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Coupling of HPLC with ^{19}F- and ^{1}H-NMR spectroscopy to investigate the human urinary excretion of flurbiprofen metabolites

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Abstract: Results of an on-line HPLC–NMR analysis of human urine from a volunteer administered the anti-inflammatory drug flurbiprofen are reported. The two major human urinary metabolites, namely the glucuronides of flurbiprofen and of 4′-hydroxyflurbiprofen, have been identified using ^{1}H- and ^{19}F-NMR spectroscopy. *In vivo* conjugation of the racemic drug and its metabolites with D-glucuronic acid results in diastereomeric molecules which give resolved NMR spectra thereby permitting the diastereomeric proportions to be evaluated. The cause of the observed deviation from equal proportions is discussed. This study represents the first use of both ^{19}F- and 600 MHz ^{1}H-NMR spectroscopy coupled to HPLC.

Keywords: *HPLC–NMR; ^{19}F; flurbiprofen; metabolism; human.*

Introduction

Analysis of biological fluids using high resolution NMR spectroscopy has been very successfully applied for both the study of altered levels of endogenous substances in pathological conditions [1] and for the identification of xenobiotics and their metabolites [2–4]. The high complexity of biofluid NMR spectra necessitates the use of NMR spectral editing techniques including the use of two-dimensional NMR methods to assign resonances [5, 6]. A successful approach has been to simplify the NMR spectra by partial separation of the components using solid-phase extraction methods and these are very effective where the drug metabolites have a range of polarities [7]. However, the ultimate goal is to separate each component in the biofluid sample and to measure its NMR spectrum in a linked on-line chromatographic system.

Progress towards this goal was provided by the attempts to develop coupled HPLC–NMR spectroscopy probes in the early 1980s [8, 9]. However, because of technical difficulties such as the need for expensive deuterated HPLC solvents and the lack of NMR sensitivity, these methods did not find widespread application for practical metabolite detection and characterization. Recent advances in magnetic field strength, developments in NMR probe technology and solvent suppression methods have resulted in major practical improvements in HPLC–NMR sensitivity. There is also much less reliance on the use of deuterated solvents in the measurement of ^{1}H-NMR spectra and hence the feasibility of the approach has been re-examined for biochemical applications. We recently reported the successful application of the HPLC–NMR method using ^{1}H-NMR at 500 MHz to the detection and identification of metabolites of the anti-inflammatory drug ibuprofen (2-(4-isobutylphenyl)-propionic acid) in human urine [10, 11].

We have now extended the biochemical applications of HPLC–NMR by considering the therapeutically-related fluorinated drug flurbiprofen ([±]-2-fluoro-α-methyl-4-

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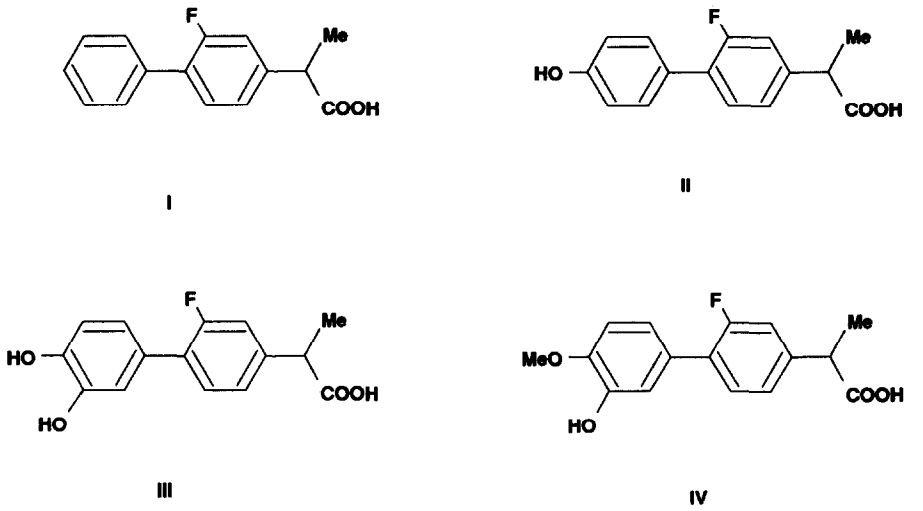

Figure 1
The structures of flurbiprofen and its principal metabolites. In man, the carboxyl group is usually conjugated with β-D-glucuronic acid.

biphenylacetic acid) which, like ibuprofen is subject to a range of metabolic transformations (see Fig. 1 and below). The widespread use of fluorinated substituents in drug molecules has encouraged us to investigate the utility of HPLC coupled with ^{19}F-NMR spectroscopy and in the present study we were able to take advantage of the presence of a ^{19}F-atom in each of the metabolites and use the ^{19}F-NMR probe as a specific HPLC detector. Furthermore, we report for the first time the use of HPLC–NMR at a ^{1}H frequency of 600 MHz.

The metabolism of flurbiprofen, as a racemic mixture, has been studied in a variety of species [12] including man. In man the metabolic fate includes glucuronidation, and ring hydroxylation together with glucuronidation. In addition the profen non-steroidal anti-inflammatory drugs have been shown to undergo a stereoselective interconversion of the inactive enantiomer into the active form [13]. It has been shown that in man 60–70% of the drug is excreted as glucuronides or sulphates. On multiple dosing, with urine collection for several days of dosing, 23% of the drug remained unchanged (**I**), 44% was converted to **II**, 5% to **III** and 25% to **IV** [12]. It appears probable that in the human over short time periods after dosing, the principal excretion product will be unchanged drug, or the glucuronide conjugate, and that it might be possible to observe metabolite **II** (also as the glucuronide). The structures of these compounds are given in Fig. 1.

We have previously used flurbiprofen as a model fluorinated drug with which to test a number of ^{1}H/^{19}F-NMR spectral editing techniques for the detection of metabolites in urine [14] and we have also reported ^{19}F- and ^{1}H-NMR data for all of the major metabolites of this compound.

Experimental

Urine was collected from a healthy male volunteer 4 h after oral dosing with 200 mg flurbiprofen. Aliquots of the sample were freeze-dried and reconstituted at a 10-fold increase in concentration into D_2O containing 2% acetonitrile for the HPLC–NMR investigation. The HPLC system comprised a Bruker LC22C pump, an LC53 autosampler and an LC313 variable wavelength UV detector. The UV detector was connected to the NMR probe via a 0.25 mm i.d. PEEK capillary line. HPLC analysis were carried out using a 50 μl injection onto a 250 × 4.6 mm i.d. Spherisorb ODS-2 column. After 5 min of isocratic flow, gradient elution using D_2O–acetonitrile–phosphate was employed with UV monitoring of the eluent peaks at 254 nm, ending after 50 min at D_2O–acetonitrile (1:1). On-flow ^{19}F-NMR detection of the LC eluent was carried out using a Bruker AMX-500 spectrometer equipped with a flow-through probe of 2 mm i.d. with a cell volume of 60 μl. Typical parameters were frequency, 470.60 MHz, 16 scans per experiment, acquisition time 0.25 s,

recycle time 1.25 s giving a resolution of 20.0 s per increment, FID apodization using a shifted sine-bell function, size 4 k data points, proton decoupling using the WALTZ16 method. For ^{1}H-HPLC–NMR experiments, a Bruker AMX-600 spectrometer was used with a similar probe but with an active volume of 250 μl. Parameters for stopped-flow ^{1}H-NMR spectra were number of scans 64, acquisition time 1.34 s, relaxation delay 3 s, spectral width 12195 Hz, no line-broadening or zero filling, 16 k FT size. Spectra of whole urine samples were also measured at 600 MHz for ^{1}H and 564.6 MHz for ^{19}F. Typical parameters were for ^{1}H, 64 scans, spectral width 7042 Hz, 32 k time domain points, no zero filling, 0.30 Hz line-broadening, 4.65 s recycle time including acquisition and for ^{19}F the acquisition and processing parameters were 1024 scans, acquisition time 1.64 s, 64 k time domain points, spectral width 20 kHz, line-broadening 2.0 Hz before Fourier transformation and ^{1}H decoupling using WALTZ16. ^{1}H chemical shifts were reference to internal acetonitrile at δ2.00 and ^{19}F chemical shifts were referenced to external trifluoroacetic acid at −76.8 ppm.

Results

The 600 MHz ^{1}H-NMR spectrum of a human urine sample from a volunteer who had ingested 200 mg of flurbiprofen is shown in Fig. 2 with an expansion of the aromatic region as an inset. Clearly, the ability to discern resonances from the aromatic protons of flurbiprofen related substances is severely limited because of spectral complexity due to extensive overlap from endogenous metabolites. Figure 3 shows the corresponding ^{1}H-decoupled ^{19}F-NMR spectrum at 564 MHz indicating that four major fluorine containing species are present. The observed chemical shifts of the major components are indicative of modifications to flurbiprofen distant to the fluorine containing phenyl ring. In all, 24 separate fluorine containing species can be detected.

Figure 4 shows the two-dimensional contour plot of an on-flow ^{19}F detected HPLC–NMR experiment with conditions as described in the Experimental section, demonstrating four ^{19}F resonances, appearing as a pair at each of two retention times (30.5 and 36.6 min). The shorter retention time peaks correspond in chemical shift to the two largest resonances

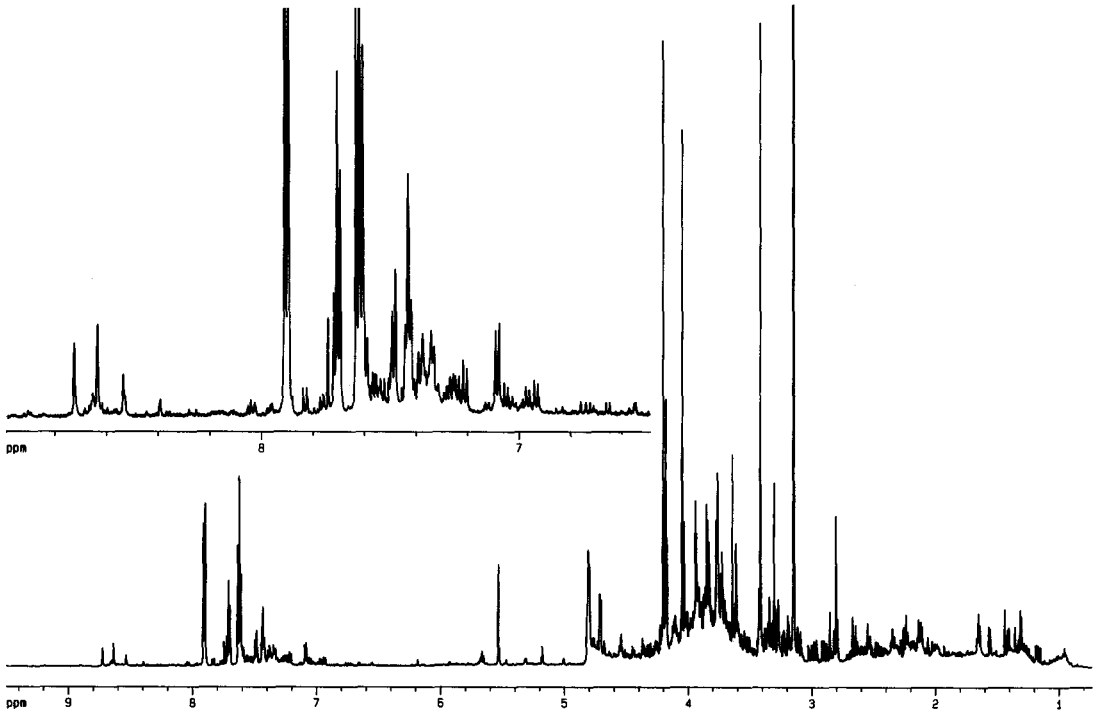

Figure 2
A 600 MHz ^{1}H-NMR spectrum from a sample of human urine collected after the oral ingestion of 200 mg flurbiprofen.

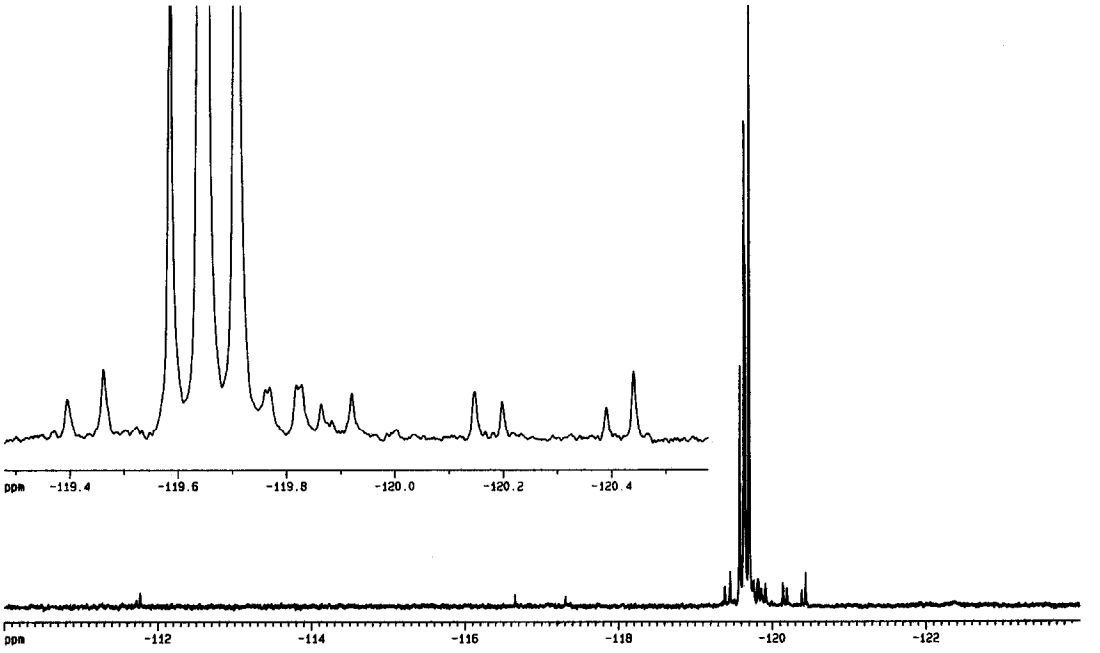

Figure 3
A 576 MHz ^{19}F-NMR spectrum from a sample of human urine collected after the oral ingestion of 200 mg flurbiprofen.

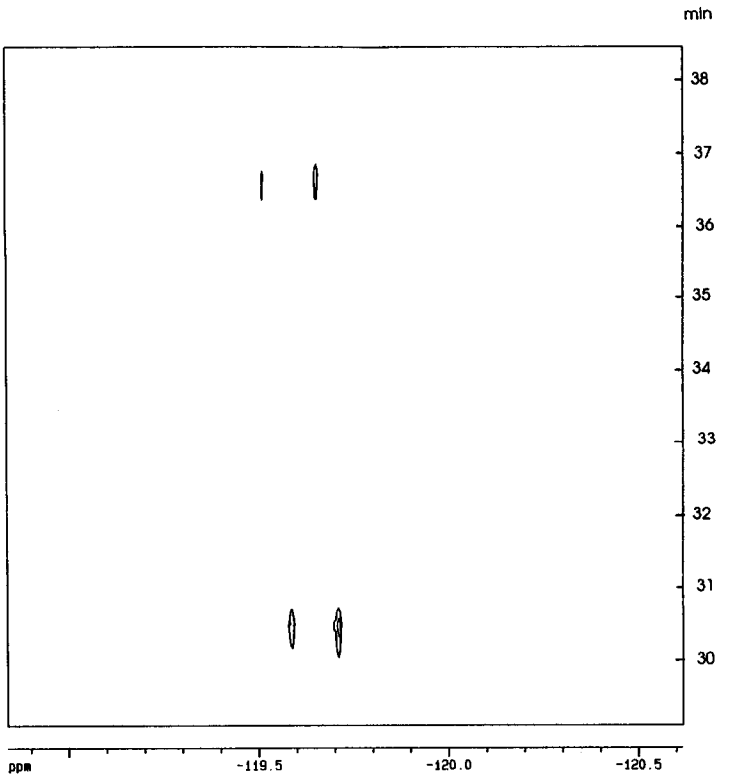

Figure 4
Two-dimensional contour plot of an on-flow 470 MHz ^{19}F-NMR-detected HPLC separation. The horizontal axis represents ^{19}F-NMR chemical shifts and the vertical axis is time.

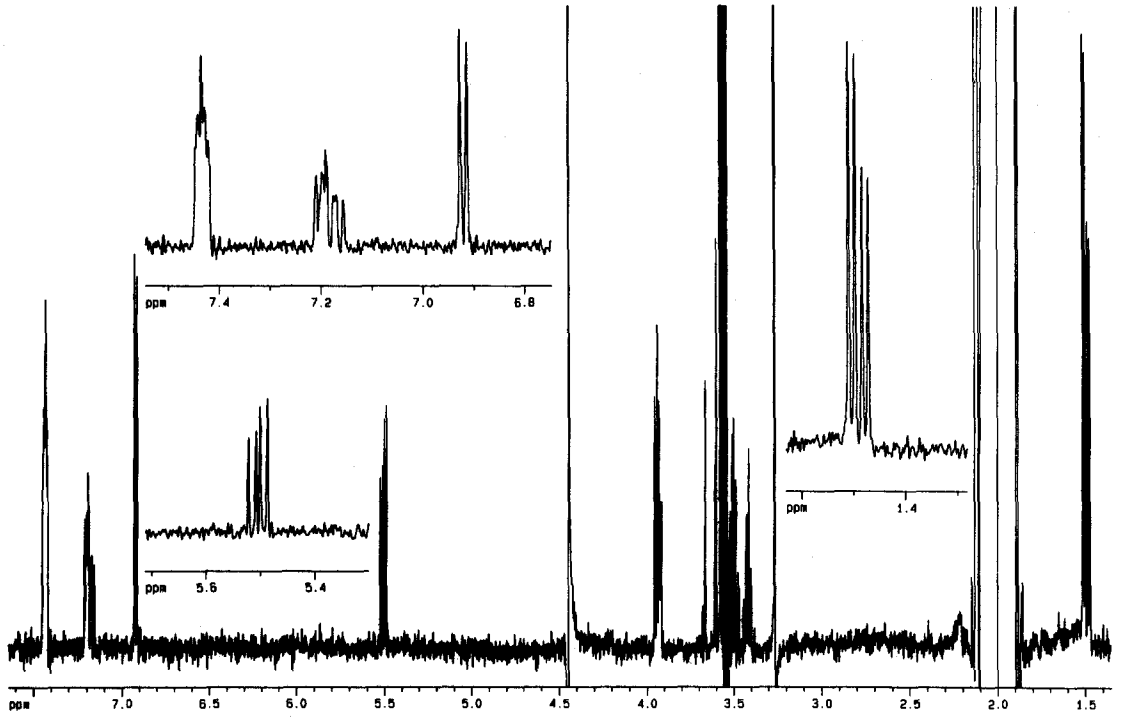

Figure 5
600 MHz ^{1}H-NMR spectrum, measured in stopped-flow mode, of the 30.5 min retention time species shown in Fig. 4 corresponding to the β-D-glucuronic acid conjugate of **II**. Residual solvent signals are at approximately δ4.4 and δ2.0.

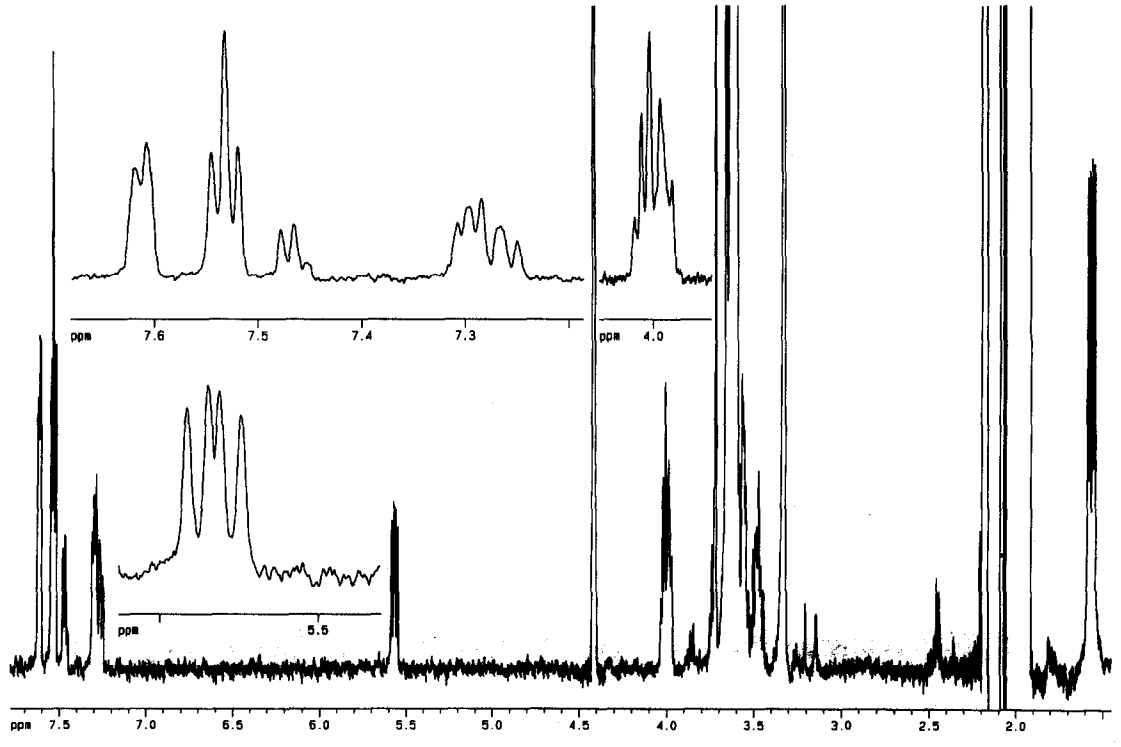

Figure 6
A 600 MHz ^{1}H-NMR spectrum, measured in stopped-flow mode, of the 36.6 min retention time species shown in Fig. 4 corresponding to the β-D-glucuronic acid conjugate of **I**.

seen in Fig. 3 and the longer retained peaks correspond to the other two major peaks seen in Fig. 3. The doubling of the peaks in the on-flow experiment is probably due to the formation of a diastereomeric pair of molecules because of conjugation of β-D-glucuronic acid with both *R* and *S* isomers of the racemic flurbiprofen. The intensities of the two components in each pair of resonances are not equal and this may be a consequence of differential pharmacokinetics or metabolism of the two isomers of the racemic drug. Alternatively, there may be some enantiomeric conversion of *R* to *S* form *in vivo*.

Re-examination of the sample using the same conditions by ^{1}H-NMR spectroscopy at 600 MHz was then carried out. A stopped-flow experiment at the retention times afforded by the ^{19}F-detected experiment gave the ^{1}H spectra shown in Figs 5 and 6. Figure 5 shows a ^{1}H-NMR spectrum of the shorter retained component consistent with the β-D-glucuronic acid conjugate of metabolite **II** with the resonances of the *p*-substituted phenyl ring appearing at δ6.91 (*ortho* to oxygen) and δ7.42 and the other three aromatic resonances as a complex multiplet at δ7.19 and also at δ7.42. The β-D-glucuronic acid H-1 proton appears as two resonances at δ5.49 and δ5.52 with proportions of 57:43 because of the diastereomeric nature of the products. The proportions obtained from the ^{19}F-NMR spectra obtained in the on-flow experiment differed somewhat because, at the recycle times used in that experiment, incomplete ^{19}F spin-lattice relaxation affected the relative signal intensities. The remaining β-D-glucuronic acid resonances appear between δ3.4 and δ3.6. The methyl resonances are clearly visible appearing as two doublets with proportions of 59:41 at δ1.49 and δ1.51. The two methine signals are observed at about δ3.94 as overlapped multiplets. Figure 6 shows the spectrum of the longer retained ^{19}F-containing fraction and is consistent with the β-D-glucuronic acid conjugate of flurbiprofen itself, **I**. Thus the aromatic chemical shifts now show no low frequency signals consistent with a lack of ring hydroxylation, these aromatic signals now integrating to eight protons. The β-D-glucuronic acid H-1 protons now appear at δ5.65 and δ5.67 as equal intensity doublets with the rest of the glucuronide signals appearing between about δ3.4 and 3.6. The methine resonances

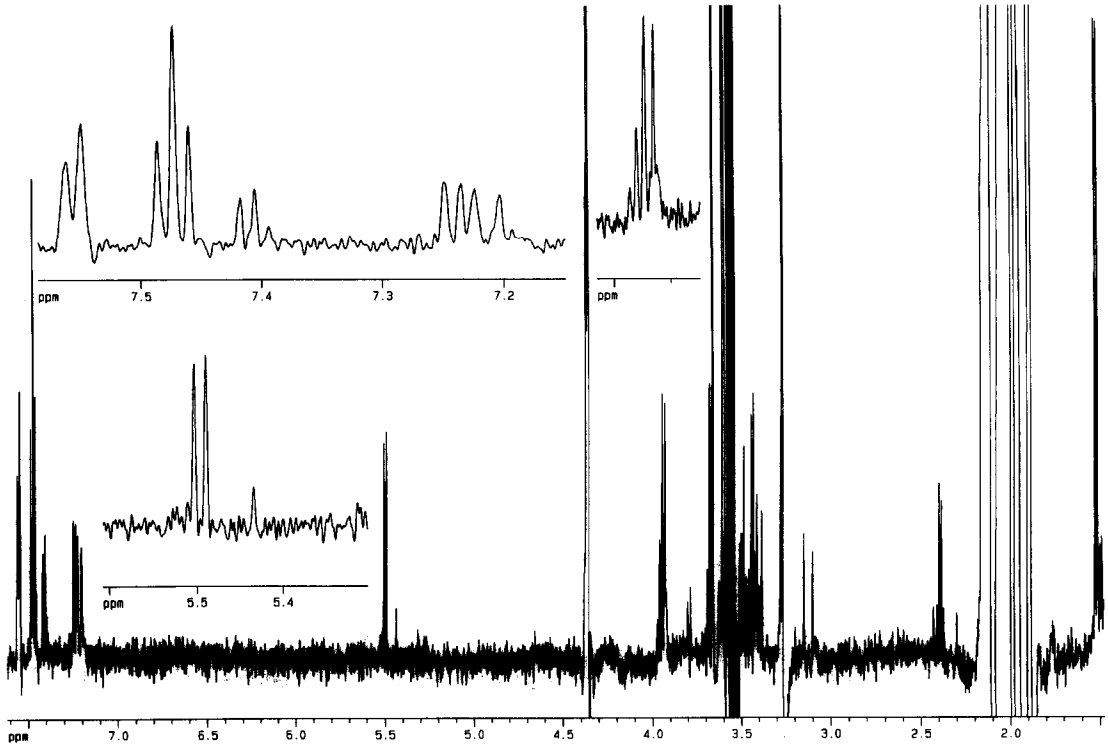

Figure 7
A 600 MHz ^{1}H-NMR spectrum, measured in stopped-flow mode, at a retention time 30 s after that of Fig. 6, showing separation of one diastereoisomer of the β-D-glucuronic acid conjugate of **I**.

are observed as two quartets at about δ4.0 and the corresponding methyl signals appear as two doublets at δ1.55 and δ1.58 with an intensity ratio of 47:53. The ^{1}H-NMR spectrum measured in stopped-flow mode at a retention time of 30 s after that which produced the spectrum in Fig. 6, is shown in Fig. 7. By comparison with Fig. 6, it can be seen that a good separation of one of the diastereoisomers of flurbiprofen-β-D-glucuropyrosiduronate has been achieved.

In addition, examination using ^{1}H-NMR in stopped-flow mode has detected one other flurbiprofen related substance in the urine. This was identified at a retention time of 54.3 min as unconjugated 4′-hydroxyflurbiprofen, **II**, on the basis of the observed chemical shifts, integrals and spin coupling patterns.

Whereas the usefulness of ^{1}H-NMR spectroscopy of urine as a means of detecting drug metabolites is greatly affected by the presence of endogenous compounds, few such limitations apply when the drug or xenobiotic is fluorinated and ^{19}F-NMR can be used. Clearly the simplicity of the ^{19}F-NMR spectra greatly eases the task of identifying which of the peaks in the chromatogram are drug related. This enables effort to be rapidly focused only on the components of the sample related to the analyte. The ability of NMR spectroscopy in this case to also enable the chiral analysis of the diastereomeric β-D-glucuronic acid conjugates to be performed, in the absence of a chromatographic separation is also noteworthy. Following this preliminary report of the first use of HPLC coupled with ^{19}F-NMR and with 600 MHz ^{1}H-NMR, further studies to extend the observations and to further explore the usefulness of coupled HPLC–NMR for the study of xenobiotic metabolism are in progress.

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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, *Prog. Drug Res.* **31**, 427–479 (1987).
[3] 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).
[4] F.Y.K. Ghauri, C.A. Blackledge, I.D. Wilson and J.K. Nicholson, *J. Pharm. Biomed. Anal.* **8**, 939–944 (1990).
[5] P.J.D. Foxall, J.A. Parkinson, I.H. Sadler, J.C. Lindon and J.K. Nicholson, *J. Pharm. Biomed. Anal.* **11**, 21–31 (1993).
[6] B.C. Sweatman, R.D. Farrant, E. Holmes, F.Y. Ghauri, J.K. Nicholson and J.C. Lindon, *J. Pharm. Biomed. Anal.* **11**, 651–664 (1993).
[7] I.D. Wilson and J.K. Nicholson, *Anal. Chem.* **59**, 2830–2832 (1987).
[8] E. Bayer, K. Albert, M. Nieder, E. Grom, G. Wolff and M. Rindlisbacher, *Anal. Chem.* **54**, 1747–1750 (1982).
[9] H.C. Dorn, *Anal. Chem.* **56**, 747–758A (1984).
[10] M. Spraul, M. Hofmann, P. Dvortsak, J.K. Nicholson and I.D. Wilson, *J. Pharm. Biomed. Anal.* **10**, 601–605 (1992).
[11] M. Spraul, M. Hofmann, P. Dvortsak, J.K. Nicholson and I.D. Wilson, *Anal. Chem.* **65**, 327–330 (1993).
[12] P.C. Risdall, S.S. Adams, E.L. Crampton and B. Marchant, *Xenobiotica* **8**, 691–704 (1978).
[13] H.B. Hucker, K.C. Kwan and D.E. Duggan, in *Progress in Drug Metabolism* (J.W. Bridge and L.F. Chasseaud, Eds), pp. 165–253. Wiley, Chichester, UK (1980).
[14] K.E. Wade, I.D. Wilson, J.A. Troke and J.K. Nicholson, *J. Pharmaceut. Biomed. Anal.* **8**, 401–410 (1990).

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