Pergamon

Journal of Pharmaceutical & Biomedical Analysis, Vol. 12, No. 5, pp. 613–618, 1994
Elsevier Science Ltd
Printed in Great Britain
0731-7085/94 $7.00 + 0.00

0731-7085(93)E0028-L

Application of the one-dimensional TOCSY pulse sequence in 750 MHz ^{1}H-NMR spectroscopy for assignment of endogenous metabolite resonances in biofluids

MANFRED SPRAUL,† JEREMY K. NICHOLSON,‡ MICHAEL J. LYNCH§ and JOHN C. LINDON*‖

† *Bruker Analytische Messtechnik GmbH, D-76287 Rheinstetten 4, Silberstreifen, Germany*
‡ *Department of Chemistry, Birkbeck College, University of London, 29 Gordon Square, London WC1H 0PP, UK*
§ *Institute of Urology and Nephrology, University College and Middlesex School of Medicine, Riding House Street, London W1P 7PN, UK*
‖ *Department of Physical Sciences, Wellcome Research Laboratories, Langley Court, Beckenham, Kent BR3 3BS, UK*

Abstract: The complex ^{1}H-NMR spectrum arising from an intact biofluid has been simplified using a one-dimensional homonuclear polarization transfer experiment (known as TOCSY or HOHAHA). This approach establishes connectivity between sequentially coupled multiplets, and the method is illustrated by the confirmation of the chemical shifts and hence resonance assignment of a number of endogenous metabolites in the 750 MHz ^{1}H-NMR spectrum of seminal fluid. This has allowed the detection and assignment of pyroglutamate and uracil in this fluid for the first time.

Keywords: *NMR; TOCSY; biofluid; metabolites.*

Introduction

In order to maximize the biochemical information that is latent in ^{1}H-NMR spectra of biofluids, it is obviously desirable to assign as many of the resolved resonances as possible. There are two major approaches to this problem in the intact biofluid. On the one hand, it is possible to measure NMR spectra of the fluid and by simple manipulations such as varying the pH, adding metal complexants such as EDTA or ultimately by addition of authentic compounds, it is possible to make a number of assignments, at least of the major endogenous components [1]. This approach has probably reached the limit of usefulness for most biofluids even on very high field spectrometers (14.1 T or greater) and the application of new simple generic approaches to metabolite signal assignment are needed. For biofluids this approach can be extended through the measurement of two-dimensional and multidimensional NMR spectra such as COSY. In this context, ^{1}H–^{1}H J-resolved (JRES) spectra have proved very useful, as they are acquired rapidly and do not need large data matrices [2–4]. This methodology has recently been applied to provide a much augmented list of assigned resonances from the NMR spectra of human cerebrospinal fluid [3] and from human blood plasma where 750 MHz ^{1}H-NMR has been used for the first time [4]. Also, additional resonance dispersion can be obtained through the use of ^{1}H-detected ^{1}H–^{13}C correlation spectra where a combination of ^{1}H and ^{13}C chemical shifts can be used to assign resonances to compounds.

An alternative approach is to effect a simplification of the fluid using separation methods or to edit the NMR spectra of the complex mixture using selective excitation techniques. The former has been successfully achieved using solid-phase extraction chromatography methods such as Bond-Elut™ columns followed by NMR spectroscopy (SPEC–NMR) or mass spectrometry [5]. Following the recent

* Author to whom correspondence should be addressed.

advent of effective coupled HPLC–NMR spectroscopy, this has been applied *inter alia* to studies of pharmaceutical drug metabolism [6, 7] where it was suggested that it would be possible to use the technique to improve the characterization of endogenous species also.

Instead of simplifying the biofluid by chromatographic separation, in principle it is possible to use a variety of pulse sequences to edit the complex NMR spectrum of the intact biofluid to yield selective information, the advantage being that intermolecular interactions in the matrix are not perturbed. One way of achieving this is to use a one-dimensional version of the TOCSY pulse sequence which reveals spin coupling connectivity along a chain of coupled nuclei [8]. This method, also known as the homonuclear Hartmann–Hahn (HOHAHA) experiment was introduced by Bax and Davies [9] and it has become widely used in structural studies. The one-dimensional version of TOCSY requires a single well resolved resonance of a molecule so that it can be selectively inverted using a π pulse followed by a spin-locked mixing period to transfer polarization along the chain of coupled proton spins. The method is rapid (comparable to the single pulse experiment) and provides spectra with good digital resolution. This approach has now been applied to confirm the NMR spectral assignments in human seminal fluid and to identify the α-CH protons of certain amino acids in one-dimensional NMR spectra of biofluids for the first time. The method has also previously been used to characterize the different glucuronide ring systems in poorly resolved HPLC fractions containing isomeric drug glucuronides [10]. In complex biofluids there is extensive chemical shift overlap of many compounds and in order for one-dimensional TOCSY to be effective at least one signal of interest must be resolved. Therefore, the studies reported here have been carried out at the highest available magnetic field strength corresponding to a ^{1}H frequency of 750 MHz.

Experimental

The human seminal plasma samples were collected routinely from patients attending a fertility clinic and were stored frozen at −70°C until required for NMR measurement. They were diluted by 50% using D_2O before NMR measurement in order to reduce the sample

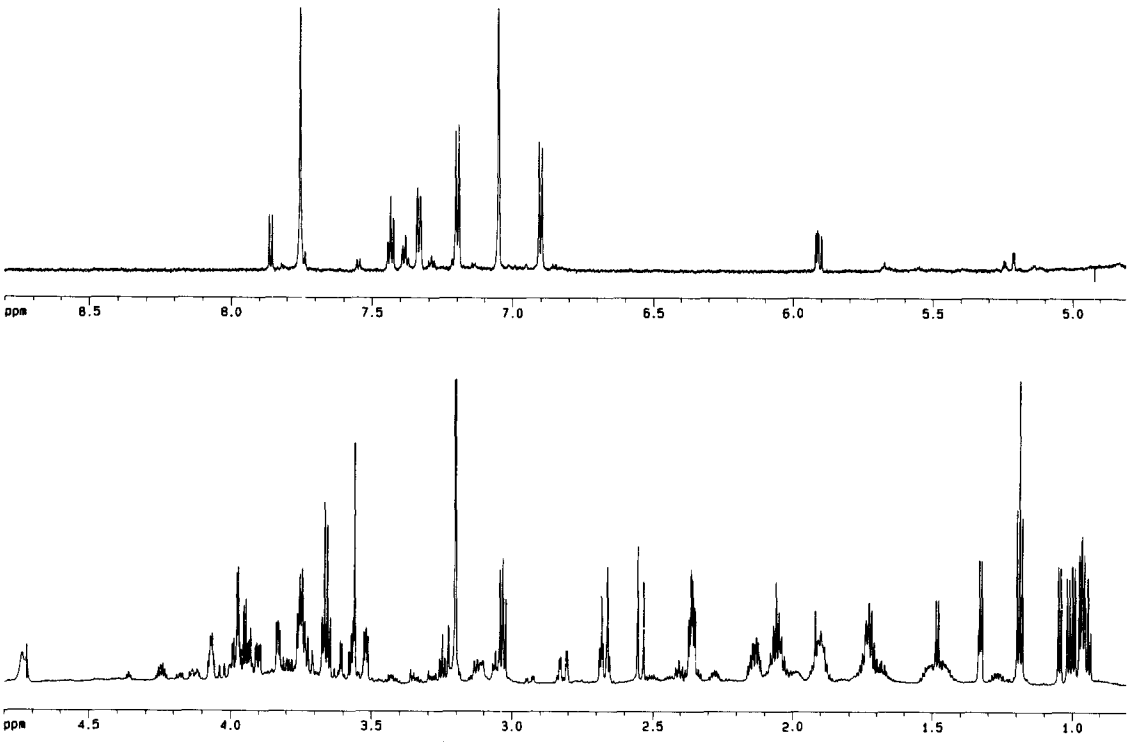

Figure 1
750 MHz ^{1}H-NMR spectrum of human seminal fluid showing the region δ0.8–8.8. The resonance assignments of the endogenous species are as given earlier [11].

viscosity and provide a field-frequency lock for the NMR experiments.

The one-dimensional TOCSY experiments were carried out at 300 K using a Bruker AMX-750 spectrometer operating at 750.13 MHz for ^1H spectroscopy with the pulse sequence of Subramanian and Bax [8] on a 5 mm triple nucleus inverse geometry probe. The water peak was suppressed by pre-irradiation with a selective pulse for 5.0 s. A Gaussian shaped selective pulse (1024 points, truncated at the 5% level, 80 ms) was used for the multiplet inversion and the spin-lock employed the MLEV-17 sequence [9] with a mixing time of 58.2 ms. Typical acquisition and processing parameters utilized 32 K time domain points filled by an equal number of zeroes before Fourier transformation, an acquisition time of 2.03 s, a relaxation delay of 3.0 s, a spectral width of 8064 Hz, four dummy scans, and line-broadening weighting factors of between 0.25 and 1.0 Hz. Chemical shifts are quoted relative to the HDO peak at δ4.72 which in turn was referenced to the chemical shift standard TSP (sodium 3-trimethylsilyl-(2,2,3,3-$2H_4$)-1-propionate at δ0.0) which was added to one sample.

Results and Discussion

Figure 1 shows the single pulse ^1H-NMR spectrum of a sample of human seminal fluid at 750 MHz, covering the range δ0.8–8.8, where the aromatic proton region is shown using a higher vertical expansion than the aliphatic proton region. Many of the resonances have been assigned on the basis of their chemical shifts and spin coupling patterns where connectivity between resonances has been inferred from two-dimensional experiments [11]. Seminal fluid contains moderate concentrations of amino acids and these, because their α-CH proton resonances fall into a narrow range of chemical shifts, contribute to the severe overlap in the NMR spectral region between δ3.5–4.2. Although it is possible to investigate this region using two-dimensional experiments such as COSY and TOCSY, these require significant data accumulation times and need large amounts of computer disk space. On the other hand, the one-dimensional TOCSY experiment is rapid and economical of disk space. Figure 2(a) shows the result of applying the selective π pulse in a one-dimensional TOCSY experiment to the less shielded methyl group resonance of valine. The coupling connectivity to the β-CH and thence to the α-CH and to the other CH_3 group is clearly visible. The α-CH lies in a region of the spectrum which is usually extremely crowded and in the past this signal has only been seen in JRES spectra [3]. However, the valine β-H resonance can be resolved in the seminal fluid NMR spectrum at 750 MHz and Fig. 2(b) demonstrates the one-dimensional TOCSY result after inverting this signal. The connectivity to both of the methyl resonances and to the α-H resonance can be seen. Figure 2(c) shows the result of selective excitation at the lactate methyl doublet. This results in observed connectivity to the lactate CH signal at δ4.15. In addition, the signal from the methyl group of threonine is obscured by the lactate methyl resonance and this experiment has also produced inversion of the threonine methyl resonance and thus results in the observation of the threonine β-CH signal at δ4.29 and in turn the α-CH resonance at δ3.61.

One of the samples examined contained a triplet resonance which was consistent with that of ethanol. The one-dimensional TOCSY spectrum which resulted from inversion of this triplet is shown in Fig. 2(d) which demonstrates the methylene quartet at δ3.68 also consistent with the chemical shift in ethanol. This is the first time to our knowledge that ethanol has been observed in seminal fluid and presumably arises from alcohol ingestion by the patient prior to attendance at the clinic.

The result of selective inversion of the complex multiplet arising from the γ-CH_2 group of glutamate at δ2.35 is depicted in Fig. 2(e), which shows conectivity to the non-equivalent β-CH_2 signals around δ2.0 and in turn to the α-CH peak at δ3.73. A different sample showed a resonance comprising a doublet of doublets at δ4.13. The one-dimensional TOCSY response observed on inversion of this multiplet is shown in Fig. 2(f) indicating the presence of a non-equivalent methylene group with shifts of δ2.45 and δ1.98 and a second methylene group at a shift of δ2.33. These resonances are all consistent with the known chemical shifts and multiplet patterns of pyroglutamate (B.C. Sweatman and J.C. Lindon, unpublished results). This is the first time that this substance has been detected in seminal fluid.

In the full ^1H-NMR spectrum of the seminal fluid, two doublets are observed at chemical

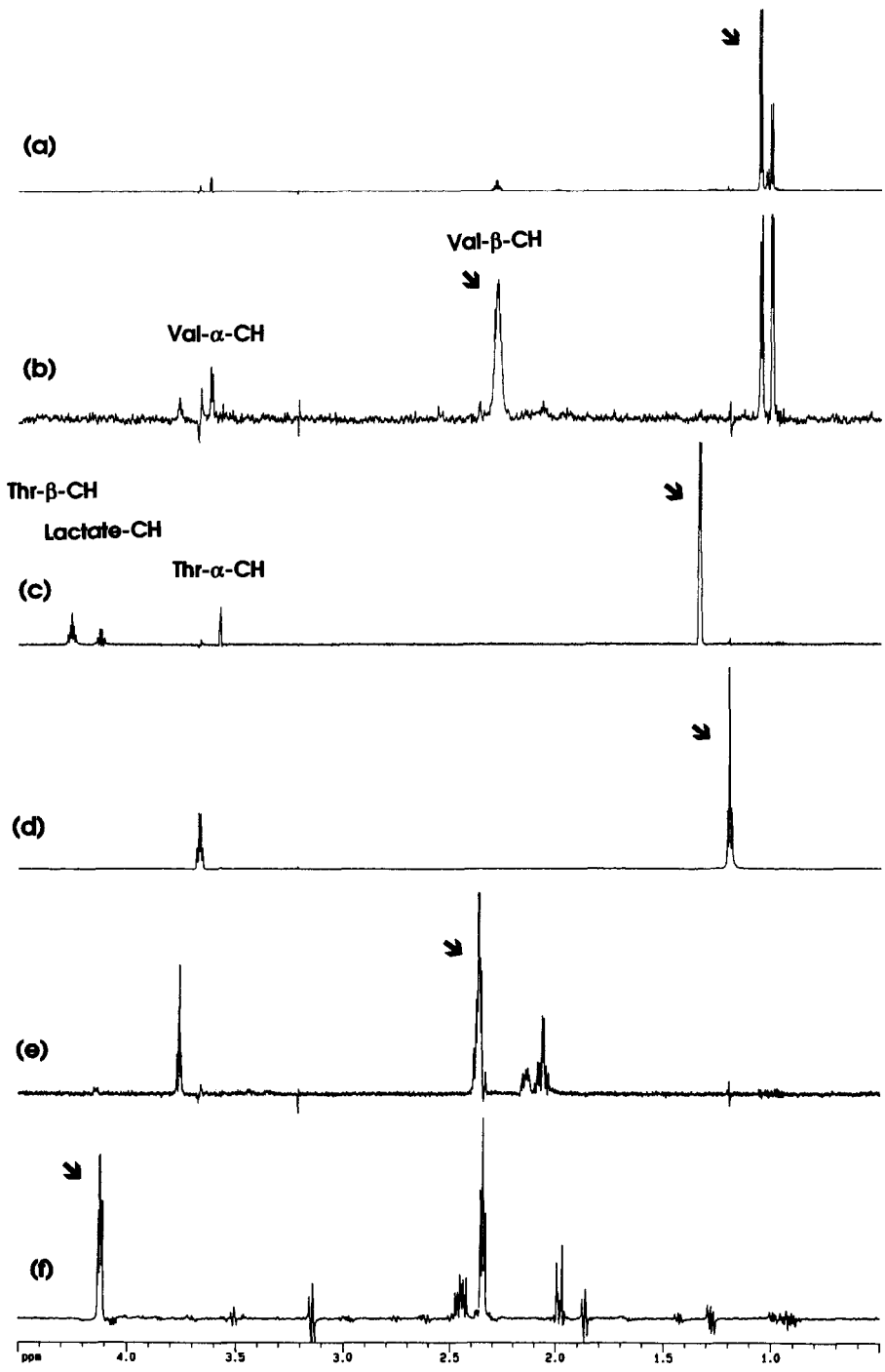

Figure 2
750 MHz ^{1}H one-dimensional TOCSY NMR spectra of human seminal fluid. (a) inversion of valine methyl resonance; (b) inversion of valine β-CH resonance; (c) inversion of lactate and threonine methyl resonances; (d) inversion of the ethanol methyl resonance; (e) inversion of glutamate γ-methylene resonance and (f) inversion of pyroglutamate α-CH resonance. The arrows indicate the resonances which were inverted.

shifts at δ5.90 and δ5.91. These two proton resonances have been assigned to the H-5 proton and to the ribose H-1′ proton in uridine [11]. These doublets were inverted in a one-dimensional TOCSY experiment and the result is shown in Fig. 3(a). The corresponding H-6 hydrogen resonance is clearly observed and two peaks are observed at δ4.37 and δ4.25

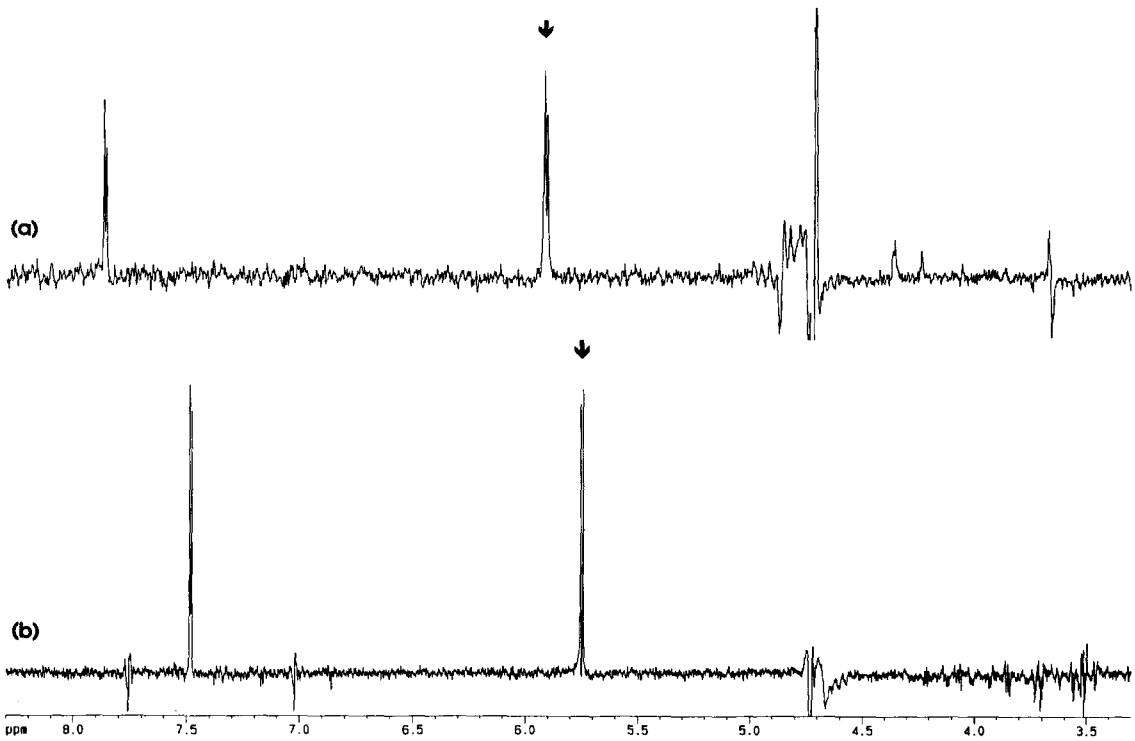

Figure 3
750 MHz ^{1}H one-dimensional TOCSY NMR spectra of human seminal fluid (a) inversion of uridine H-5 olefinic resonance and uridine ribose H-1′ resonance and (b) inversion of uracil H-5 resonance. The arrows indicate the resonances which were inverted.

corresponding to H-2′ and H-3′ of the ribose ring at the correct chemical shifts as measured using authentic uridine (B.C. Sweatman and J.C. Lindon, unpublished results). Clearly, the mixing time in the pulse sequence was not sufficient to trace out all of the ribose ring proton coupling pathway, but nevertheless all of the observed resonances add weight to the assignment as uridine. Figure 3(b) shows the results of a one-dimensional TOCSY experiment on a seminal fluid sample from a different subject. In this case the H-5 and H-6 signals are at different chemical shifts and this is consistent with free uracil and in addition no ribose proton resonances are observed. In this sample, enhanced hydrolysis of any uridine has taken place to yield uracil and free ribose. This latter substance will now have been converted from the single furanose form found in uridine into an equilibrium mixture of the α- and β-forms of both pyranose and furanose ring isomers, the β-pyranose predominating [12]. This mixture of species results in a loss of signal-to-noise ratio for the ribose proton NMR signals as well as a change in chemical shifts such that they are no longer visible in the one-dimensional ^{1}H-NMR spectrum.

The simplification of the biofluid ^{1}H-NMR spectrum which is effected by the application of this pulse sequence is dramatic and highly informative. The success of the method, which is rapid and of high sensitivity, relies on the molecule under study having a clearly resolved resonance which is spin coupled to others in an unbroken chain of couplings. The method should be applicable to other biofluids for the assignment of endogenous species and is best suited to those molecules which, because of extended couplings are most difficult to observe in the highly overlapped one-dimensional NMR spectrum. The approach is also applicable to the identification of drug metabolites in intact biofluids [10] and will be most selective when combined with the maximal resolving power afforded by ultra-high field NMR spectrometers.

References

[1] J.K. Nicholson and I.D. Wilson, *Prog. NMR Spectrosc.* **21**, 449–501 (1989).
[2] P.J.D. Foxall, J.A. Parkinson, I.H. Sadler, J.C. Lindon and J.K. Nicholson, *J. Pharmaceut. Biomed. Anal.* **11**, 21–31 (1993).
[3] B.C. Sweatman, R.D. Farrant, E. Holmes, F.Y.

Ghauri, J.K. Nicholson and J.C. Lindon, *J. Pharmaceut. Biomed. Anal.* **11**, 651–664 (1993).
[4] P.J.D. Foxall, M. Spraul, R.D. Farrant, J.C. Lindon, G.H. Neild and J.K. Nicholson, *J. Pharmaceut. Biomed. Anal.* **11**, 267–276 (1993).
[5] I.D. Wilson and J.K. Nicholson, *Anal. Chem.* **59**, 2830–2832 (1987).
[6] M. Spraul, M. Hofmann, P. Dvortsak, J.K. Nicholson and I.D. Wilson, *Anal. Chem.* **65**, 327–330 (1993).
[7] M. Spraul, M. Hofmann, I.D. Wilson, E. Lenz, J.K. Nicholson and J.C. Lindon, *J. Pharmaceut. Biomed. Anal.* **11**, 1009–1015 (1993).
[8] S. Subramanian and A. Bax, *J. Magn. Reson.* **71**, 325–330 (1987).
[9] A. Bax and D.G. Davies, *J. Magn. Reson.* **65**, 355–360 (1985).
[10] G.C. Leo and W.-N. Wu, *J. Pharmaceut. Biomed. Anal.* **10**, 607–613 (1992).
[11] M.J. Lynch, E.P.N. O'Donoghue, J.P. Pryor, J.C. Lindon, P.J.D. Foxall and J.K. Nicholson, *J. Pharmaceut. Biomed. Anal.* **12**, 5–19 (1994).
[12] E. Breitmaier and U. Hollstein, *Org. Magn. Reson.* **8**, 573–575 (1976).

[*Received for review 11 November 1993*]