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750 MHz HPLC-NMR spectroscopic identification of rat microsomal metabolites of phenoxypyridines

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Abstract

Directly coupled 750 MHz HPLC-^{1}H NMR spectroscopy has been applied to the characterisation of low level metabolites of 3-amino-2-(2-fluorophenoxy)pyridine (AP) and 3-nitro-2-(2-fluorophenoxy)pyridine (NP) in rat microsomes. In stop-flow HPLC-NMR mode, the direct injection of microsomal extracts enabled the separation and characterisation of minor metabolites. NP is converted into AP to an extent of 93.4% and this is further metabolised to 4- and 6-hydroxy-AP (6 and 0.6% respectively). Unequivocal identification of these metabolites was achieved without the use of a radiolabel or synthetic standards and thus demonstrates the applicability of directly coupled HPLC-NMR to metabolite identification in in vitro systems. The potential exists for HPLC-NMR and HPLC-NMR-MS to provide rapid metabolic information within the timescale of high throughput lead optimisation exercises in drug discovery. © 1997 Elsevier Science B.V.

Keywords: Fluorophenoxypyridines; Metabolism; Rat microsomes; 750 MHz HPLC-NMR

1. Introduction

Directly coupled HPLC-^{1}H NMR spectroscopy has been shown to provide an effective non-destructive approach for separating and identifying drug metabolites in biofluids such as urine. Such characterisation can often be achieved without the need for radiolabelled materials, synthetic standards or the presence of a UV chromophore and in cases where there are other NMR active nuclei in the drug such as ^{19}F or ^{31}P, metabolite detection can be achieved using the X nucleus signal [1–5]. The majority of metabolic HPLC-NMR studies have been from in vivo studies where urinary excretion of metabolites is extensive. In contrast, the metabolite concentrations generated in in vitro systems can be very significantly lower. Shockcor et al. [6] have recently described the successful identification of human microsomal metabolites of a momoamine oxidase inhibitor BW1370U87 using directly coupled HPLC-NMR.

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As this compound is metabolised extensively to several products by the human hepatic fraction, the concentrations produced were such that it was possible to confirm rapidly the structures of the metabolites previously identified [7]. In the present study we have investigated the use of HPLC-NMR to determine the in vitro metabolic fate of two model drugs (fluorophenoxypyridines) for which there is no published metabolism data.

Microsomes are widely used in drug research and new methods for studying microsomal metabolism are always being sought. The hepatic microsomal fraction is used in in vitro preclinical drug metabolism studies [8] since the most clinically important phase I drug-metabolising enzyme family, the Cytochrome P450 family, is located in this fraction. Incubation of novel therapeutic agents with reconstituted microsomes from rat, dog and human liver tissue can give an indication of the propensity of the agent to undergo P450-mediated metabolism in these species [9]. Certain P450 isozymes, most notably CYP2D6 [10] and CYP2C8 [11] also exhibit a genetic polymorphism of expression in different clinical populations and there is increasing regulatory pressure to determine the risk of new drug entities to individuals who would be susceptible to clinical drug–drug interactions. Genetically engineered organisms such as *E. Coli*, yeasts and V79 cells are used to express individual human P450 isozymes to determine which are responsible for metabolising such novel therapeutic agents [12]. We have thus investigated the use of HPLC-NMR for characterisation of low level metabolites produced by one such in vitro system, that of the rat hepatic microsomal fraction.

Sensitivity is a key issue for consideration where low level microsomal metabolites are being detected. Very high frequency spectrometer operation allows increased sensitivity as well as spectral frequency dispersion which is important in the analysis of complex and overlapped systems. The highest field strength NMR spectrometers currently available operate at 17.6 T (750 MHz ^{1}H observation frequency) and 18.8 T (800 MHz ^{1}H observation frequency) and both can be directly coupled to HPLC systems for drug metabolite analysis [4,13,14]. The purpose of the study undertaken here was to apply such ultra high field HPLC-NMR spectroscopy to the characterisation of minor metabolites of two novel phenoxypyridine substrates 3-amino-2-(2-fluorophenoxy)pyridine (AP) and 3-nitro-2-(2-fluorophenoxy)pyridine (NP) and thus to investigate the feasibility of using HPLC-NMR spectroscopy to identify metabolites of relatively stable model drugs in a representative in vitro system.

2. Experimental

2.1. Standards and reagents

AP, NP and 5-nitro-2-(2-fluorophenoxy)-pyridine (5NP) (Fig. 1 for structures and numbering) were obtained from Fluorochem, UK. Pestanal grade (pesticide analysis grade) acetonitrile was obtained from Riedel de Haen, Germany, and deuterated water was obtained from Fluorochem, UK.

2.2. Rat hepatic microsomal experiments

In vitro metabolism experiments were carried out using hepatic microsomal fractions obtained from male rats. Microsomes were prepared by homogenisation of the liver followed by ultracentrifugation at 20 000 × *g* and 105 000 × *g*. The in-

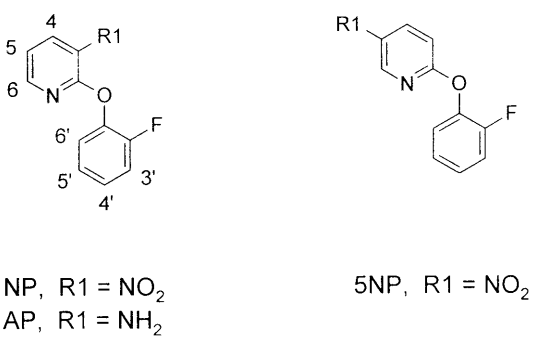

Fig. 1. Structures and numbering scheme for phenoxypyridines: 3-amino-2-(2-fluorophenoxy)pyridine (AP); 3-nitro-2-(2-fluorophenoxy)pyridine (NP); 5-nitro-2-(2-fluorophenoxy)-pyridine (5NP).

cubations were carried out at 37°C, 1 mM substrate and 1 mg ml^{-1} protein in a total incubation volume of 6 ml. The incubation volume was made up of 50 mM Tris–HCl (pH 7.4), 5 mM magnesium chloride and 5 μM manganese chloride. The reducing equivalents required by cytochrome P450 were provided by NADPH (1 mM) which was regenerated using an isocitrate–isocitrate dehydrogenase system. Substrate dissolved in DMSO, was added at the start of a 5 min pre-incubation time and NADPH was added at time zero to start the enzymic reaction. Metabolic activity was terminated 120 min after addition of NADPH by adding the incubation mixture to 30 ml of tert-butyl-methyl ether. Samples were extracted on a rotary mixer for 10 min followed by centrifugation at $1700 \times g$ for 5 min. The ether layer was removed and evaporated to dryness under nitrogen; the residue was stored at −20°C until required for analysis; samples were resuspended in 100 μl mobile phase prior to HPLC analysis.

2.3. HPLC assay of rat microsomal extracts

As metabolite standards and radiolabelled parent compound were not commercially available, a reverse phase HPLC system was developed to separate extracted parent from metabolites on the assumption that the metabolites produced are more polar than the parent molecule. Thus the HPLC assay was tailored to retain the parent on the column for a period long enough to resolve the metabolites which elute earlier. The choice of internal standards for determination of extraction efficiency and measuring reproducibility between injections was based on less polar analogues of the parent molecules so the later elution would not interfere with the metabolites. NP was used as internal standard for the AP extraction and 5NP was the internal standard for the NP extraction.

Chromatographic separation was achieved using UV detection to monitor the eluent. The system consisted of a Bruker LC22C pump (Rheinstetten, Germany) and a Bruker LC33 variable wavelength UV detector operating at 300 nm. The outlet of the UV detector was connected to the HPLC-NMR flow probe via an inert polyether-(ether)ketone capillary (0.25 mm i.d.). Data were collected using a Bruker Chromstar HPLC data system. Analytical chromatography was performed on a 150 mm × 4.6 mm i.d. HPLC column packed with Spherisorb C6 of 5 μm particle size. Separation of the parent molecules, metabolites and internal standards was achieved within 25 min using acetonitrile–sodium perchlorate (pH 2.5; 20 mM) in deuterated water (35:65 v/v) with a flow rate of 1 ml min^{-1}.

2.4. Stop-flow HPLC-NMR spectroscopy

The HPLC-NMR data were acquired using a Bruker DMX-750 spectrometer equipped with a dedicated ^{1}H-^{13}C inverse geometry HPLC-NMR flow probe (cell 4 mm i.d. with a volume of approximately 120 μl). ^{1}H NMR spectra were obtained in the stop flow mode at 750.13 MHz. Spectra were acquired using the NOESYPRESAT (Bruker, Rheinstetten, Germany) pulse sequence for solvent suppression with time-shared double presaturation of the water and acetonitrile frequencies. Free induction decays (FIDs) were collected into 16 K data points with a spectral width of 15 015 Hz, an acquisition time of 1.09 s and the spectra were acquired by accumulation of between 32 and 512 FIDs, depending on the concentration of the compound in the NMR flow probe. Prior to Fourier transformation, an exponential line broadening function was applied to each spectrum to improve the signal-to-noise ratio.

3. Results

3.1. AP metabolism

Successive 50 μl injections of reconstituted extracts were made to check reproducibility of the chromatography and stop-flow HPLC-NMR spectroscopy was then used. The UV-detected chromatogram is shown in Fig. 2. UV-absorbing peaks corresponding to putative metabolites and parent material were transferred from the UV detector cell to the NMR flow probe cell and ^{1}H NMR spectra were acquired. ^{1}H NMR spectroscopy identified the peak eluting at 4.6 min

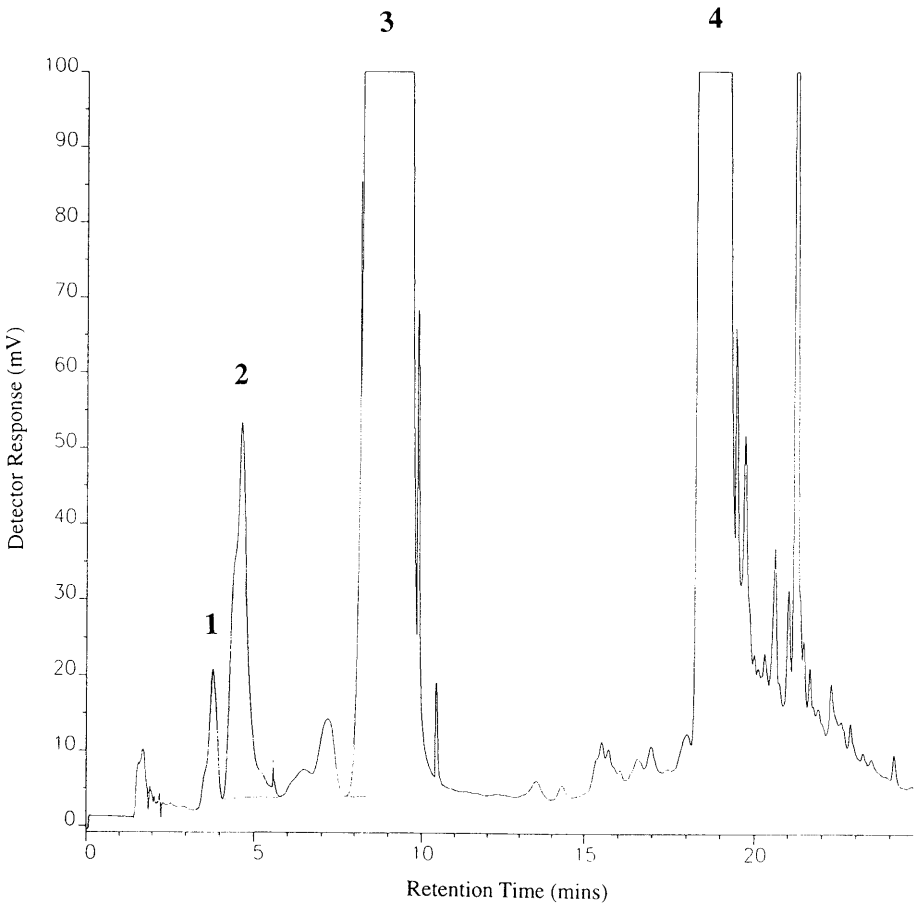

Fig. 2. UV-detected stop-flow HPLC chromatogram following injection of a rat microsomal extract from AP incubation. Whilst the flow was stopped, ^{1}H NMR spectra were obtained for peaks denoted 1, 2, 3 and 4.

(Peak 2) as a metabolite representing 6% conversion of AP and the peak eluting at 9.5 min (Peak 3) as AP itself. The internal standard for the extraction of AP was NP (Peak 4) which eluted at 18.8 min. Peak 1 shown in Fig. 3A which eluted at 3.7 min gave no NMR spectrum and is assumed to arise from a very low level endogenous species which has a high UV extinction coefficient.

The ^{1}H NMR spectrum of AP shown in Fig. 3C was assigned according to the numbering system given in Fig. 1. The proton *meta* to the amino group (H5) experiences a shielding effect and appears as a doublet of doublets at δ7.10 with an *ortho* coupling of 7.4 Hz to H4 and of 5.5 Hz to H6. The proton *ortho* to the pyridine nitrogen (H6) is deshielded and results in a doublet at δ7.69 with a typical pyridine *ortho* coupling of 5.5 Hz to H5 and a *meta* coupling of 1 Hz to H4. Proton H4 appears as a doublet at δ7.62 with an *ortho* coupling to H5 of 7.4 Hz and a *meta* coupling to H6 of 1 Hz. The protons in the fluorophenoxy-ring appear as a complex multiplet, integrating as four protons, between δ7.25 and 7.29. The peak co-chromatographed with AP standard and the ^{1}H NMR spectrum was identical to that acquired using a standard 5 mm NMR probe.

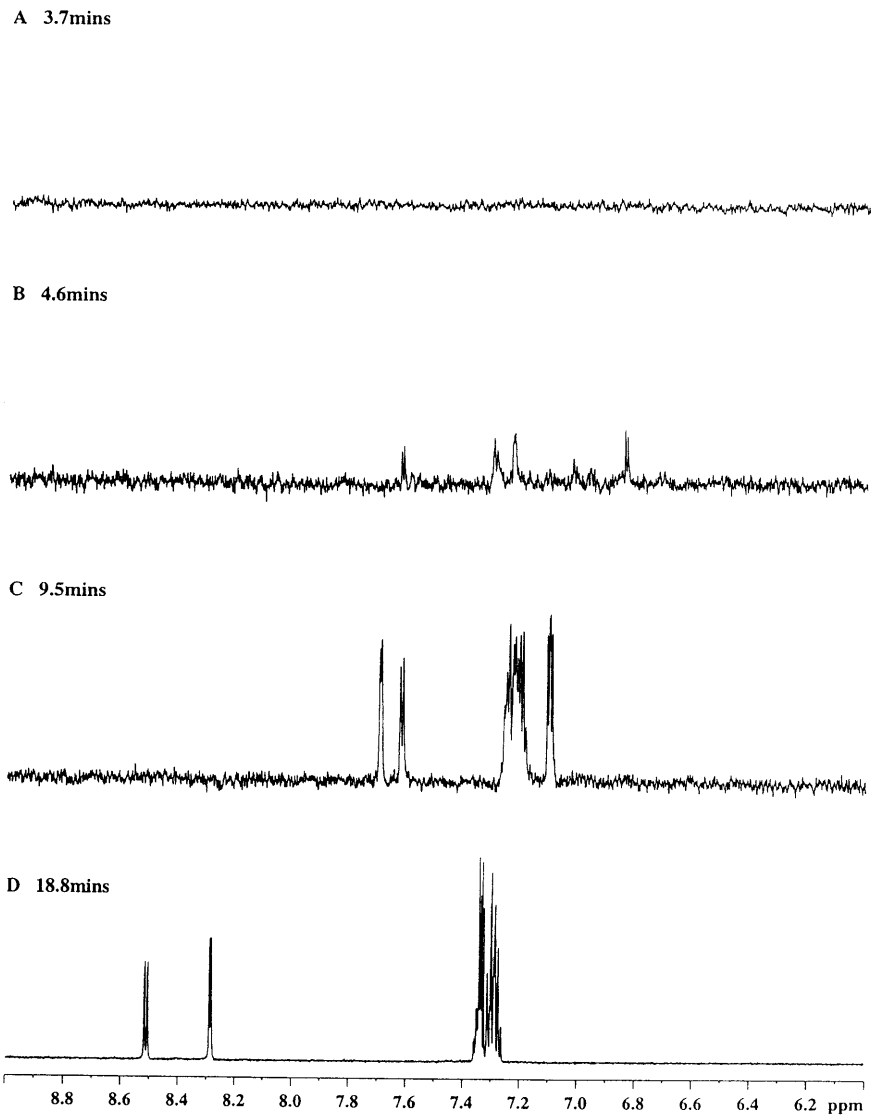

Fig. 3. 750 MHz ^{1}H NMR spectra obtained from stop-flow HPLC-NMR spectroscopy of AP and metabolites obtained at the retention times of peaks 1, 2, 3 and 4 shown in Fig. 2.

The structure of the minor metabolite eluting at 4.6 min was elucidated as 4-hydroxy-3-amino-2-(2-fluorophenoxy)pyridine (4HAP). Its ^{1}H NMR spectrum is shown in Fig. 3B. A doublet at δ7.62 is coupled to a doublet at δ6.83 with an *ortho* coupling of 5.5 Hz, typical of a pyridine moiety. The absence of the triplet at δ7.10 indicates a substitution in the pyridine ring at position 4. Evidence for H4 substitution is also provided by the fact that the H6 chemical shift is similar to that observed for the parent molecule. In addition, the H5 proton chemical shift experiences a low frequency shift from δ7.10 to δ6.84 which is consistent with the presence of a hydroxyl substituent. The protons of the fluorophenoxy ring are still centred at approximately δ7.25 showing

that hydroxy substitution in the pyridine ring has little effect on the chemical shifts of protons in the fluorophenoxy ring. These ^{1}H NMR resonances also integrate to four protons which proves that the fluorophenoxy ring is still intact. The spectrum is consistent with that of 4-hydroxy-3-amino-2-(2-fluorophenoxy)pyridine. The monohydroxylated structure is also consistent with the rapid elution time of this polar metabolite compared to the parent molecule and represents 6% conversion of the parent.

NP, the internal standard used for the extraction of AP and its metabolites, eluted at 18.8 min under the above HPLC conditions. The NMR spectrum for NP (Fig. 3D) was assigned as follows: the most deshielded proton in the molecule is the proton *ortho* to the nitro group (H4). This gives rise to a doublet of doublets at δ8.51 with an *ortho* coupling of 7.8 Hz to H5 and a *meta* coupling of 1.8 Hz to H6. The proton *ortho* to the pyridine nitrogen (H6) gives a doublet of doublets at δ8.29 with an *ortho* coupling of 4.5 Hz to H5 and a *meta* coupling of 1.8 Hz to H4. The doublet of doublets for H5 appears downfield from that observed for the AP molecule and overlaps with the protons of the fluorophenoxy ring at δ7.33 with the *ortho* pyridine coupling of 4.5 Hz and an *ortho* coupling of 7.8 Hz to H4. The protons H3′ to H6′ give rise to a multiplet centred at δ7.3 which integrate to four protons. This peak co-chromatographed with NP standard and the NMR spectrum was identical to that acquired using a standard 5 mm NMR probe.

3.2. NP metabolism

The chromatogram shown in Fig. 4 represents the UV-absorbing profile of the rat microsomal metabolites of NP. The parent molecule elutes at 18.8 min on injection of standard but there was a negligible amount of the parent observed on injection of the extract. The major peak in the chromatogram eluted at 11 min, with minor metabolites representing 0.6 and 6% conversion of parent compound eluting at 4 and 5 min respectively. The internal standard used for this compound was the less polar 5NP which eluted at 20 min.

The ^{1}H NMR spectrum obtained for the peak eluting at 11 min is identical to that of AP, as described above (compare Fig. 3C Fig. 5C). The absence of parent NP material in the extract and the presence of AP as the major component indicates the reduction of NP to AP. Thus the peaks at 4 and 5 min represent minor metabolites of AP.

The metabolite eluting at 4 min gave the spectrum shown in Fig. 5A and was identified from its ^{1}H NMR spectrum as follows. The doublet at δ7.76 is coupled to the doublet at δ6.51 with an *ortho* coupling of 8.25 Hz. This larger coupling and the chemical shift is consistent with a hydroxy substitution at position H6. The chemical shifts of the protons of the fluorophenoxy ring are still centred at δ7.25 similar to the parent AP molecule indicating that 6-hydroxylation on the pyridine ring has little effect on the chemical shifts of the protons of the fluorophenoxy ring. The resonances from this ring also integrate to four protons which proves that the fluorophenoxy ring structure is still intact. This metabolite is therefore 6-hydroxy-3-amino-2-(2-fluorophenoxy)pyridine and represents 0.6% conversion of the parent substrate.

The spectrum of the peak eluting at 5 min is identical to that observed for the metabolite eluting at 4.6 min from the AP chromatogram (compare Fig. 3B Fig. 5B), indicating 4HAP is a common hydroxylated metabolite for both compounds. This peak represents 6% conversion of the parent substrate.

4. Discussion

Aromatic amino groups are generally stable to hepatic P450-mediated metabolism and both the chromatography results and the NMR spectra of the AP metabolites produced support this trend. As the function of the P450 family of enzymes is to catalyse the metabolism of mostly lipophilic compounds, the low turnover of parent AP to 4HAP (6%) is ascribed to the low lipophilicity of the AP molecule. The incubation of NP with rat hepatic microsomes results in extensive, rapid reduction of the nitro group to the amino group as is evidenced by the absence of NP in the chro-

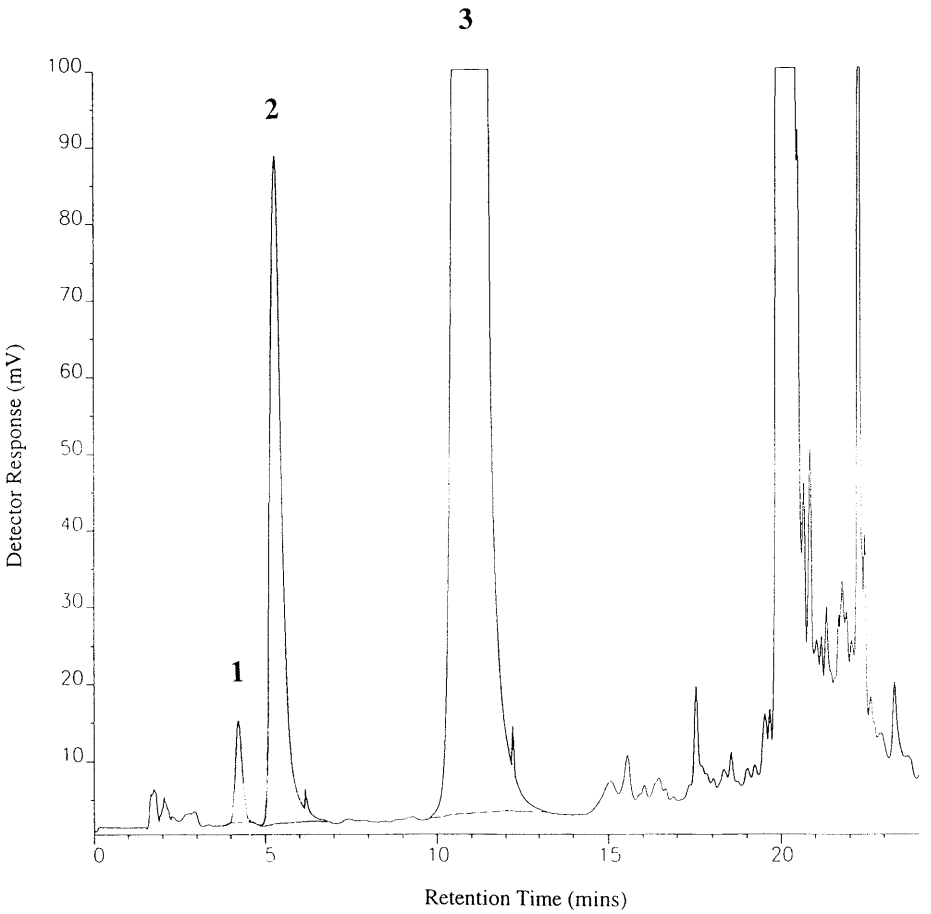

Fig. 4. UV-detected stop-flow HPLC chromatogram following injection of a rat microsomal extract from NP incubation. Whilst the flow was stopped, ^{1}H NMR spectra were obtained for peaks denoted 1, 2 and 3.

matogram and the presence of the amino compound peak. The metabolism of AP then proceeds to 4HAP (6%) and 6HAP (0.6%). The initial reduction of NP to AP may be P450 3A4 mediated [15]

Using ^{1}H HPLC-NMR spectroscopy it has been possible to demonstrate the in vitro microsomal metabolism of two fluorophenoxypyridine compounds. The facile and almost quantitative reduction of NP to AP was demonstrated and the 750 MHz ^{1}H NMR spectroscopy allowed identification of two minor metabolites of AP at 6 and 0.6% conversion. HPLC-NMR spectroscopy is therefore a realistic technique for identifying in vitro metabolites at low conversion factors. However, in many cases, parts of a metabolite structure have no proton-containing functional groups and then NMR spectroscopy alone may not be sufficient for full characterisation. In these circumstances, recent studies have shown that HPLC-NMR-MS provides a complementary technique for the unequivocal structural identification of such novel drug metabolites [16,17].

As automation of in vitro methodology including hepatocyte and liver slice systems becomes available, information on metabolic propensities of novel candidate drugs within a

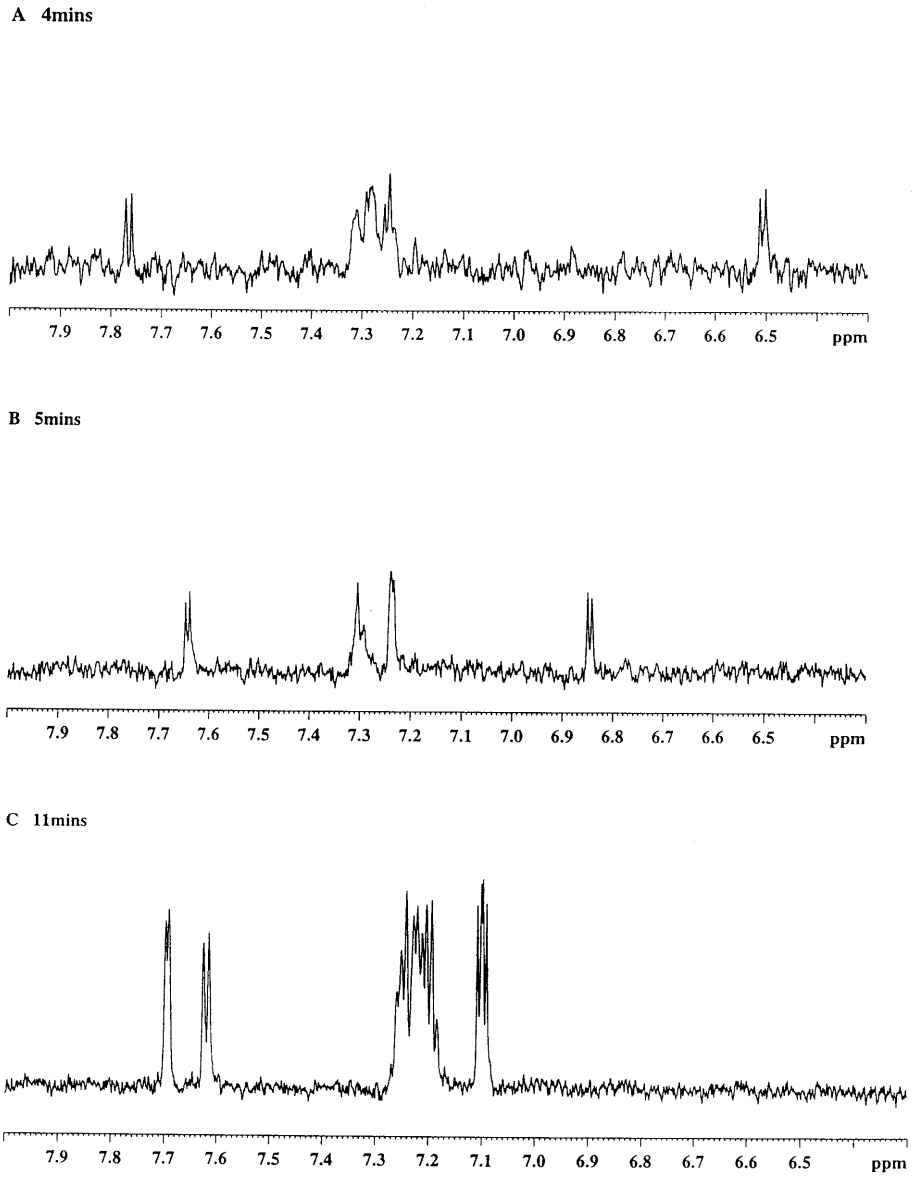

Fig. 5. 750 MHz ^{1}H NMR spectra obtained from stop-flow HPLC-NMR spectroscopy of NP metabolites obtained at the retention times of peaks 1, 2 and 3 shown in Fig. 4.

given structural series could provide information on structure–metabolism relationships. HPLC-NMR-MS will be well placed to provide the rapid on-line interactive analysis required to support such high-throughput methodology at the discovery stage thus influencing rational drug design strategies.

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