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Short Communication

Investigation of the human metabolism of antipyrine using coupled liquid chromatography and nuclear magnetic resonance spectroscopy of urine

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ABSTRACT

The potential of coupled high-performance liquid chromatography–nuclear magnetic resonance spectroscopy for the detection and identification of drug metabolites has been investigated by direct analysis of human urine collected following administration of antipyrine. This approach provided a rapid method of characterizing the major human urinary metabolites of this drug and promises to be of widespread value in structural studies of xenobiotic metabolites.

INTRODUCTION

Antipyrine is frequently used as a probe of drug metabolism, in particular of the P450 family of enzymes in studies of enzyme induction. The metabolism of antipyrine and the pattern of metabolic conjugation have therefore been studied extensively in rat and man [1–4], in which a pattern of N-dealkylation, hydroxylation, glucuronidation and sulphation is observed. Whilst the rat principally forms sulphate conjugates, it appears that only glucuronides are formed in man. The major urinary metabolites detected in man are the glucuronic acid conjugates of the N-demethylated compound norantipyrine, 4-hydroxyantipyrine and 3-hydroxymethylantipyrine. The

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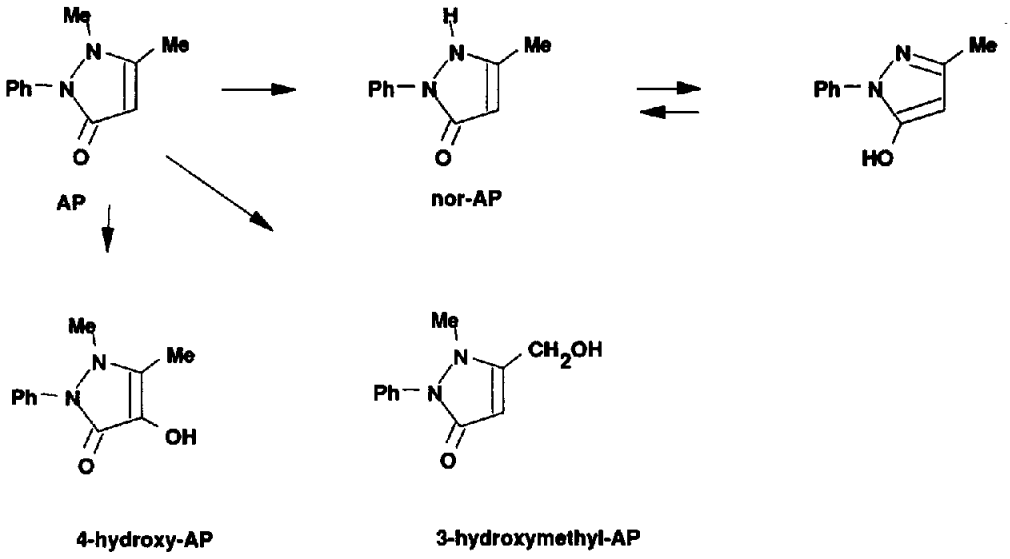

Fig. 1. Structures of antipyrine (AP) and its metabolites in man. Hydroxylated substances are mainly present as glucuronic acid conjugates.

structures of these compounds are given in Fig. 1. Norantipyrine can tautomerise to the 5-enol, and it is this O-glucuronide that is formed rather than an N-glucuronide [4].

The direct coupling of HPLC with NMR spectroscopy has waited for a number of technological developments to make it a feasible routine technique. In the late 1970s and early 1980s, a number of studies were carried out [5,6] but they suffered from the fact that the then highest possible NMR sensitivity was barely adequate for routine work and from the need to use expensive deuterated solvents for the HPLC because the solvent suppression pulse sequences available in NMR could not cope with 100% protio solvents. Since then, major advances have promised to make the routine use of on-line NMR detection of HPLC fractions a useful adjunct to the armoury of analytical methods. Experiments can be carried out in one of three modes, direct on-line NMR detection of the HPLC eluent (on-flow), a stopped-flow approach or finally the eluted fractions can be stored in capillary tubes for later recall for detailed NMR spectroscopic studies. No compromise needs to be made in the chromatographic conditions, and programmed gradient elution profiles can be accommodated. There have been improvements in sensitivity due to increases in magnetic field strength and advances in NMR receivers, as well as improved solvent suppression techniques and modifications to the NMR flow-cell design. All of these factors have combined to yield a sensitivity level of detection with double solvent suppression of about 10 μg of compound in the on-flow mode and in the nanogram range in stopped-flow mode.

Recently, the first application of this methodology to the identification of drug metabolites was described [7,8]. Thus, it was possible to structure the major human urinary metabolites of the anti-inflammatory drug, ibuprofen [2-(4-isobutylphenyl)propionic acid] and, under stopped-flow conditions, NMR spectra were obtained for ibuprofen glucuronide and for the glucuronides of the hydroxylated and side-chain oxidised metabolites of ibuprofen. In addition, evidence was gained for the presence of unconjugated forms of these metabolites from the NMR spectra.

Antipyrine, a widely used standard material, provides a further useful test of the coupled HPLC–NMR approach as it should allow a rapid unambiguous determination of the structures of the major metabolites of this drug and their pattern of conjugation to be determined rapidly and without any pretreatment of the human urine at a conventional therapeutic dose level.

EXPERIMENTAL

A single 1-g dose of antipyrine was taken orally by a healthy volunteer and urine collected over 48 h in time periods of 0–2, 2–4, 4–8, 8–12, 12–24 and 24–48 h. The sample investigated here was from the 4–8 h period and was freeze-dried, and for the HPLC–NMR experiments was reconstituted into 2.5 times its original concentration using ^2H_2O–$C^2H_3O^2H$ (1:1). The freeze-drying was carried out principally for ease of sample transportation and the concentration step allowed a smaller sample injection. Also, the use of the small amount of deuterated solvent was convenient for any NMR measurements on whole urine samples. None of these factors was necessary for the success of the study. No chemical shift reference compound was added. A 30-μl aliquot of this concentrate was used for the analysis.

Chromatography was carried out at 303 K, using a Bruker (Rheinstetten, Germany) LC22C pump, an LC53 autosampler and an LC313 variable wavelength UV detector with a Spherisorb ODS-II 5 mm column of dimensions 250 × 4.6 mm (Merck, Poole, UK) and gradient elution. The solvents were ^2H_2O (Cambridge Isotope Labs., Wessel, Germany), containing 0.05 *M* KH_2PO_4 at pH 2.47 and acetonitrile (Riedel de Haen, Germany) with a gradient changing from 1% to 55% acetonitrile in 35 min at a flow rate of 1 ml/min. Detection was by UV at 210 nm and ^1H NMR using a 60-μl active volume cell. The NMR spectroscopy was carried out using a Bruker AMX-500 instrument operating at a ^1H NMR frequency of 500 MHz. Solvent suppression was achieved through the use of a standard one-dimensional solvent suppression sequence irradiating at both the water and acetonitrile resonance positions. During the gradient elution these resonance frequencies change, and a blank run was carried out first in order to obtain these frequencies. Acquisition parameters were: time domain points 32K with no zero-filling, a spectral width of 12 195 Hz and a recycle time of 3.7 s. Typically, 128 transients were acquired for each spectrum, leading to a total acquisition time of about 470 s, although longer acquisitions up to 20 min were also measured. A line broadening of 1.0 Hz was applied to the free induction decay before Fourier transformation. Chemical shifts were referenced to that of acetonitrile at δ2.00. In our experience, stopped-flow operation does not cause any problems of band broadening due to diffusion if the flow is halted for less than 2 h, even with several stops.

RESULTS

In this investigation, the stopped-flow technique was used. In this technique, when a peak is detected on the UV trace, the solvent flow is halted and the fraction corresponding to the UV peak is transferred to the in-line NMR probe for measurement of the ^1H NMR spectrum. This approach, rather than on-flow, was used in order to obtain sufficient NMR spectral digital resolution and a suitable signal-to-noise ratio for full characterisation of the metabolites. On-flow detection is possible but, because of the time resolution needed, both parameters would have had to be restricted. The fractions have been investigated by NMR, and many of the UV-absorbing peaks correspond to natural metabolites which have been identified; these results will be published separately. However, the object of this study was to detect and identify metabolites of antipyrine, and therefore three peaks were studied in more detail by ^1H NMR because their high UV absorbance suggested aromatic species which may be drug-related.

The first peak, with a retention time of 20.95 min, gave an NMR spectrum (Fig. 2, upper trace) consistent with a single species comprising the ether glucuronide of 4-hydroxyantipyrine. The 2′-, 3′- and 4′-hydrogens from the phenyl ring resonate at δ7.33, δ7.55 and δ7.50 respectively, consistent with N-substitution; the 2-N-methyl resonance is observed at δ3.11. The C1–H resonance of the anomeric proton of the glucuronide is visible as a doublet at δ4.74 with a coupling constant of about 7 Hz, indicating a β-glucuronide, and the H2, H3 and H4 glucuronic acid protons resonate as a set of overlapping bands between

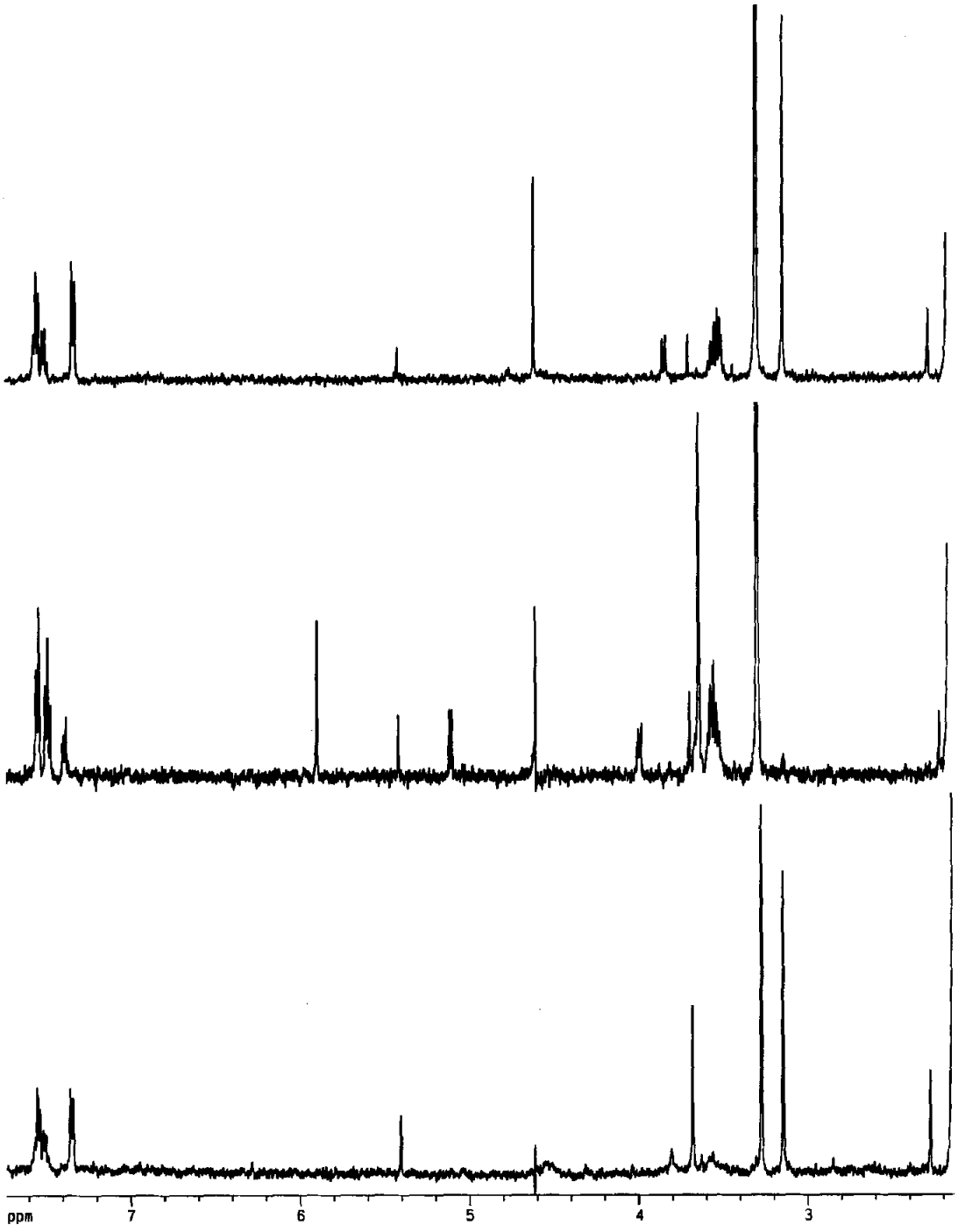

Fig. 2. ^{1}H NMR spectra at 500 MHz corresponding to the drug-related chromatographic peaks. Measurements were made in stopped-flow mode: upper trace = 4-hydroxyantipyrine glucuronide; middle trace = norantipyrine glucuronide: lower trace = 4-hydroxyantipyrine and 3-hydroxymethylantipyrine glucuronide.

δ3.47 and 3.58, with H5 showing as a separate resonance at δ3.82 as a doublet as expected. The 3-methyl resonance can be observed as a singlet at δ2.25. The intensity of this signal and that of the glucuronic acid H1 proton was reduced because of partial suppression caused by the saturation of the closely adjacent acetonitrile and water solvent resonances, respectively. The 4-hydroxy substitution is also confirmed by the absence of a resonance for the 4-H olefinic hydrogen.

Fig. 2, middle trace, shows the 500-MHz proton NMR corresponding to a second peak eluting at 21.41 min, which is also of a single species and is consistent with norantipyrine glucuronide. Thus, the aromatic protons have chemical shifts of δ7.55, δ7.48 and δ7.38 for the 2′-, 3′- and 4′-protons, respectively. These are quite different to those observed for the 4-hydroxyantipyrine because the Ph–N.C=O functionality has been replaced by Ph–N.COR. The glucuronide resonances are also shifted somewhat relative to those shown in Fig. 2, upper trace, with the C1–H being at δ5.09 as a doublet with a coupling constant of 7.5 Hz indicating β-glucuronidation. The 2′-, 3′- and 4′-protons appear in a complex envelope between δ3.47 and 3.58 with the H5′ proton distinct at δ3.97 as a doublet. The signal from the 2-methyl group appears at δ2.19, again partially suppressed by its close proximity to the acetonitrile peak. The olefinic hydrogen gives rise to a singlet at δ5.89. The broad singlet which appears at about δ3.6 and which could possibly be confused with an N-methyl resonance appears in many fractions in the HPLC separation and may arise from the column or the solvents used.

A third chromatographic peak with a longer retention time gave the 500-MHz ^{1}H NMR spectrum shown in Fig. 2, lower trace. This appears to contain two components. The major resonances are consistent with unconjugated 4-hydroxyantipyrine itself. Thus, the aromatic protons resonate at δ7.35 for the H2′ protons, δ7.55 for the H3′ protons and δ7.50 for H4′. The N-methyl group gives a resonance at δ3.15 and the 3-methyl group appears at δ2.28, and this major component does not contain a resonance for an olefinic hydrogen. This compound could arise from hydrolysis of the glucuronide or be simply unmetabolised drug. In addition, a minor component can be observed which shows an olefinic hydrogen resonance at δ6.28, glucuronide proton resonances at δ3.81 (H5) and δ3.5–3.6 (H2–H4). These could arise from the other known metabolite, namely 3-hydroxymethylantipyrine glucuronide. The methylene resonance which would be very diagnostic for this structure is not observed and would be expected at about δ5.4 and may be obscured by the artefact peak always observed at about this chemical shift.

CONCLUSIONS

Using standard HPLC conditions on a C_{18} column, it has proved possible to detect and characterise the major human metabolites of a drug using the coupled HPLC procedure with stopped-flow NMR spectroscopy. This is a rapid method and promises to be of great value in identifying drug metabolites non-destructively and without the need to use radiolabelled compounds.

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