Journal of Pharmaceutical & Biomedical Analysis
Vol. 6, No. 2, pp. 151–165, 1988
Printed in Great Britain

0731-7085/88 $3.00 + 0.00

Solid phase extraction chromatography and NMR spectroscopy (SPEC–NMR) for the rapid identification of drug metabolites in urine

IAN D. WILSON*† and J. K. NICHOLSON‡

**Drug Development, Hoechst Pharmaceutical Research Laboratories, Walton Manor, Walton, Milton Keynes, Bucks MK7 7AJ, UK*
‡Department of Chemistry, Birkbeck College, University of London, Malet Street, London WC1E 7HX, UK

Abstract: The use of solid phase extraction onto disposable columns containing a C18 bonded silica gel provides a rapid and simple procedure for the removal of interfering endogenous components from urine samples containing drug metabolites prior to detection and identification by ^{1}H NMR spectroscopy. In addition, these columns can be used to retain and concentrate the compounds of interest, thus improving the effective sensitivity of the NMR detection method. Using simple step gradients chromatographic separations can be performed, and metabolites may be rapidly fractionated. This approach (solid phase extraction chromatography with NMR or SPEC–NMR) utilises the multiparametric metabolite detection facility of a Fourier transform NMR spectrometer to monitor a chromatographic separation, as such it has some of the beneficial properties of a directly linked liquid chromatography–NMR system without any of the disadvantages. Applications of the SPEC–NMR method in the investigation of drug metabolism are illustrated here by reference to excretion studies on the drugs ibuprofen, paracetamol, aspirin, oxpentifylline and naproxen.

Keywords: *Urinalysis; high resolution proton NMR; drug metabolite identification; solid phase extraction; SPEC–NMR.*

Introduction

There is considerable utility in the application of high resolution ^{1}H nuclear magnetic resonance (NMR) spectroscopy to the direct detection, identification and quantification of drugs, drug metabolites and endogenous components in urine and plasma samples [1–13]. The advantage of such an approach is that rapid multi-component metabolite determinations of a complex biological sample can be effected with a minimum of physical or chemical pre-treatment of the matrix. Furthermore, it is not necessary to make assumptions as to the identity of compounds present in the sample prior to determination. This contrasts markedly with most analytical methods (particularly

†Present address: Department of Safety of Medicines, ICI Pharmaceuticals Division, Meerside, Alderley Park, Macclesfield, Cheshire SK10 4TG, UK.

chromatographic procedures) where sample preparation and detection are deliberately optimised to select a restricted range of closely related compounds. However, NMR is an insensitive technique when compared with most chromatographic detection methods, and for ^1H NMR to be effective, analytes must be present in relatively large amounts (>50 μM) and must also possess suitable resonances, usually from CH, CH_2 or CH_3 protons which can be readily identified and quantified [1–4]. Ideally, the resonances from drug metabolites should lie in regions of the proton NMR spectrum of a biological sample which are relatively free of signals from endogenous components (previously described as being low in "chemical noise" [5]). One of the factors limiting the more widespread use of proton NMR in this way is that, hitherto, such studies have required the use of spectrometers operating at very high magnetic field strengths e.g. 9.4 or 11.75 T corresponding to proton resonance frequencies of 400 and 500 MHz, respectively. Although such instrumentation is becoming more common in research laboratories, it is still not widely available mainly due to the very high capital costs involved. Studies have, therefore, been undertaken to explore the applications of the technique to study the metabolism and urinary excretion of drugs using "routine" instruments operating at much lower frequencies such as 250 MHz [5, 6].

A number of problems are encountered when low to medium strength spectrometers are used for ^1H NMR analysis of biological fluids. High field spectrometers i.e. operating at 9.4 T/400 MHz or more, apart from having high sensitivities, give better frequency dispersion of signals which help to simplify the interpretation of spectra obtained from complex biochemical mixtures in which there may be resonances from many magnetically similar protons. At lower operating frequencies (e.g. 200–250 MHz) the resonances from endogenous compounds are usually extensively overlapped, and these may interfere with the signals from drug metabolites [5, 6]. Observation of signals from compounds in low concentration (low mM range) in the presence of the very large solvent water signal (>100 M in protons) can also pose severe dynamic range problems in Fourier transform spectrometers, which all utilise analogue to digital converters [4]. Therefore, without adequate suppression of the water signal, spectral information on metabolites is greatly reduced due to inadequate digitisation of their proton signals [4]. Recently, spin-echo methods have been used to reduce the intensity of the large water signal present in all biological samples [14–16]. This method requires some type of chemically induced exchange broadening of the water signal in combination with a T_2 pulse sequence, to effect water suppression on the basis of the short water T_2 relaxation time [16]. These solvent suppression methods are also less effective at lower magnetic field strengths because of field dependent relaxation effects [15, 16]. The simple expedient of freeze drying urine followed by reconstitution in a smaller volume of ^2H_2O, largely overcomes the dynamic range problem, but may in turn result in the loss of volatile components and deuteration of exchangeable protons. Nevertheless, the major acidic metabolite of the drug oxpentifylline in freeze dried urines was detected and quantified without difficulty using a 250 MHz spectrometer [5]. However, the interpretation of spectra of these freeze dried urine samples was still complicated by interferences from endogenous urinary components. There are also limitations to the amount by which urine samples may be concentrated before the high ionic strength of the solution results in salting-out of some compounds. This is clearly undesirable for most quantitative procedures as losses of this type are not generally reproducible. High ionic strengths of solutions for NMR may also adversely affect the tuning of the radio-frequency circuitry in the NMR probe with consequent loss in sensitivity. To overcome

these problems, the use of straightforward sample preparation techniques that can rapidly and selectively remove interfering endogenous metabolites, and also allow sample solvent deuteration and concentration of metabolites have been investigated. Experience with sample preparation for chromatographic analysis suggested that solid phase extraction methods were likely to produce the desired results. Using this approach, compounds of interest are selectively retained on an adsorbent (contained in a small disposable column) and then eluted with a suitable solvent, the spectrum of the resulting eluate being measured directly by ^{1}H NMR spectroscopy [6]. Preliminary results with naproxen showed that in addition it was also possible to concentrate, fractionate and purify the drug metabolites from urine using this method [6]. The technique has now been extended to a wider range of compounds and the results described here show the application of solid phase extraction chromatography with nuclear magnetic resonance (SPEC–NMR) for the analysis of urine samples containing a range of commonly used drugs, including in particular analgesics and anti-inflammatory drugs such as paracetamol, aspirin, ibuprofen and naproxen, and the anti-anginal drug oxpentifylline.

Materials and Methods

NMR spectroscopy

^{1}H NMR spectra were measured at ambient probe temperature (25°C) on a Bruker WM250 spectrometer operating at 250 MHz with quadrature detection. 350 Free induction decays (FIDs) were collected after 90° pulses into 16,384 computer points, using a sweep width of 3400 Hz and a data acquisition time of 2.4 s. A further delay of 5 s was also added between successive pulses, to allow the nuclear spins to return to equilibrium magnetization via T_1 relaxation. The signal from the residual water protons was suppressed by the application of a pre-saturating secondary irradiation field at the water resonance frequency, this being gated off during acquisition of the FID.

The ^{13}C NMR spectrum of naproxen glucuronide in deuterated dimethyl sulphoxide (DMSO) was obtained on a Bruker AM200 spectrometer operating at 50.3 MHz ^{13}C frequency using a sweep width of 12,000 Hz and an acquisition time of 1.4 s. 9100 FIDs were collected into 32,768 computer points, and an exponential function corresponding to 3 Hz line broadening applied prior to Fourier transformation.

Subjects

A healthy, normal male subject (aged 32) was dosed orally with single therapeutic doses of aspirin (acetyl salicylic acid, 600 mg), ibuprofen [2-(4-isobutylphenyl)-propionic acid, 400 mg] or paracetamol (500 mg). Urine samples, including a pre-dose sample, were collected as voided into pre-weighed plastic containers and stored frozen (−20°C) until required. Samples of urine containing metabolites of the drug naproxen [d-2(6-methoxy-2-naphthyl)-propionic acid] were obtained (as above) from a female subject (aged 42) with mild osteoarthritis, following a single oral dose of 500 mg. In another study (described in detail elsewhere [5]) samples were obtained from a healthy male volunteer dosed orally with 600 mg of oxpentifylline.

Sample preparation

250 MHz ^{1}H NMR spectra were obtained from all samples by freeze drying 2 ml of urine and reconstituting in 1 ml of ^2H_2O. The following procedure was adopted for solid

phase sample preparations: Samples of urine (2 ml) were loaded onto 3 ml capacity C18 Bond-Elut columns (Analytichem International, purchased from Jones Chromatography Ltd., Glamorgan, UK), containing 500 mg of sorbent, which had been activated by washing with 5 ml of methanol and then 5 ml of water. In most experiments the urine samples were acidified with 10 μl ml^{-1} of 99% formic acid. At this point the column was washed with 5 ml of $C^2H_3O^2H$ or mixtures of $C^2H_3O^2H$ and ^2H_2O. Column eluates were collected into 20 ml scintillation vials. Solvents were removed using a stream of nitrogen and freeze drying if necessary.

Treatment of solid phase C18 columns with ion pair reagent to modify retention characteristics

Where necessary the solid phase cartridges were washed first with methanol (5 ml) followed by 1 ml of a 0.05 g ml^{-1} solution of cetyltrimethyl ammonium bromide (CAB) in methanol, and then washed with 1 ml of distilled water. This treatment resulted in the retention of the ion pair reagent and so modified the extraction properties of the column. A 2 ml sample of urine containing paracetamol metabolites was made alkaline by the addition of 100 μl of 2 M NaOH and loaded onto a CAB treated C18 cartridge, which was then washed with dilute alkali, and the retained paracetamol metabolites eluted with methanol as above.

Stepwise elutions

In order to obtain selective recovery of metabolites in some samples, stepwise elution procedures were employed. Urine samples were applied to the C18 columns as previously described and after washing with acidified water were eluted with methanol–water mixtures (5 ml washes) starting with 20:80 and progressing through 40:60, 60:40, and 80:20 steps to 100% methanol. These fractions, after removal of methanol using a stream of nitrogen and freeze drying to remove the residual water, were then redissolved in ^2H_2O.

Results

The 250 MHz ^1H NMR spectrum of a freeze dried control urine sample with the signals from many endogenous metabolites assigned is shown in Fig. 1. The spectrum contains several frequency "windows" in which there are few resonances from endogenous compounds and in which signals from drug metabolites may be observed with relatively little interference (e.g. 0–1 ppm; 1.5–1.9 ppm; 5–7 ppm and 7.8–12 ppm [5]). NMR spectra measured at much higher field strengths (e.g. corresponding to 400 or 500 MHz observation) are less prone to interferences from endogenous compounds or chemical noise [5] as signals show greater frequency dispersion and so overlap less with each other. After loading an aliquot of the urine sample shown in Fig. 1 onto a C18 Bond-Elut column, only urinary pigments were retained on the column. However, after acidification with formic acid to approximately pH 2, the bulk of the urinary aromatic acids (e.g. hippurate, indoxyl sulphate, etc.) were selectively retained on the column, whilst most of the non-aromatic amino acids and neutral molecules such as glucose, passed through the column into the eluate. The aromatic compounds were then recovered on washing the column with methanol. Subsequent experiments showed that as little as 20% methanol was sufficiently elutropic to give complete recovery of these components. Solid phase extraction chromatography was then applied to acidified urine samples containing a variety of drugs and their metabolites (see below).

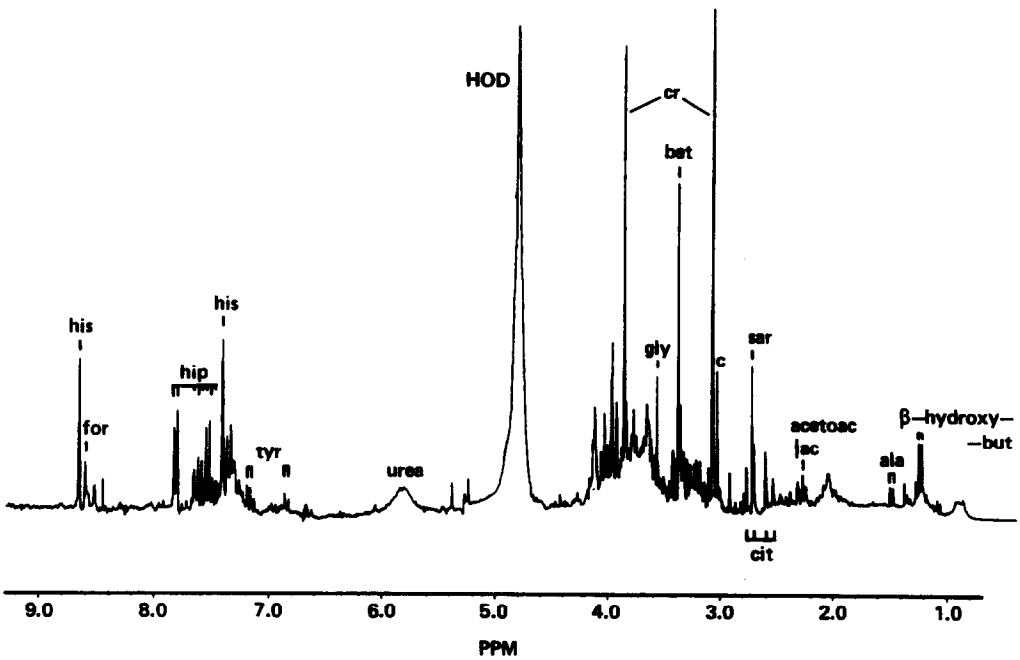

Figure 1
A 250 MHz ^1H NMR spectrum of a sample of freeze dried control urine (2 ml) redissolved in ^2H_2O for spectroscopy. Key: ac, acetate; ACETOAC, acetoacetate; ala, alanine; bet, betaine; β-hydroxybut, β-hydroxybutyrate; c, creatine; cit, citrate; cr, creatinine; for, formate; gly, glycine; hip, hippurate; his, histidine; sar, sarcosine; tyr, tyrosine.

Oxpentifylline

The quantitative determination of 1-(3′-carboxypropyl)-3,7 dimethylxanthine, CPDX, the major acidic metabolite of oxpentifylline in urine, following oral dosing with 600 mg of the drug has recently been investigated [5]. A typical ^1H NMR spectrum of a urine sample containing CPDX is shown in Fig. 2a, in which signals for the two *N*-methyl groups at the N3 and N7 positions and the C8H proton are well resolved. However, signals for the side chain methylene groups are subject to considerable overlap with resonances from endogenous metabolites. A very significant improvement in this respect was obtained by concentrating the metabolite onto a C18 cartridge and then eluting with methanol (Fig. 2b). Whilst the degree of purification achieved by this simple procedure might suffice for unequivocal assignment in most cases, even better results could be obtained by washing the cartridge with methanol–water 20:80 (v/v) to elute hippuric acid and indoxyl sulphate, prior to eluting the CPDX with 100% methanol. This treatment gave a >95% pure preparation of CPDX in which all the proton resonances of the molecule could be unequivocally assigned (Fig. 2c).

Ibuprofen

The NMR spectrum shown in Fig. 3a was obtained from a urine sample collected 2–4 h after a single oral dose of 400 mg of ibuprofen. The spectrum of the control pre-dose urine sample is shown in Fig. 1. After drug treatment many new resonances were observed. These included a broad envelope of overlapping aromatic resonances centred

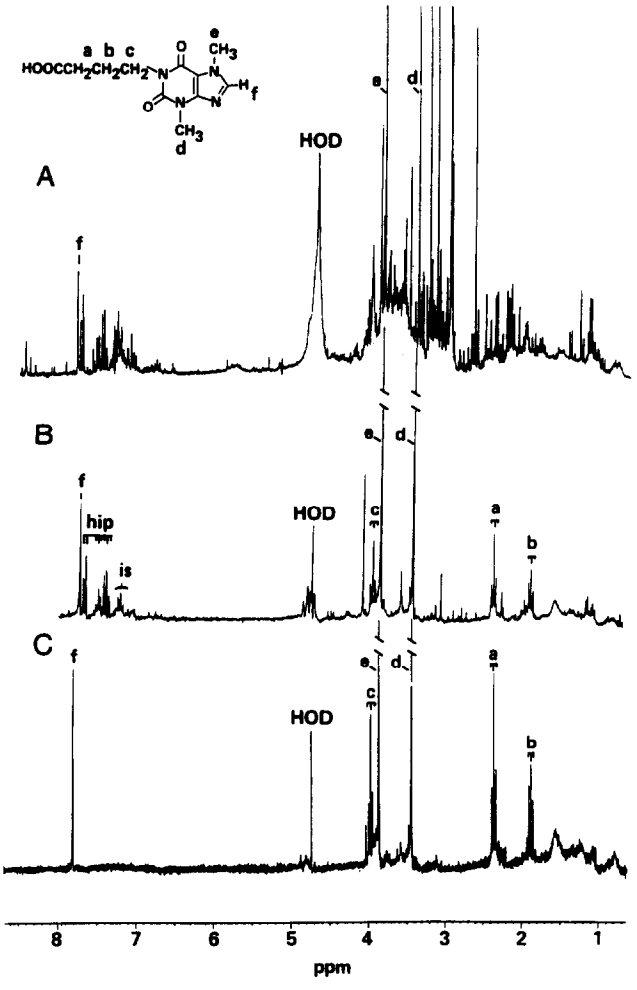

Figure 2
(A) A 250 MHz ^1H NMR spectrum of a sample of freeze dried urine (2 ml) obtained from a subject following an oral dose of oxpentifylline (600 mg). The sample was redissolved in ^2H_2O for spectroscopy. (B) A 250 MHz ^1H NMR spectrum obtained following solid phase extraction of the sample illustrated in (a) onto a C_{18} Bond-Elut cartridge and subsequent elution with methanol. Methanol was removed and the residue dissolved in ^2H_2O for spectroscopy. (C) A 250 MHz ^1H NMR spectrum of a sample treated as described for (b) but with a methanol–water wash (20:80) of the cartridge to elute endogenous contaminants prior to elution of the oxpentifylline metabolite with methanol. Following removal of methanol the residue was dissolved in ^2H_2O for spectroscopy. Key: as for Fig. 1.

at about 7.25 ppm, and a prominent doublet at 5.4 ppm from the β-anomeric proton of ibuprofen and its metabolites present as glucuronide conjugates. The spectral region from 0.8 to 1.5 ppm also contained several signals of drug origin including a large singlet at 1.18 ppm {methyl group from a metabolite with a hydroxylated isobutyl side chain i.e. 2-[4-(2-hydroxy-2-methylpropyl)-phenyl] propionic acid, HMPPP}. Application of 2 ml of acidified urine to a C18 column, followed by elution of the retained material with methanol produced the result shown in Fig. 3b. As seen with the CPDX metabolite of oxpentifylline, the signals from the ibuprofen metabolites were much more prominent in

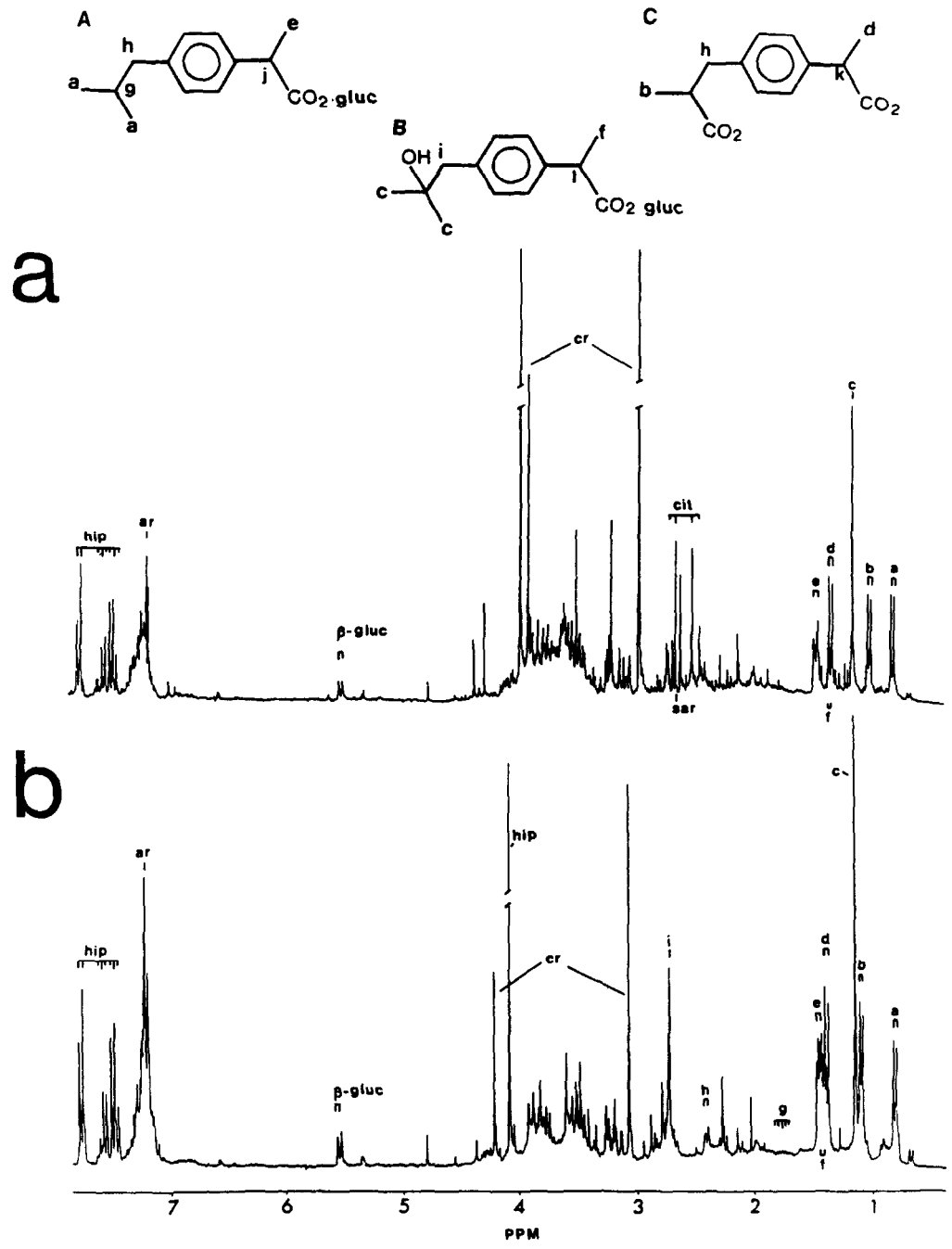

Figure 3
(a) A 250 MHz ^1H NMR spectrum of a sample of freeze dried urine (2 ml) obtained from a subject following oral dosing with 400 mg of ibuprofen. The sample was redissolved in ^2H_2O for spectroscopy. Structures of the metabolites present are shown above the figure with provisional assignments on the metabolite signals as indicated. (b) A 250 MHz ^1H NMR spectrum of the sample shown in (3a) following solid phase extraction onto a C_{18} Bond-Elut cartridge and elution with methanol. Methanol was removed and the residue dissolved in ^2H_2O for spectroscopy. Key: As for Fig. 1.

this spectrum, Using the stepwise gradient elution technique, it was possible to fractionate further the ibuprofen metabolites into two groups of metabolites, both essentially free of endogenous compounds. The first group, eluting with 40% methanol, appeared by NMR to contain a glucuronide of HMPPP, and the side chain oxidised metabolite 2-[4-(2-carboxy-2-methylpropyl)-phenyl] propionic acid (CMPPP). Elution with 60% methanol, led to the recovery of further material consisting predominantly of ibuprofen glucuronide. A small amount of contamination of the 60% fraction with metabolites predominantly eluting in the 40% methanol wash was also observed. Thus, as was seen for the oxpentifylline metabolite, CPDX, using the C18 cartridge in this way allows the metabolites to first be concentrated and partially purified from the crude urine sample, whilst subsequent gradient elution then enables a conclusive structural identification of the metabolites to be made.

The capacity of the C18 cartridge to retain ibuprofen metabolites, by gradually increasing the amounts of urine (containing 1–2 mg ml^{-1} of metabolites) loaded onto the column until no further retention of drug related substances occurred, was also investigated. There was no detectable breakthrough of ibuprofen metabolites until about 15 ml of urine had been loaded to a 3 ml C18 Bond-Elut column. When 20 ml of urine was loaded some breakthrough of drug related substances occurred. However, elution of the retained material with methanol gave very concentrated samples containing primarily drug metabolites. This procedure allowed high quality NMR spectra with very good signal to noise ratios to be obtained after the collection of 16–32 FIDs. The spectrum shown in Fig. 4 was obtained following the elution with methanol of a cartridge to which

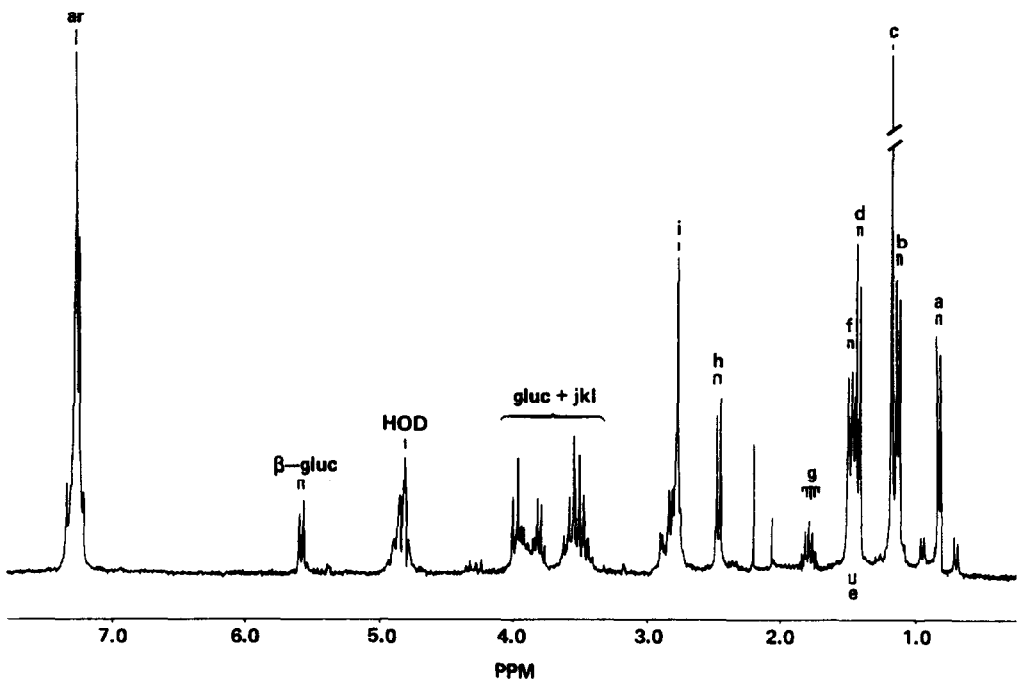

Figure 4
A 250 MHz ^1H NMR spectrum of the concentrate obtained following the solid phase extraction of 12 ml of the sample shown in (Fig. A) onto a C_{18} Bond-Elut cartridge. The ibuprofen metabolites were eluted with methanol. After removal of the solvent the residue was dissolved in ^2H_2O for spectroscopy.

12 ml of urine had been applied (i.e. insufficient to cause metabolite breakthrough). In the case of urine, columns appear to have a greater capacity or affinity for acidic drug metabolites of this type than the endogenous components. Thus few signals from endogenous components were observed, even though no attempt was made to remove them by the use of stepwise elution procedures. So in this instance, a high column loading was beneficial both in terms of purification and ease of NMR detection.

Naproxen

The spectrum shown in Fig. 5a was obtained from a urine sample covering the period 0–5.5 h after a single oral dose of the non-steroidal anti-inflammatory drug naproxen. A prominent doublet is observed at 5.5 ppm corresponding to the β anomeric proton of the naproxen glucuronides. Signals from the α methyl protons from the propionyl side chain together with a number of drug related aromatic signals between 7 and 8 ppm were also present. However, the signals from the naproxen metabolites were weak in comparison to those of the endogenous urinary components. Concentration of the metabolites onto a

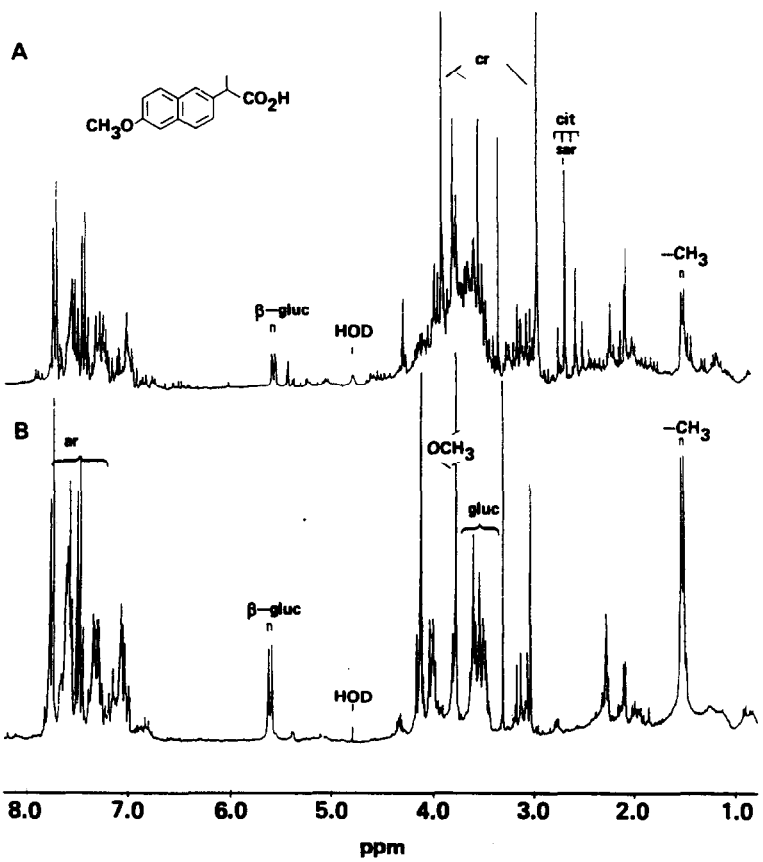

Figure 5
(A) A 250 MHz ^{1}H NMR spectrum of a sample of freeze dried urine (2 ml) obtained following oral dosing with naproxen. The sample was redissolved in ^2H_2O for spectroscopy. (B) A 250 MHz ^{1}H NMR spectrum of the sample shown in (a) after solid phase extraction onto a C_{18} Bond-Elut cartridge and elution with methanol. Methanol was removed and the sample redissolved in ^2H_2O for spectroscopy. Key: As for Fig. 1.

C18 cartridge, followed by elution with methanol, gave the spectrum shown in Fig. 5b (after removal of the solvent and redissolution in ^2H_2O). Stepwise elution with a gradient of increasing methanol concentration, allowed the complete removal of most endogenous components, and the separation of the drug metabolites into fractions that gave readily interpretable ^1H NMR spectra, i.e. *O*-desmethyl plus parent Fig. 6a (as glucuronides) for the 40% MeOH wash, whilst the 60% wash gave just naproxen glucuronide (Fig. 6b).

As shown by the experiments on urines containing ibuprofen metabolites, solid phase extraction columns can be used to concentrate compounds from crude biological samples very rapidly. In order to test the effectiveness of the method for obtaining compounds from urine on a larger scale, we chose to extract purified naproxen glucuronide and its *O*-desmethyl metabolite from urine collected from 0 to 5.5 h after ingestion of drug. After passing 20 ml of urine through a 3 ml C18 Bond-Elut column and eluting with a stepwise methanol/water gradient as described above, the individual fractions containing the

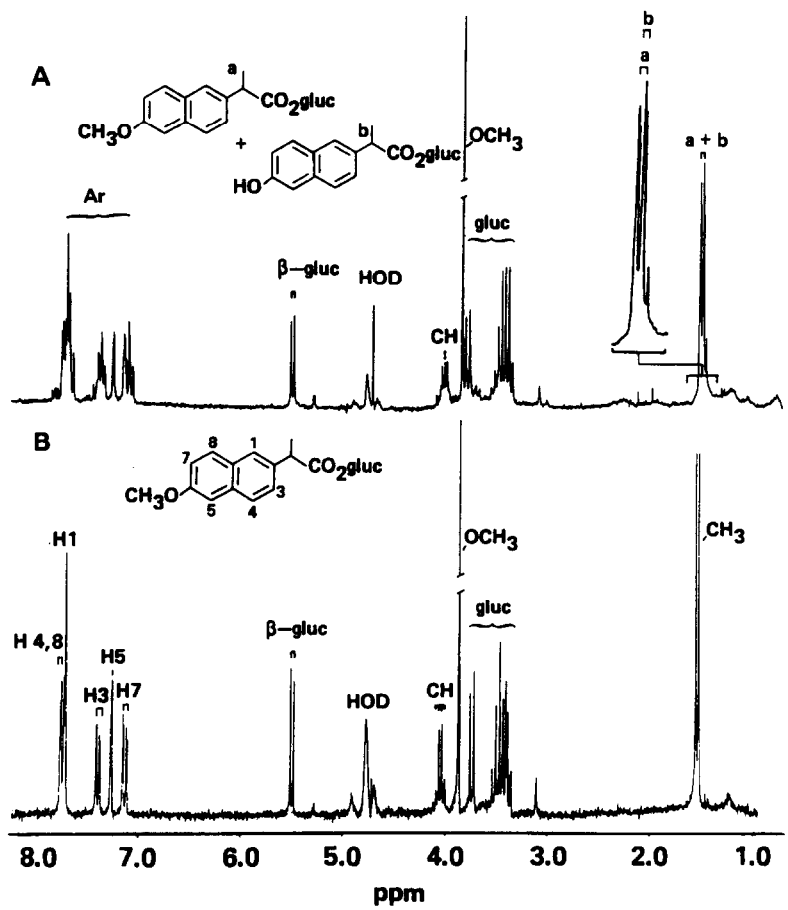

Figure 6
The 250 MHz spectrum obtained following the sequential elution of naproxen metabolites adsorbed from urine (A) onto a C_{18} Bond-Elut column. The fraction eluting with methanol–water (40:60) (A) contains *O*-desmethyl naproxen and naproxen glucronides whilst the fraction eluting with methanol–water 60:40 (B) contains naproxen glucuronide. Provisional assignments of the signals are as indicated.

required metabolites (as shown by ^{1}H NMR) were purified further by passage through a second column under the same conditions. This process resulted in the preparation of >95% pure naproxen and *O*-desmethyl naproxen glucuronides as judged by both mass spectrometric and ^{1}H NMR criteria. The naproxen glucuronide was isolated in sufficient quantity for a ^{13}C NMR spectrum to be obtained (Fig. 7). Fast atom bombardment mass spectrometry of both metabolites confirmed the structures proposed on the basis of NMR measurements (Wilson and Nicholson, unpublished).

Aspirin

NMR spectra obtained from urine samples from a subject treated with aspirin contained extra signals (Fig. 8a) from salicyluric acid (the glycine conjugate of salicylic acid), which is the major metabolite of acetyl salicylic acid in man [17]. These assignments were confirmed by reference to spectra of the authentic compound. A small doublet was also observed at 5.8 ppm corresponding to the β anomeric proton of a glucuronide conjugate. Concentration of this urine onto a C18 Bond-Elut column and elution with methanol gave rise to fraction with a much simplified NMR spectrum (Fig. 8b) in which the signals for salicyluric acid were enhanced. As expected the major contaminant observed in this extract is huppuric acid (the glycine conjugate of benzoic acid), which is structurally and chromatographically similar to salicyluric acid.

The metabolism of aspirin in man is highly variable [17] and in a recent study involving 129 volunteers, salicyluric acid was found to form between 19.8 and 65% of the total. In a study of aspirin overdose, Ogunbona [18] found that between 13.6 and 76% of the dose

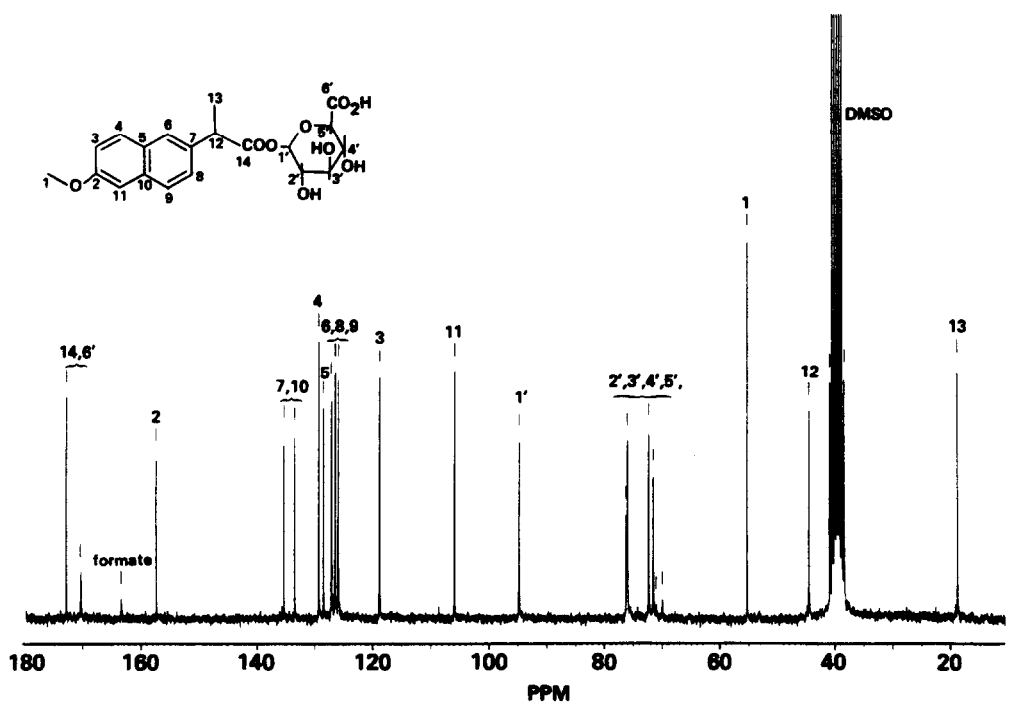

Figure 7
A 50 MHz ^{13}C NMR spectrum of naproxen glucuronide (in $^2H_6$ DMSO) obtained following solid phase extraction onto a C_{18} Bond-Elut cartridge and stepwise elution with methanol–water as described in the text.

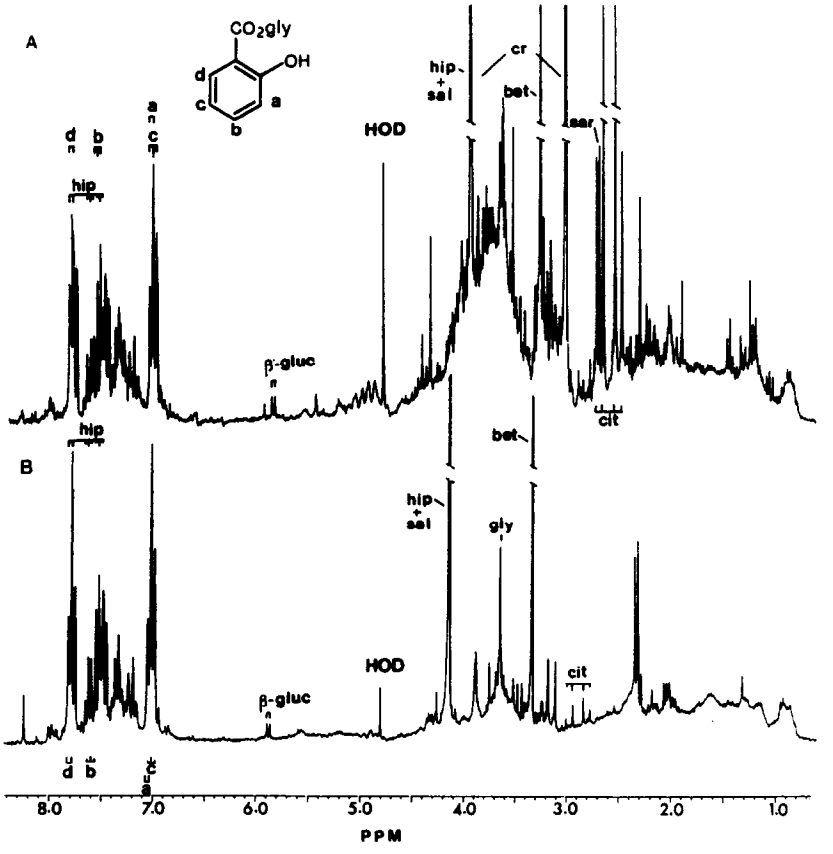

Figure 8
(A) A 250 MHz ^{1}H NMR spectrum of a sample of freeze dried urine (2 ml) obtained following an oral dose of aspirin (600 mg). The sample was redissolved in ^2H_2O for spectroscopy. (B) A 250 MHz ^{1}H NMR spectrum of the same sample following solid phase extraction onto a C_{18} Bond-Elut cartridge and elution with methanol. The methanol was removed and the residue dissolved in ^2H_2O for spectroscopy. Key: as for Fig. 1.

was excreted as salicyluric acid. Some subjects excrete a significant amount of aspirin in metabolites such as glucuronides, but again this is subject to large variations, and to date the samples examined by us have contained mainly salicyluric acid and not glucuronides.

Paracetamol

Signals from the glucuronide and sulphate metabolites of paracetamol in the urine of dosed subjects are readily observed in NMR spectra of urine (Fig. 9a, reference 4). These compounds are not retained on a C18 column under the conditions described above. However, most major endogenous aromatic compounds (such as hippurate) are retained, and thus the aromatic signals of the paracetamol metabolites can be assigned more easily in 250 MHz ^{1}H spectra (Fig. 9b). Paracetamol metabolites may be extracted onto a C18 cartridge, if prior to loading a basified sample, the column is first modified by washing with a solution of a long chain quaternary ion-pair reagent e.g. cetyltrimethyl-ammonium. The loading of the C18 cartridge with an ion-pair reagent in this way converts it into a temporary ion-exchange column, from which the paracetamol metabolites may be removed by washing with methanol.

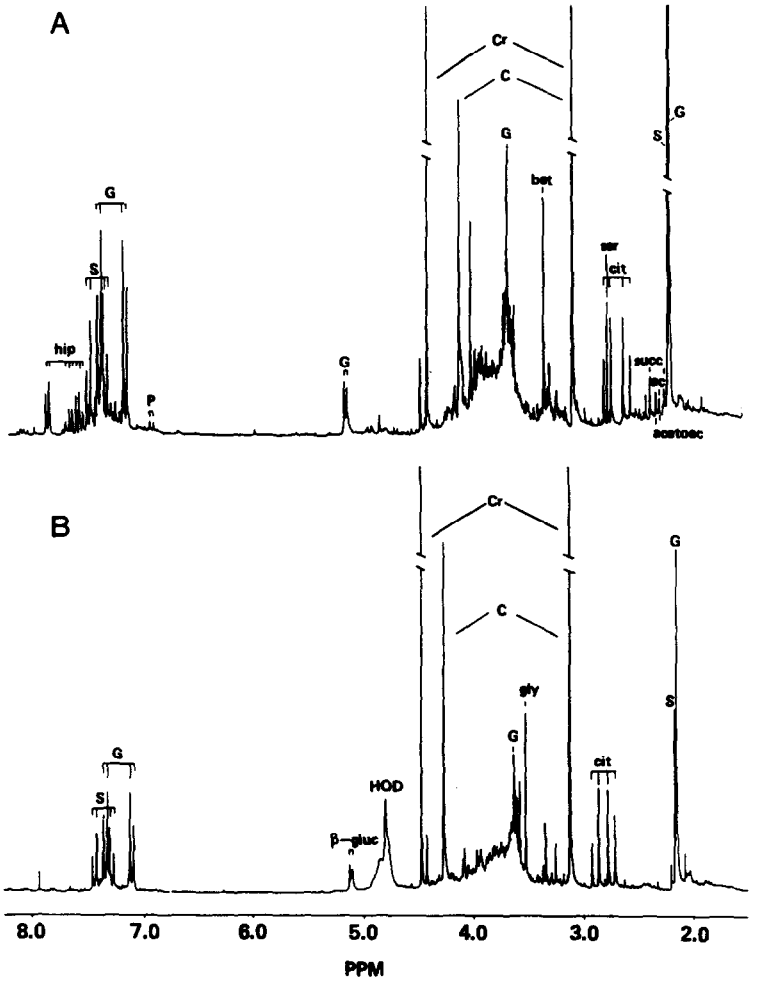

Figure 9
(a) A 250 MHz ^{1}H NMR spectrum of a sample of freeze dried urine (2 ml) obtained following an oral dose of paracetamol (500 mg). The sample was redissolved in ^2H_2O for spectroscopy. (b) A 250 MHz ^{1}H NMR spectrum of the unretained aqueous portion of the sample shown in (9a) after application to a C_{18} Bond-Elut solid phase extraction cartridge. The eluate was freeze dried and redissolved in ^2H_2O for spectroscopy. Key: as for Fig. 1.

Discussion and Conclusions

In previous studies it was shown that high field ^{1}H NMR spectroscopy of urine can provide qualitative and quantitative data on the drug and endogenous metabolites present [4, 7, 8]. Most of this work employed very high frequency NMR measurements on biological samples, so as to allow the interpretation of complex patterns of partially overlapping resonances from structurally similar metabolites. Lower frequency measurements on such matrices normally require some type of preliminary clean-up of the sample, before resonances from drug metabolites contained in the sample can be identified or assigned [5, 6]. The present study using solid phase extraction chromatography and NMR (SPEC–NMR) has extended the methodology to include a simple

and rapid procedure for sample preparation leading to NMR analysis of drug metabolites in urine samples after therapeutic doses of several commonly used, and sometimes abused, drugs. This strategy can also be adopted for novel therapeutic agents in the early stages of drug development and can allow drug metabolism data to be collected at a much earlier stage in the development programme than has previously been possible (I. D. Wilson, unpublished).

SPEC–NMR appears to offer several useful analytical features. At the simplest level this method can greatly simplify NMR spectra of urine (and possibly other biofluids) samples by the selective or controlled removal of many endogenous components which are not retained on the column. Considerable concentration of metabolites can be achieved very quickly and easily, which is of particular importance as NMR spectroscopy is an inherently insensitive technique in comparison to most other analytical methods. A more sophisticated use of C18 columns involves stepwise elution procedures to fractionate complex mixtures of metabolites, thus further simplifying interpretation of the NMR spectra; this is of particular value if only modest (e.g. 200 MHz) NMR instrumentation is available. Under certain circumstances this approach has been used to obtain essentially pure metabolite preparations (e.g. naproxen glucuronide) very rapidly, as such SPEC–NMR may be competitive with preparative HPLC for certain types of application. The main source of analytical power in this method is the ability of NMR to provide multi-frequency (or multi-component) monitoring of the chromatographic process, essentially providing an off-line detector. In order to obtain information in this way the compound under study must have observable protons, present in sufficient concentrations in the final measured sample (typically $>50\ \mu M^{-1}$), these criteria are easily met with a large number of drug metabolism applications.

SPEC–NMR has many of the advantages of directly linked HPLC–NMR systems with few of the disadvantages. For example HPLC–NMR is intrinsically difficult as the two techniques have very different optimal operational characteristics and requirements e.g. HPLC: mixed solvents, continuous flow, and relatively low concentrations of analytes; NMR: single deuterated solvent, static sample (in the flow sense) but spinning to obtain best possible resolution, and compounds present in the millimolar concentration range for rapid detection. Direct matching of these requirements is currently not possible for most desired drug metabolite separation procedures. To obtain worthwhile results using on-line HPLC detection by NMR requires modifications to the NMR probe, an HPLC column placed within the magnet and a very small (e.g. 28 μl) cell, with consequent reduction in sensitivity [19–21]. Spectral acquisition conditions must also be modified according to the separation being undertaken, for example if analytes under separation have very different T_1 or T_2 relaxation rates. In the absence of a completely deuterated solvent system, the dynamic range problems associated with NMR detection of small amounts of analyte may also be insuperable for anything other than preparative scale HPLC, as solvent suppression factors of $>10^3$ are usually required in order for signals from analytes at sub-millimolar concentrations to be adequately digitized (15), factors of 10^2 have so far only been obtained for HPLC–NMR [21].

Using the SPEC–NMR approach to drug metabolite detection and characterization, complete separation of metabolites may be unnecessary for unequivocal assignment of drug metabolite resonances in many cases, as the NMR "detector" effectively "separates" the compounds on the basis of the magnetic properties of their protons. There may also be applications of SPEC–NMR in "preparative scale" chromatographic separations of metabolites, as (unlike conventional preparative HPLC) SPEC–NMR

method development is fast and relatively inexpensive. The isolation of naproxen glucuronide, in sufficient quantity for natural abundance ^{13}C NMR spectroscopy to be performed in a short time, is an excellent example of this approach where more conventionally a method such as preparative scale HPLC or TLC would normally have been used.

To date, these studies with SPEC–NMR have been limited to C18 bonded phases and to urine samples. However, the extension of this approach to other biological fluids such as plasma and also to tissue extracts, and to the use of other types of silica-bonded SPEC phases to extend the range of drug classes that can be studied is currently being performed.

In conclusion, SPEC–NMR offers many advantages over both conventional chromatographic and NMR spectroscopic methods of drug metabolite detection and characterization. At the same time it is inexpensive, easy to implement and applicable to a wide range of desired separations or analyses. As all the drugs and their metabolites that we have studied so far have their own ^{1}H NMR fingerprints, it should be possible in many instances to make positive identification of the parent or metabolite in unknown samples, and so there may be important clinical applications of this technique in drug overdose situations, and in forensic toxicology. Furthermore, the SPEC–NMR approach is clearly applicable to any analytical problem where the concentration of low amounts of organic solute (e.g. water pollutants) from a complex matrix is necessary in order to enable their characterization.

Acknowledgements — We thank the National Kidney Research Fund for supporting this and related work. We are grateful to Dr. P. J. Sadler and Dr. I. M. Ismail for their advice, and Mr. R. Pickford for technical assistance.

References

[1] J. K. Nicholson, M. J. Buckingham and P. J. Sadler, *Biochem. J.* **211(3)**, 605–615 (1983).
[2] J. K. Nicholson, M. O'Flynn, P. J. Sadler, A. Mcleod, S. M. Juul and P. H. Sonksen, *Biochem. J.* **217**, 365–375 (1984).
[3] J. K. Nicholson, J. R. Bales, P. J. Sadler, S. M. Juul, A. Macleod and P. H. Sonksen, *Lancet* **ii**, 751–752 (1984).
[4] J. R. Bales, D. P. Higham, I. Howe, J. K. Nicholson and P. J. Sadler, *Clin. Chem.* **30**, 426–432 (1984).
[5] I. D. Wilson, I. M. Ismail and J. K. Nicholson, *J. Pharm. Biomed. Anal.* **(5)**, 157–163 (1987).
[6] I. D. Wilson and I. M. Ismail, *J. Pharm. Biomed. Anal.* **(4)**, 663–665 (1986).
[7] J. R. Bales, P. J. Sadler, J. K. Nicholson and J. A. Timbrell, *Clin. Chem.* **30(10)**, 1631–1636 (1984).
[8] J. K. Nicholson, J. Timbrell and P. J. Sadler, *Mol. Pharmacol.* **27**, 644–651 (1985).
[9] J. K. Nicholson, J. Timbrell, D. P. Higham and P. J. Sadler, *Human Tox.* **3(4)**, 334–335 (1984).
[10] J. R. Bales, J. K. Nicholson and P. J. Sadler, *Clin. Chem.* **31**, 757–762 (1985).
[11] K. Tulip, J. K. Nicholson and J. A. Timbrell, *Proceedings of the 3rd International Symposium on Chemical and Biological Reactive Intermediates.* Plenum Press, New York (1986).
[12] J. R. Everett, K. Jennings and G. Woodnut, *J. Pharm. Pharmacol.* **37**, 869–873 (1985).
[13] K. Tulip, J. A. Timbrell, J. K. Nicholson, I. D. Wilson and J. Troke, *Drug. Met. Disp.* **(14)**, 746–749.
[14] J. Bell, J. Brown and P. J. Sadler, *J. Comp. Asst. Tomog.* (1986).
[15] S. C. Connor, J. E. Everett and J. K. Nicholson, *Mag. Reson. Med.* **(4)**, 461–470 (1987).
[16] T. M. Eads, S. D. Kennedy and R. G. Bryant, *Anal. Chem.* **58**, 1752–1756 (1986).
[17] A. J. Hutt, J. Caldwell and R. L. Smith, *Xenobiotica* **16**, 239–249 (1986).
[18] F. A. Ogunbona, *J. Chromatogr.* **377**, 471–474 (1986).
[19] D. A. Laude and C. L. Wilkins, *Anal. Chem.* **56**, 2471–2475 (1984).
[20] D. A. Laude, R. W. K. Lee and C. L. Wilkins, *Anal. Chem.* **57**, 1464–1469 (1985).
[21] D. A. Laude and C. L. Wilkins, *Trends in Analytical Chemistry* **5(9)**, 230–235 (1986).

[*Received for review 27 May 1987*]