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Short Communication

Liquid chromatography coupled with high-field proton NMR for profiling human urine for endogenous compounds and drug metabolites

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Introduction

A number of applications of high-field NMR spectroscopy have previously been described for the analysis of biological fluids (e.g. blood plasma, urine, bile, cerebrospinal fluid) both for endogenous compounds and for xenobiotics and their metabolites [1–4]. In the field of xenobiotic metabolism it has been amply demonstrated that multinuclear NMR spectroscopy of such biological fluids can provide a rapid and convenient approach to the detection of metabolites [1–8]. Similarly ^1H NMR spectroscopy has proved to be of value in the study of clinical samples and in studies on the mechanisms and time course of toxicity [9–11]. However, the complexity of the biological fluid spectra obtained has often been such that identification of sample components from the single-pulse ^1H NMR spectrum has not been straightforward.

In such circumstances two orthogonal approaches to the identification of unknowns can be used, singly, or in combination. In the first, sophisticated multipulse (and multidimensional) NMR methods can be used to extract structural information directly. In the second, the problem can be simplified by the selective removal of endogenous contaminants followed, if necessary, by the separation and isolation of a particular analyte. To this end strategies have been devised based on solid-phase extraction–chromatography monitored by NMR spectroscopy (SPEC–NMR) [12–14]. The extraction and purification of target compounds is achieved by stepwise gradient elution from a suitable SPE phase (e.g. octadecylsilane bonded silica). This methodology has proved to be robust, convenient, inexpensive and rapid. However, despite the practical success of the SPEC–NMR approach it remains a 'low resolution' technique (in terms of chromatographic separation), the deficiencies of which are compensated for, to a significant extent, by the NMR spectrometer which serves as the 'detector'.

For very complex mixtures, such as intact biological fluids, the hyphenation of NMR with liquid chromatography might be expected to offer clear benefits. Such systems have already been described [15–20] but, due to the technical difficulties involved, such as insufficient dynamic range and sensitivity and problems with solvent suppression, LC–NMR has not been widely exploited. However, given the significant recent technical advances in this field including increases in NMR spectrometer field strengths, methods for ensuring adequate solvent suppression together with the design of dedicated LC–NMR probes, the authors have

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been encouraged to examine the utility of LC–NMR for the profiling of biological fluids such as urine.

The present work reports data from preliminary studies by LC–NMR on a human urine sample, obtained following the ingestion of ibuprofen (2-(4-isobutylphenyl)-propionic acid). This report demonstrates, for the first time, the utility of LC–NMR for the detection and identification both of drug metabolites and of endogenous components.

Materials and Methods

Biological samples

Urine was obtained from a healthy male volunteer for the period 0–4 h after a single oral dose of ibuprofen (400 mg). The sample was acidified to pH 2 with HCl on collection in order to prevent the breakdown of ester glucuronide metabolites. An aliquot of this sample (10 ml) was freeze-dried and later reconstituted in 1.5 ml deuterium oxide–deuteroacetonitrile (^2H_2O–CD_3CN) (2:1, v/v) prior to chromatography. A 10-ml aliquot of the urine sample was also subjected to solid-phase extraction onto C18-bonded silica gel (Bond Elut, 6 ml cartridge, 500 mg of adsorbent, Jones Chromatography, Hengoed, UK), followed by elution with 6 ml of methanol, as described earlier [13].

Chromatographic conditions

LC was performed on a 5-μm Nucleosil 100 C-18 bonded silica column (125 × 4.6 mm i.d., with a 20 × 4.6 mm i.d., precolumn). The LC system consisted of a Bruker LC22C pump, an LC53 autosampler and an LC33 variable wavelength UV detector (operated at 210 and 240 nm as appropriate). The outlet of the UV detector was connected to the HPLC–NMR probehead via an inert polyethylether ketone (PEEK) capillary (0.25 mm i.d.).

Gradient elution was performed using: (A) 0.05 M potassium dihydrogenphosphate (pH 2.45) in ^2H_2O (in order to provide a field-frequency lock for the spectrometer); and (B) acetonitrile (CH_3CN) (Pestanal grade, Riedel de Haen, Germany). Linear gradient elution was employed for sample analysis (typically 1–45% acetonitrile over 80 min) at a column temperature of 35°C. For stopped-flow experiments 100 μl of sample was injected and a flow rate of 1 ml min^{-1} was used. In the case of continuous flow experiments 70 μl of sample was used with a solvent flow rate of 0.5 ml min^{-1}.

NMR spectroscopy

LC–NMR was performed using a Bruker AMX-500 NMR spectrometer equipped with a dedicated ^1H flow-probe (probe flow cell of 2 mm i.d., with a volume of 60 μl). Continuous and stopped-flow ^1H NMR spectra were obtained at 500 MHz using a one-dimensional version of the nuclear Overhauser effect spectroscopy (NOESY) pulse sequence, which included presaturation and resulted in conditions for ensuring optimal double solvent suppression.

Continuous-flow spectra were obtained after performing a preliminary run in order to obtain the correct presaturation frequencies for each time increment of this two-dimensional experiment where the proton resonance position corresponded to one dimension and time the other. This preliminary experiment was performed without solvent suppression, using the pulse program to simulate all the pulses and time delays of the NOESY experiment, in order to obtain data with the same time profile as that for gradient LC. Each time increment was subjected to peak-picking, to yield two frequency lists which represented the change in resonance frequency for each of the solvent components. In the actual gradient LC run after injection of the sample the frequency values for suppression of each solvent suppression were read in from the corresponding time increment in the respective list. With 16 scans acquired per increment a time resolution of *ca* 12 s was obtained for the LC–NMR experiment. Stopped-flow spectra were acquired by the accumulation of 256 or 512 transients under high-resolution conditions, i.e. the time domain data set contained 64 K data points and the recycle time was *ca* 5 s.

Results and Discussion

The 500 MHz single-pulse ^1H NMR spectra of the 10 ml aliquot of the total 0–4 h post-dose urine sample after freeze drying and reconstitution in ^2H_2O, contained resonances both for the normal range of endogenous compounds and for a number of signals for ibuprofen metabolites (cf. [13–14]). A typical chromatogram is shown in Fig. 1 illustrating sequential UV-absorption and ^1H-NMR detection with double solvent suppression. As

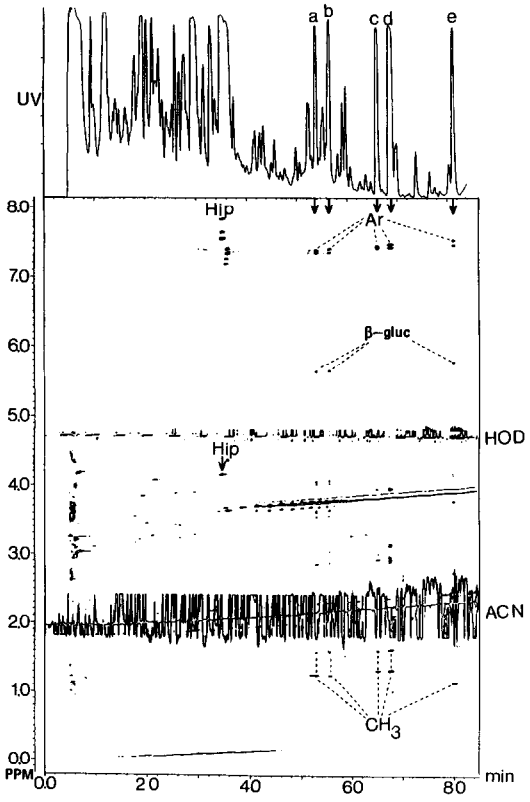

Figure 1
HPLC, using gradient elution, of an ibuprofen-metabolite containing urine with UV (210 nm) and ^{1}H NMR detection (details in Materials and Methods). Hip, hippuric acid resonances; Ar, aromatic signals for ibuprofen metabolites; B-gluc, signals for anomeric protons of glucuronides; CH_3, signals for ibuprofen metabolite methyl groups. The signals resulting from the solvent are indicated by HOD (water) and ACN (acetonitrile). The peaks labelled a–e correspond in retention time to the indicated ibuprofen metabolites (a, HMMPA-glucuronide; b, CMPP-glucuronide; c, HMPPA; d, CMPPP; e, ibuprofen glucuronide).

can be seen the chemical shift of the methyl resonance in acetonitrile changed as gradient elution proceeded, while that of the water signal remained stable because the spectrometer was locked to the ^2H_2O resonance, as noted above. This two-dimensional plot of chemical shift versus retention time represents the spectrum observed at successive time increments. The selectivity of the method is such that resonances typical of hippuric acid are observed, together with methyl and aromatic protons for ibuprofen metabolites, and the anomeric protons of their β-glucuronides. This two-dimensional map enables all detectable protons signals to be visualized simultaneously and allows the sample to be rapidly screened for compounds of interest. Although it is possible to extract an information-bearing row from this two-dimensional matrix, and thus obtain a one-dimensional spectrum, the digital resolution is reduced due to the fast repetition time needed for the single increments.

Clearly both the UV and NMR 'chromatograms' contain signals from a considerable number of compounds, including endogenous substances and ibuprofen metabolites. Stopped-flow ^{1}H NMR spectroscopy was performed on a number of these peaks in order to facilitate their identification. A typical example of the results obtained for an endogenous compound is illustrated in Fig. 2, which shows the spectrum obtained for hippuric acid (t_R 35.93 min). Such spectra clearly demonstrate the ability of LC–NMR under the stopped-flow conditions employed to provide an unambiguous identification of endogenous urinary metabolites. It was also possible in the continuous flow studies to observe a number of signals at *ca* 5.6 ppm for the anomeric protons of the glucuronides of ibuprofen and some of its metabolites. Under stopped-flow conditions spectra were obtained for ibuprofen glucuronide and for the glucuronides of the hydroxylated and side-chain oxidized metabolites of ibuprofen (2-[4-(2-hydroxy-2-methylpropyl)-phenyl]propionic acid, HMPPA) and (2-[4-(2-carboxy-2-methylpropyl)phenyl]propionic acid, CMPPP). In addition unconjugated HMPPA and CMPPP were also readily identified in the chromatograms based on their NMR spectra. An example of one such spectrum for HMPPA is shown in Fig. 3. This spectrum clearly shows the typical pattern for a *p*-disubstituted aromatic ring at *ca* 7.2 ppm together with signals corresponding the side chain and propionic acid methyls (*ca* 1.1 and 1.4 ppm respectively, see Fig. 3).

In addition to single-pulse one-dimensional NMR experiments a number of more complex two-dimensional experiments (e.g. 2-D TOCSY) can be performed under stopped-flow conditions (to be reported elsewhere) enabling further structural information to be obtained.

The use of LC–NMR as described here can clearly enable the detection and identification of unknowns in complex biological fluid samples with minimal sample pretreatment and with a considerable saving in time compared to conventional methods. The on-column detection limits for LC–NMR depend on both

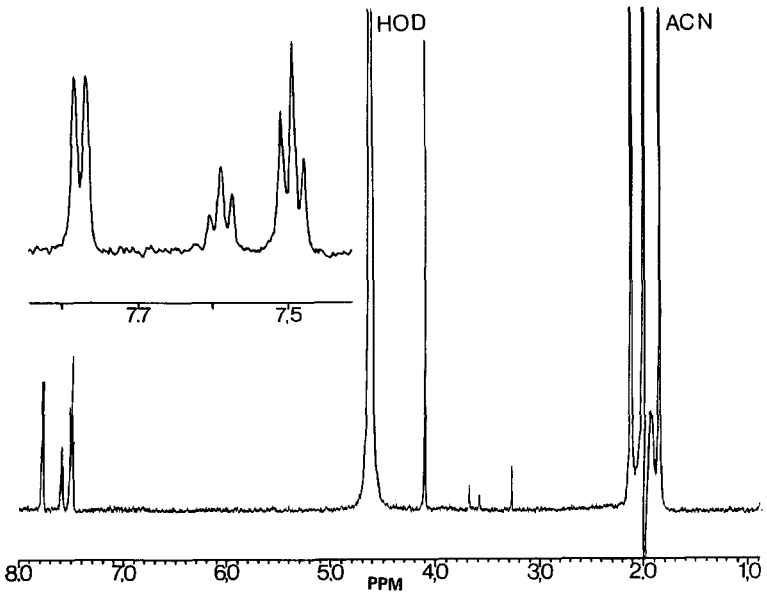

Figure 2
^{1}H NMR spectra of the endogenous urinary component hippuric acid (corresponding to the peak with a retention time of 35.93 min in Fig. 1) obtained by gradient HPLC–NMR with stopped-flow NMR spectroscopy.

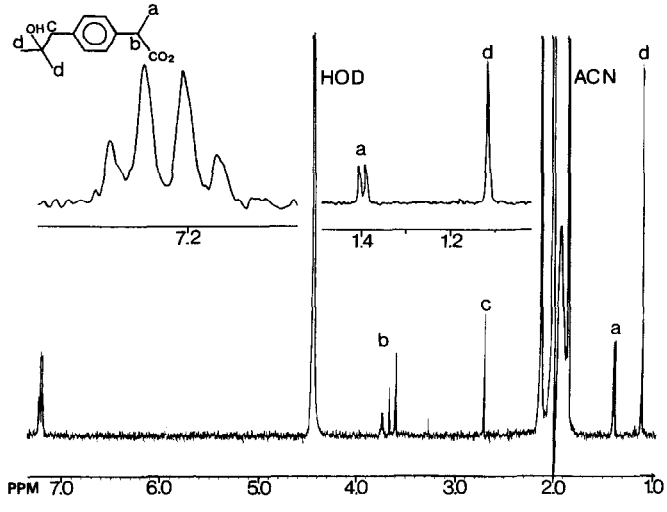

Figure 3
A ^{1}H NMR spectrum of the ibuprofen metabolite HMPPA (corresponding to the peak with a retention time of 65.70 min) excreted in the 0–4 h post-dose (400 mg) urine sample obtained by gradient HPLC–NMR with stopped-flow NMR spectroscopy.

the analyte and the experimental conditions employed. For continuous-flow studies detection limits were of the order of *ca* 10 μg on-column, while for stopped-flow experiments they corresponded to *ca* 1 μg on-column. Although LC–NMR does not have the sensitivity of LC–MS, nevertheless it is sufficiently sensitive for the rapid profiling of bioloigcal fluids such as urine.

It is to be expected that continuing improvements in HPLC–NMR technology should result in further improvements in sensitivity. Based on this preliminary evaluation of LC–NMR for biological analysis it is clear that this hyphenated system has considerable potential for investigations in drug metabolism, toxicology and clinical biochemistry. Further studies on a variety of biological fluids and

sample types to further explore the benefits of this emerging technology are being conducted in the authors' laboratories.

References

[1] J.K. Nicholson and I.D. Wilson, in *Drug Metabolism — from Molecules to Man* (D.J. Benford, J.W. Bridges and G.G. Gibson, Eds), pp. 189–207. Taylor and Francis, London (1987).

[2] J.K. Nicholson and I.D. Wilson, *Prog. Drug Res.* **31**, 427–479 (1987).

[3] J.K. Nicholson and I.D. Wilson, *Prog. NMR Spectros.* **21**, 449–501 (1989).

[4] I.D. Wilson, K.E. Wade and J.K. Nicholson, *Trends Anal. Chem.* **8**, 368–374 (1989).

[5] K. Tulip, J.A. Timbrell, J.K. Nicholson, I.D. Wilson and J. Troke, *Drug Met. Disp.* **14**, 746–749 (1986).

[6] K.E. Wade, I.D. Wilson, J.A. Troke and J.K. Nicholson, *J. Pharm. Biomed. Anal.* **8**, 401–410 (1990).

[7] T.D. Spurway, K.P.R. Gartland, A. Warrander, R. Pickford, J.K. Nicholson and I.D. Wilson, *J. Pharm. Biomed. Anal.* **8**, 969–973 (1990).

[8] F.Y.K. Ghauri, C.A. Blackledge, I.D. Wilson and J.K. Nicholson, *J. Pharm. Biomed. Anal.* **8**, 939–944 (1990).

[9] J.K. Nicholson, M. O'Flynn, P.J. Sadler, A. Macleod, S.M. Juul and P.H. Sonksen, *Biochem. J.* **217**, 365–375 (1984).

[10] P. Foxall, M. Bending, K.P.R. Gartland and J.K. Nicholson, *Human Toxicol.* **8**, 491–496 (1989).

[11] J.K. Nicholson and I.D. Wilson, *Anal. Proc.* **28**, 183–184 (1991).

[12] I.D. Wilson and I.M. Ismail, *J. Pharm. Biomed. Anal.* **4**, 663–665 (1986).

[13] I.D. Wilson and J.K. Nicholson, *Anal. Chem.* **59**, 2830–2832 (1987).

[14] I.D. Wilson and J.K. Nicholson, *J. Pharm. Biomed. Anal.* **6**, 151–165 (1988).

[15] E. Bayer, K. Albert, M. Nieder, E. Grom, G. Wolff and M. Rindlisbacher, *Anal. Chem.* **54**, 1747–1750 (1982).

[16] H.C. Dorn, *Anal. Chem.* **56**, 747–758 A (1984).

[17] D.A. Laude, Jr and C.L. Wilkins, *Trends Anal. Chem.* **5**, 230–235 (1986).

[18] K. Albert and E. Bayer, *Trends Anal. Chem.* **7**, 288–293 (1988).

[19] K. Albert, M. Kunst, E. Bayer, M. Spraul and W. Bermel, *J. Chromatogr.* **463**, 355–363 (1989).

[20] K. Albert, M. Kunst, E. Bayer, H.J. de Jong, P. Genissel, M. Spraul and W. Bermel, *Anal. Chem.* **61**, 772–775 (1989).

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