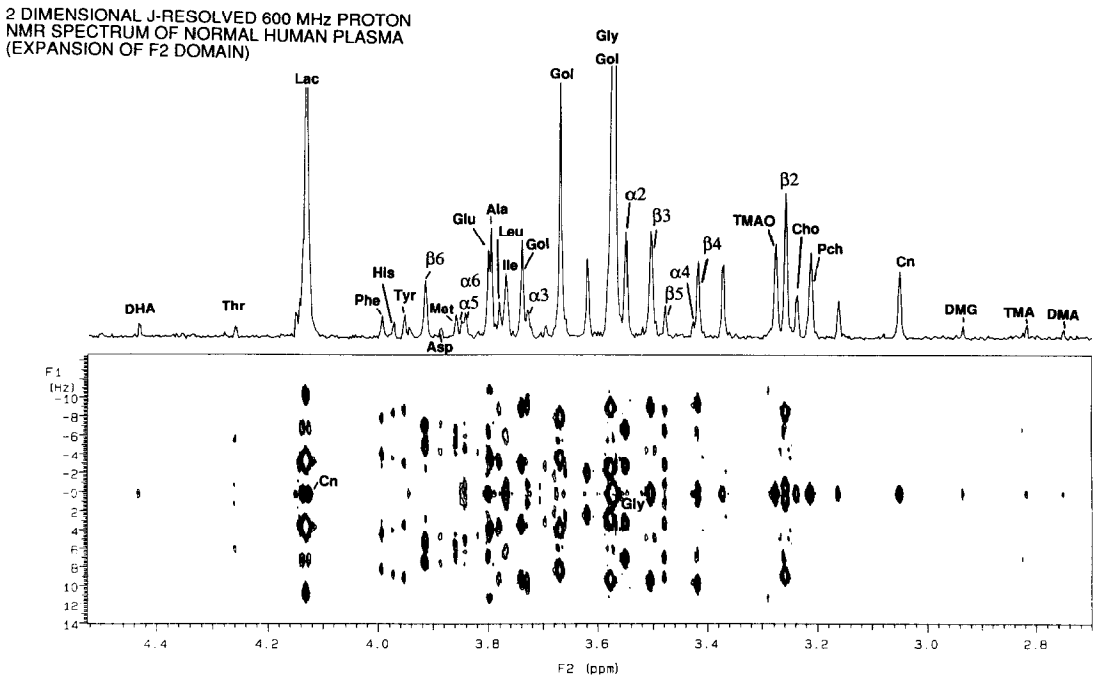

Figure 6
Expanded 600 MHz ^{1}H NMR spectrum of region B shown in Fig. 5 with assignments as before, plus: α2–α6, signals from α-D-glucose; β2–β6, signals from β-D-glucose; Pch, phosphorylcholine; Gol, glycerol; Leu, leucine; Asp, aspartate; His, histidine; Tyr, Tyrosine; Phe, phenylalanine; Thr, threonine.

biochemically-important metabolites, including several amino acids, glycerol, TMAO, choline and phosphorylcholine, are well resolved and are assigned for the first time (Fig. 6). Their signals are undetectable in the single-pulse spectrum due to overlap with glucose. Indeed this region of the spectrum of any biofluid is perhaps the most complex (i.e. richest in biochemical information) and has hitherto been closed to detailed NMR study because of spectral interference due to glucose. Thus JRES spectroscopy offers an efficient means of simplifying this region of the spectrum.

Conclusion

At ultra-high field ^{1}H JRES spectroscopy offers an efficient means of reducing peak overlap from low molecular weight compounds (including drug metabolites) in untreated biological fluids. It also allows the detailed assignment of complex regions of the biofluid spectrum containing many overlapped and highly coupled spin-systems. Good signal-to-noise ratios can be achieved within a short total spectral accumulation time (<1 h) for both plasma and urine. The method is an effective aid to peak assignment, as chemical shift and coupling constant information can be readily extracted and also because the method is not directly affected by the presence of high concentrations of high molecular weight species, the resonances of which are eliminated via T_2 relaxation during the JRES pulse sequence. The authors would advocate this method for routine use in the collection of metabolic data from NMR spectra of biological fluids and as a first step in resonance assignment for unknowns.

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