ELSEVIER

Journal of Chromatography B, 748 (2000) 281–293

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Mass directed peak selection, an efficient method of drug metabolite identification using directly coupled liquid chromatography–mass spectrometry–nuclear magnetic resonance spectroscopy

G.J. Dear[a], R.S. Plumb[a], B.C. Sweatman[a], J. Ayrton[a], J.C. Lindon[b], J.K. Nicholson[b], I.M. Ismail[a,*]

[a] *LC/NMR/MS Laboratory, Discovery 2, Bioanalysis and Drug Metabolism Division, GlaxoWellcome Research and Development, Park Road, Ware, Herts. SG12 0DP, UK*

[b] *Biological Chemistry, Division of Biomedical Sciences, Imperial College of Science, Technology and Medicine, Sir Alexander Fleming Building, South Kensington, London SW7 2AZ, UK*

Abstract

Mass spectrometry (both MS and MS–MS) has been used to determine which eluting chromatography peaks in an LC–MS–nuclear magnetic resonance (NMR) experiment should be selected for extended NMR spectroscopic measurement. This mass directed selection of chromatographic peaks has been applied to test mixtures and urine samples for identification of drug metabolites. It was used to simultaneously determine when drug-related material was eluting and provided molecular mass information on these components. Stop-flow LC–NMR was used to acquire data for structural characterisation of drug-related components. This work further serves to demonstrate the potential of coupling tandem mass spectrometry using an ion trap spectrometer with LC–NMR spectroscopy, to provide an extremely powerful tool in structural elucidation.

Keywords: Peak selection; Drug metabolites

1. Introduction

The coupling of liquid chromatography (LC) and nuclear magnetic resonance (NMR) spectroscopy is now a well established technique which has become an essential tool for molecular structure determination with a wide range of applications including chemical impurities, combinatorial chemistry libraries, natural products and drug metabolites [1–5]. The further coupling of several mass spectrometry (MS) techniques to provide LC–NMR–MS has been shown to be synergistic with NMR and MS giving complementary information [6–9].

LC–NMR and LC–NMR–MS can be performed in the on-flow mode or the stop-flow mode (either directly or indirectly via a loop-storage arrangement). One advantage of on-flow operation is that there is no requirement for the independent detection (say by UV) of eluting peaks and subsequent timed transfer to the active volume of the NMR detector. However, on-flow operation suffers from the drawback that data acquisition times are restricted to the

*Corresponding author. Tel.: +44-1920-884-424; fax: +44-1920-884-374.

E-mail address: imi2682@glaxowellcome.co.uk (I.M. Ismail).

0378-4347/00/$ – see front matter
PII: S0378-4347(00)00291-7

period that the chromatographic peak remains within the NMR transmitter/receiver coil. The detection limits of on-flow operation are consequently much higher compared to stop-flow or loop-storage modes of operation. Thus the method is only practicably applicable in those instances where the concentrations of analyte being analysed are relatively high [10,11] and this limitation has restricted the use of on-flow LC–NMR and on-flow LC–NMR–MS for the analysis of drug metabolites in biological fluids.

There are developments such as microcoil NMR probes [12,13] and probes operating at cryogenic temperatures [14] that will improve the sensitivity of detection for NMR experiments such that on-flow LC–NMR may become a more useful experiment and therefore used more frequently. However until these developments are implemented stop-flow LC–NMR will continue to be the primary mode of operation for most routine applications.

Stop-flow LC–NMR operation necessitates the interruption of the chromatographic separation so that eluting peaks of interest may be transferred to the active volume of the NMR spectrometer. This allows one- and two-dimensional NMR spectra with good signal-to-noise to be acquired over a longer time period than would be possible using on-flow techniques [15].

The most common method of detecting peaks as they elute from a high-performance liquid chromatography (HPLC) column is by their UV absorbance and this has been adopted as the standard for LC–NMR. The apex of the UV peak is used to calculate the timings for peak transfer either directly into the NMR probe for immediate analysis or into storage loops for subsequent analysis. However, the absence of any UV chromophore in a molecule can restrict the use of LC–NMR to on-flow operation, using the NMR as the chromatographic peak detector. Even where an UV chromophore is present in a molecule, the peak of interest may often be completely or partially obscured by other masking peaks in the chromatogram, rendering the operation of stop-flow LC–NMR difficult. This is a problem commonly encountered in the direct analysis of metabolites in biological fluids such as urine and bile using LC–NMR, especially for low concentrations of metabolites. Sample pre-concentration and semi-preparative chromatographic isolation cannot eliminate this possibility entirely when minor metabolites are to be characterised [16].

To overcome this problem so that stop-flow LC–NMR can be carried out for substances with low or no UV chromophore we describe here a reconfiguration, which replaces UV detection with mass spectrometric detection for the screening of drug-related components and for the initiation of stop-flow NMR data acquisition. This is exemplified by the mass-detected LC–NMR structural elucidation of urinary metabolites of novel drug candidates, GW420867 and GI265080 (potential compounds in the treatment of HIV infections and bipolar disorders, respectively) directly from untreated samples.

2. Experimental

2.1. Chemicals

HPLC-grade acetonitrile was purchased from Rathburn (Walkerburn, UK) and formic acid (analytical-reagent grade) was obtained from Fisons (Loughborough, UK). Deuterium oxide and Riedel-de Haen Pestanal-grade acetonitrile were purchased from Fluorochem (Old Glossop, UK).

GW420867 [(*S*)-2-ethyl-7-fluoro-3-oxo-3,4-dihydro-2H-quinoxaline-carboxylic acid isopropylester) was synthesised by Hoechst Marion Roussel (Frankfurt-Am-Main, Germany).

GI265080 {4-amino-1-(2,6-difluorobenzyl)-1H-1,2,3-triazolo[4,5-c]-pyridine hydrochloride} and the *N*-5 oxide of GI265080 (GI262663) were synthesised at GlaxoWellcome Research and Development (Stevenage, UK). The structures of these substances are shown in Fig. 1.

All other chemicals and reagents were of analytical grade or equivalent.

2.2. Samples

The samples used comprised control rat urine into which GI265080 and its *N*-5 oxide (G1262663) were spiked at a concentration of 100 μg/ml. In addition samples of dog urine collected over 0–24 h following a single oral 50 mg/kg dose of GW420867 were used. All samples were kept stored frozen at −20°C prior to analysis.

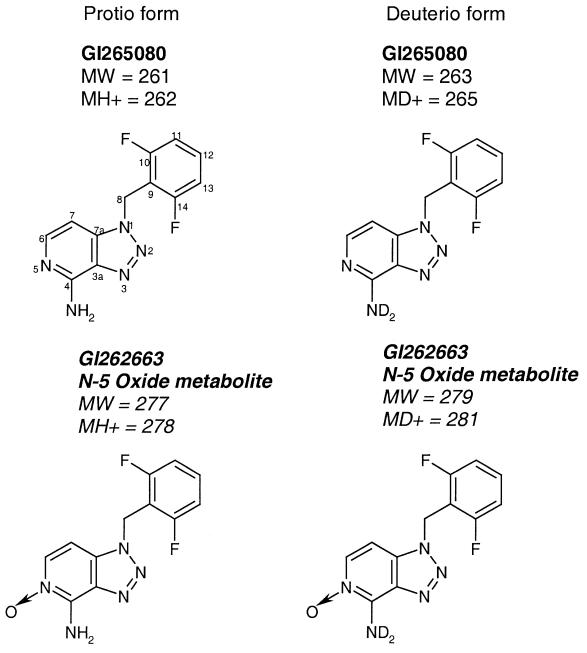

Fig. 1. GI265080 and its *N*-5 oxide.

2.3. Chromatography

Chromatographic separations were performed using a HP1050 binary pumping system connected to a HP1100 autosampler (Hewlett-Packard, Waldbronn, Germany). Samples containing GI256080 were analysed using system (i) described below, whilst samples containing GW420867 were analysed by system (ii).

System (i): 150×4.6 mm Intersil ODS3 5 μm column (Captial HPLC, Edinburgh, UK), using a mobile phase consisting of 0.1% (v/v) formic acid in deuterium oxide and 0.1% (v/v) formic acid in acetonitrile. The proportion of acetonitrile was programmed to increase linearly from 0 to 25% over 30 min. The flow-rate was maintained at 1 ml/min throughout the analysis.

System (ii): 150×4.6 mm Zorbax RxC8 5 μm column (Hichrom, Bedfordshire, UK), using a mobile phase composition identical to system (i). The proportion of acetonitrile was programmed to increase linearly from 0 to 50% over 30 min. The flow-rate was maintained at 1 ml/min throughout the analysis.

2.4. Mass spectrometry

Mass spectra were obtained using a Bruker Esquire ion trap mass spectrometer (Bruker-Daltonics, Bremen, Germany), fitted with an atmospheric pressure ionisation (API) source. Nitrogen was used as the nebuliser gas at 40 p.s.i., and as the drying gas at a flow-rate of 8 l/min and a temperature of 250°C (1 p.s.i.=6894.76 Pa). The mass spectrometer was operated in positive ion electrospray mode with a scan rate of 13 000 u per second and a range of 50–600 m/z. Generated ions were monitored by the ion trap using data dependent automatic MS–MS, in which the base peak intensity was constantly compared to a user-defined threshold (10^4 in this case). When this threshold was exceeded, the ion trap mass spectrometer first isolated the base peak over a given mass window and then induced fragmentation using resonant excitation at 1.5 V. In essence, during sample acquisition where base peak intensity exceeded the threshold, alternate single scans of MS and MS–MS were acquired. All MS–MS experiments were performed using the ion charge control (ICC) facility to automatically adjust the accumulation time as the ion abundance changed.

2.5. ^{1}H-NMR spectroscopy

^{1}H-NMR experiments were performed using a Bruker DRX-600 spectrometer (Bruker, Rheinstetten, Germany) equipped with a ^{1}H–^{19}F flow probe (cell 4 mm I.D. with a volume of approximately 120 μl) operating at 600.13 MHz for ^{1}H observation. ^{1}H-LC–NMR spectra were acquired using a standard NOESYPRESAT pulse sequence for solvent suppression with time-shared double pre-saturation of the water and acetonitrile frequencies. In these experiments typically 10K transients were acquired into 32K data points over a spectral width of 12 019 Hz (20 ppm) giving a pulse repetition time of 3.2 s. Prior to Fourier transformation, an exponential line broadening function of 0.3 Hz was applied to each spectrum to improve the signal-to-noise ratio.

2.6. LC–MS–NMR

The LC–UV–MS–NMR system was configured as

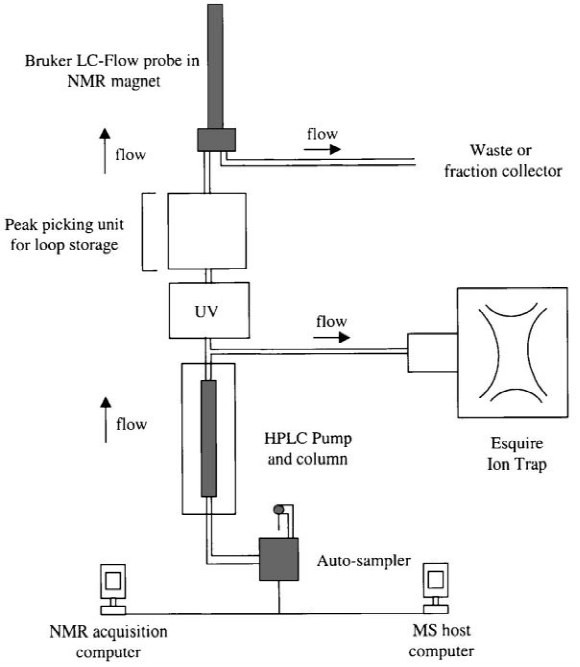

Fig. 2. Schematic diagram of the LC–UV–MS^n–NMR system.

depicted in Fig. 2 by connecting the Bruker DRX-600 NMR spectrometer and the Bruker Esquire mass spectrometer in parallel. In this configuration the mass spectrometer was operated just outside the 5 Gauss line of the NMR magnet. HPLC separations were provided using a HP1050 binary pumping system and a HP1100 autosampler (Hewlett-Packard), with on-line UV detection at 254 nm, using a Knauer VWM (Knauer, Germany). The chromatographic separations for LC–MS–NMR were performed under the control of Bruker HyStar (v 1.1) software operating in stop-flow mode.

The column effluent was split so that approximately 950 μl/min of the flow was directed to the UV and NMR flow cells with 50 μl/min directed to the electrospray source of the MS. The length of the connection tubing was adjusted such that the apex of an eluting peak reached both the MS and UV detectors simultaneously and the NMR detector later. Thereby, peaks selected by the MS output, could be held in the NMR flow cell, using the pre-programmed delay between the UV detector and the NMR flow cell.

3. Results and discussion

The coupling of MS and NMR with LC can be carried out in parallel (as described above) or in series where the MS system is placed after the NMR flow cell. The serial arrangement is the simpler and more robust set-up. Although examples of parallel operation exist none have utilised the MS data to initiate stop-flow NMR data acquisition. Serial operation allows the completion of all NMR experiments whether continuous on-flow or stop-flow prior to the start of MS analysis, but can introduce the possibility of peak dispersion before MS analysis for any peaks trapped between the NMR and MS systems when the flow is stopped. However, operation in serial mode is easier to set up, which is an important factor when the MS and NMR spectrometers, which are dedicated to hyphenated LC–NMR–MS analysis are predominantly operated as individual ''stand alone'' instruments. A quick and simple method of switching between individual and coupled operation, is therefore imperative. Additionally in serial operation where NMR data acquisition precedes MS acquisition, the opportunity exists to re-protonate exchangeable protons that have been deuterated through the use of deuterated solvents [9]. Furthermore, no problems are encountered with regards to data synchronisation when operating in serial mode, since the NMR data acquisition is completed prior to MS acquisition.

In contrast to serial operation where the entire sample is available for analysis by MS, parallel operation requires the arrangement of a defined solvent split to each instrument. Typically ≈95% of the post-column eluent is directed to the NMR flow cell (which is by far the least sensitive of the two detectors) and 5% to the MS source (usually electrospray). This consequently enables the use of stop-flow NMR with minimum degradation in the integrity of the chromatogram or sensitivity of the NMR spectrometer, compared with the serial configuration. Despite these advantages of the parallel mode, it can be a time-consuming system to configure, since the post-column split has to be very precise such that the acquired MS and NMR data are synchronised appropriately.

A major benefit of double MS and NMR hyphenation would be the ability of the MS to direct the

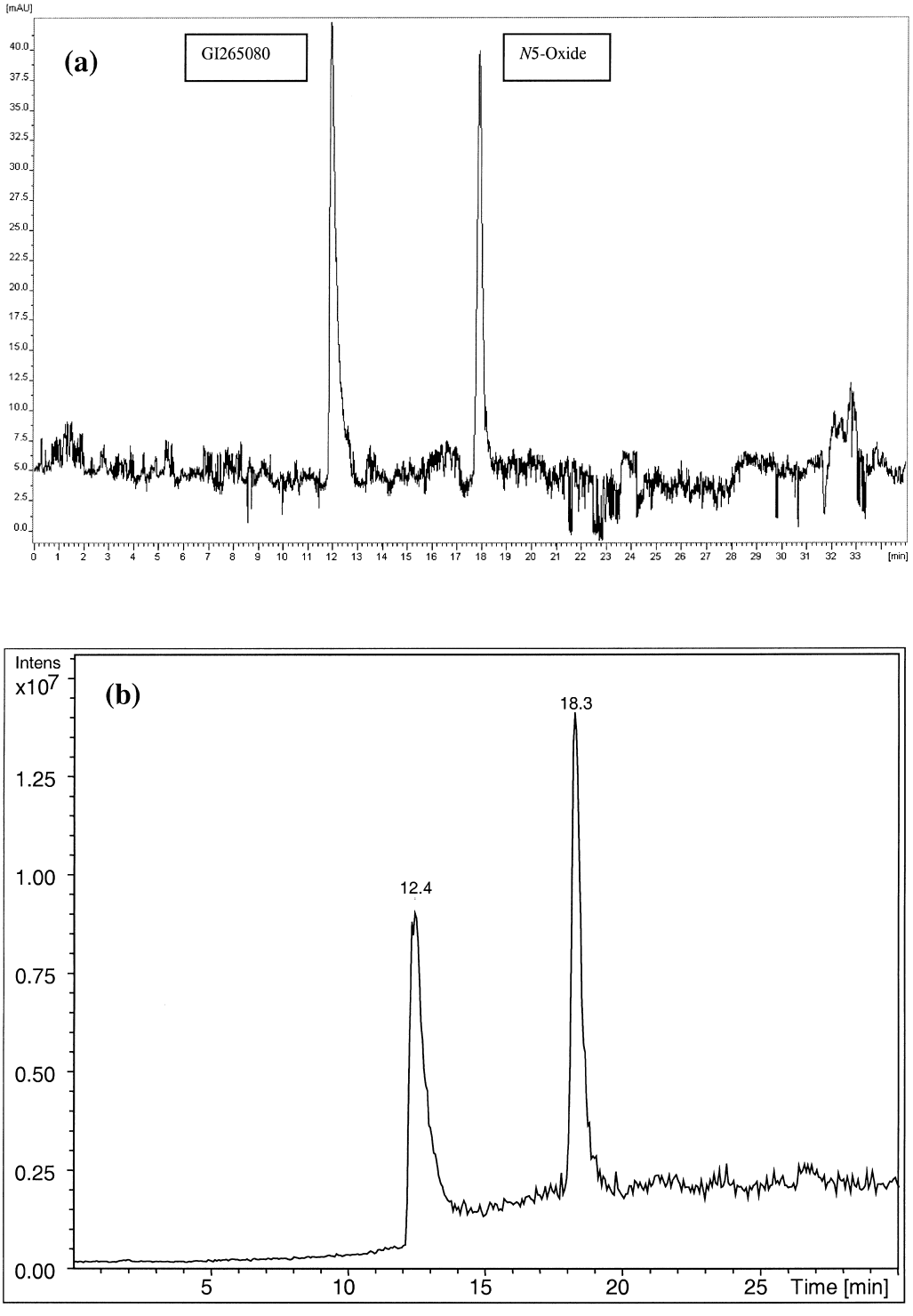

Fig. 3. UV and TIC chromatograms following injection of a standard test solution (GI265080 and its *N*-5 oxide) onto the LC–UV–MS–NMR system.

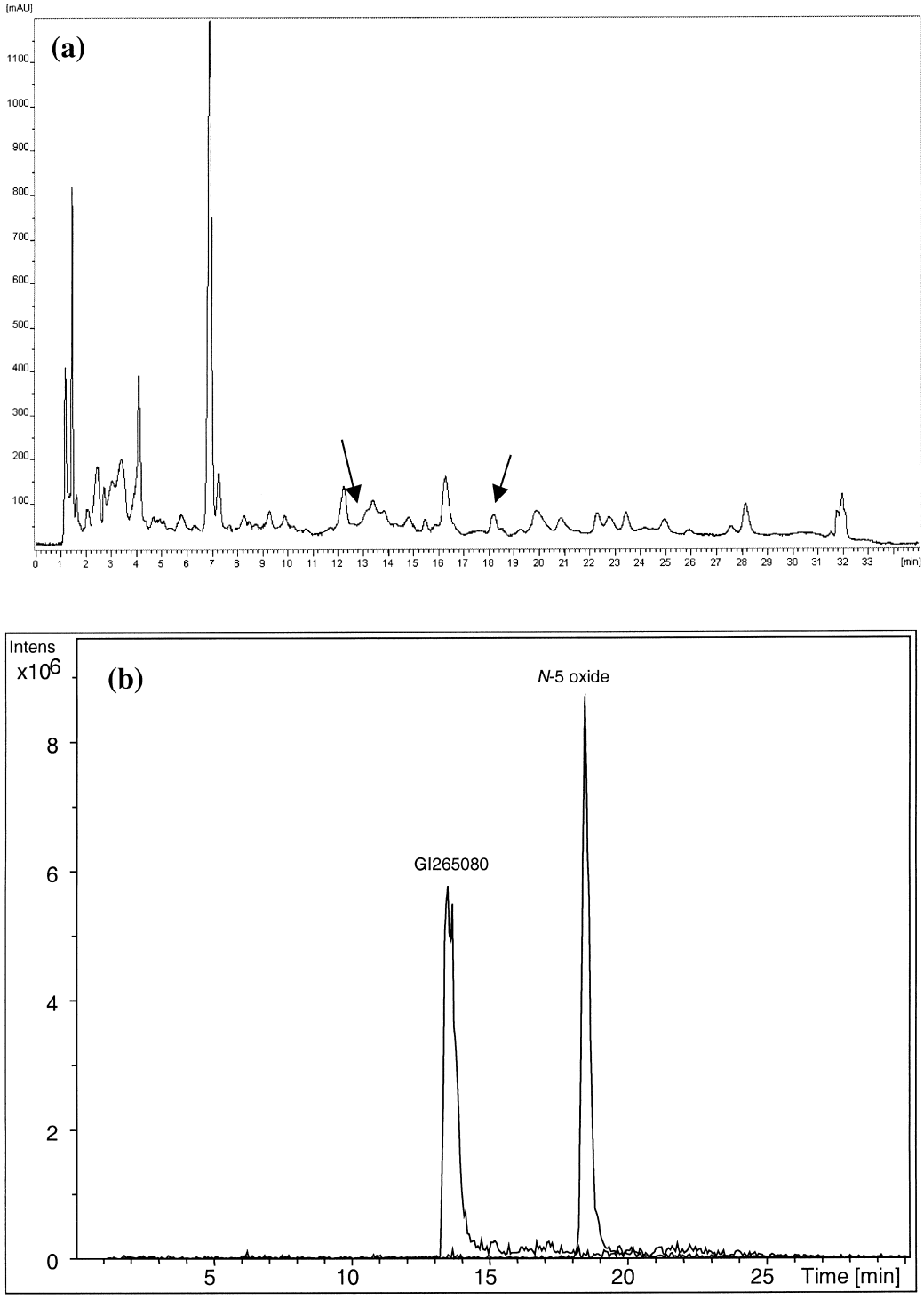

Fig. 4. UV and extracted ion chromatograms following injection of a standard test solution (GI265080 and its *N*-5 oxide) spiked into control rat urine onto the LC–UV–MS–NMR system.

NMR spectrometer in stop-flow operation, enabling LC–NMR experiments to become more selective with only peaks of the appropriate mass being analysed by subsequent NMR. This synergy could be further extended if data dependent MS–MS scanning is utilised, allowing stop-flow experiments solely on chromatographic peaks exhibiting the appropriate diagnostic product ions. The simplest way of creating a stop-flow configuration where peak selection is defined by an associated MS would be to reverse the order of the MS and NMR instruments in the serial configuration. However, this is of course impractical, since MS is a destructive technique.

To circumvent this problem, the spectrometers have to be connected in parallel, with the parallel split connection adjusted such that the chromatographic peak passes completely through the MS prior to filling the NMR flow cell. With an appropriate delay the MS response can be interrogated, and a decision made on whether to trap the eluting peak. In this way the synergistic benefits of MS directed peak selection for stop-flow NMR experiments may be achieved.

In order to test this system for its ability to use the MS to direct stop-flow NMR experiments, an aqueous test solution of GI265080 and its *N*-5 oxide metabolite was employed (100 μg/ml of each component). The solution (25 μl) was injected onto the ''pseudo'' LC–UV–MS–NMR system represented schematically in Fig. 2. The *N*-5 oxide metabolite of GI265080 was detected in the UV chromatogram eluting at approximately 18.5 min, whilst GI265080 itself was observed at approximately 12.5 min (Fig. 3a). Two peaks at similar retention times were also clearly discernible in the associated MS total ion current (TIC) chromatogram (Fig. 3b) showing good synchronisation of the detectors. The UV output can be used to direct stop-flow NMR using a manual feature of the software control. This allows operator selection of the chromatographic peak for stop-flow NMR, directly, independent of UV response and retention time. This feature employed with the on-line MS was made possible by the synchronisation of the UV and MS outputs. Once this instruction is selected from the software, a pre-programmed delay is activated to stop the solvent flow and hence trap the peak of interest in the NMR flow cell. This automatic delay, which is flow-rate dependent and

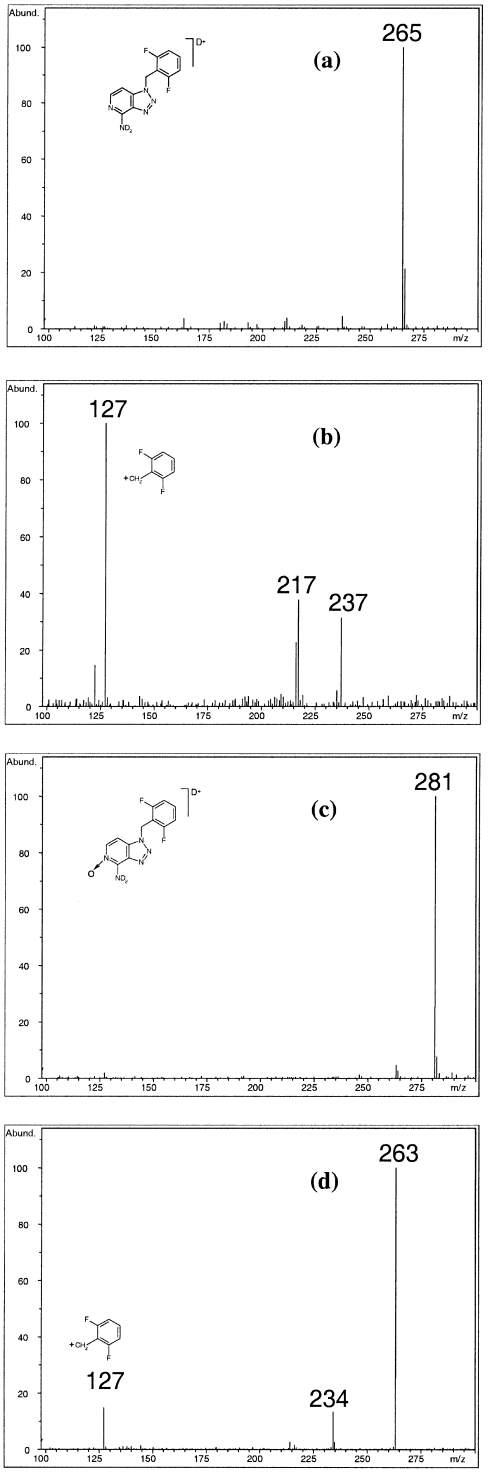

Fig. 5. MS and MS–MS spectra of GI265080 and its *N*-5 oxide metabolite obtained from the analysis of spiked urine.

software controlled, was used in conjunction with the MS to trap chromatographic peaks of interest.

Using this approach, separate ^{1}H-NMR spectra of GI265080 and its *N*-5 oxide metabolite were obtained using the MS TIC chromatogram to initiate trapping of the peak in the NMR flow cell (data not shown). However, when dealing with more complex mixtures a more selective response than that provided by either UV or TIC outputs is required in order to direct stop-flow NMR experiments. Extracted mass chromatograms can be used as selective criteria for stop-flow experiments, but only when the molecular masses of the components of interest are already known. In drug metabolism studies the exact identity of the metabolites may not always be correctly anticipated. In such instances, the use of data dependent MS–MS analysis using a modern ion trap spectrometer would allow the operator to utilise the presence of product ions diagnostic of the parent compound to direct stop-flow NMR. A similar approach has been used to enable MS–MS directed fraction collection of metabolites directly from biological samples [17].

To test the use of on-line MS–MS for selecting peaks for stop-flow LC–NMR experiments a spiked urine test solution as described in the experimental section was used. A 50-μl injection of this sample onto the LC–UV–MS–NMR system produced the UV profile displayed in Fig. 4a. Clearly, the UV response (at 254 nm) is complicated and would hence prevent simple stop-flow LC–NMR, due to the multitude of endogenous peaks and the relatively weak response of the analyte peaks of interest (arrowed). The extracted mass chromatograms corresponding to GI265080 and its *N*-5 oxide metabolite are also displayed in Fig. 4b. Fig. 5 shows the MS spectra of the two components. The *N*-5 oxide metabolite was characterised through the presence of a base peak at *m*/*z* 281, corresponding to the deuterated MD^{+} species, Fig. 5c. Under auto MS–MS, collisional activation of the base peak using a resonant excitation of 1.5 V revealed an MS–MS spectrum dominated by a product ion at *m*/*z* 263, corresponding to the loss of a hydroxyl group, Fig. 5d. A diagnostic product ion at *m*/*z* 127 was rationalised as the difluoro phenyl group. GI265080 was shown to generate an equivalent deuterated MD^{+} ion at *m*/*z* 265, Fig. 5a. The MS–MS spectrum of this peak also produced the common and characteristic product ion at *m*/*z* 127, Fig. 5b.

The formation of MD^{+} ions results from the use of deuterium oxide as the aqueous component of the

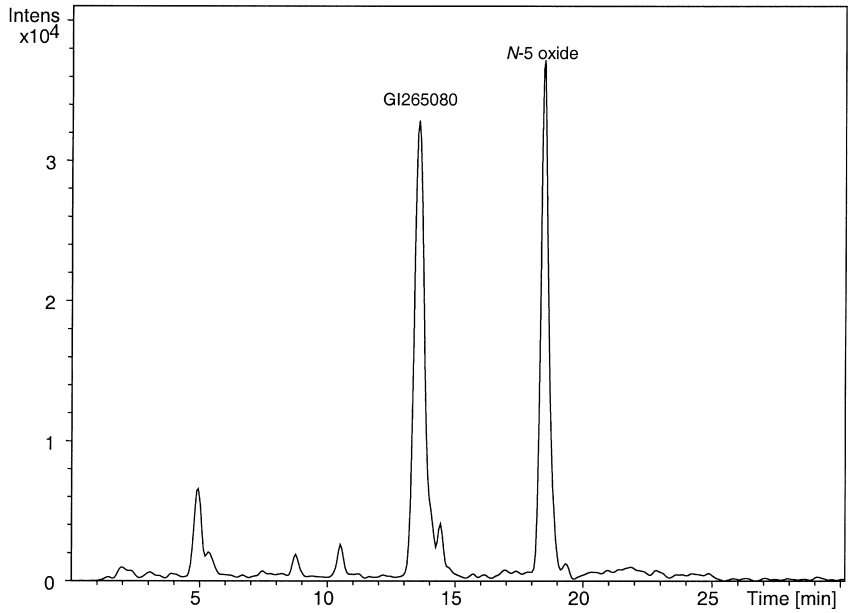

Fig. 6. The extracted product ion (*m*/*z* 127) chromatogram of GI265080 and its *N*-5 oxide metabolite.

mobile phase in the LC–NMR measurements. Since labile hydrogen atoms will be able to undergo hydrogen–deuterium exchange this factor must be considered when rationalising both parent and product ions. The structures of GI265080 and its *N*-5 oxide are shown in Fig. 1 together with their deuterio variants. Clearly in this instance the observation of a common, and therefore diagnostic product ion at m/z 127 provides a selective means of pinpointing these analytes within the complex UV chromatogram. The extracted ion chromatogram corresponding to this common product ion is shown in Fig. 6. Compared with the associated UV chromatogram (Fig. 4a) the extracted product ion data clearly provides a more specific means of initiating stop-flow NMR experiments.

The ^{1}H-NMR spectra resulting from stop-flow acquisition of GI265080 and its *N*-oxide metabolite, directly from spiked urine, using the MS in data dependent auto MS–MS mode, are presented in Fig. 7. The quality of the spectra indicates that the drug related peaks have been successfully isolated from urine with sufficient purity and concentration to enable structural characterisation. The parent drug, GI265080 and its *N*-5 oxide metabolite are readily distinguished through the diagnostic downfield NMR chemical shift of the aromatic H-6 proton of the pyridine ring which appears as a doublet at δ 7.78 in GI265080 and at δ 7.93 in the *N*-5 oxide metabolite. The remaining protons of the NMR spectra of both GI265080 and the metabolite can be readily assigned and are consistent with their structures.

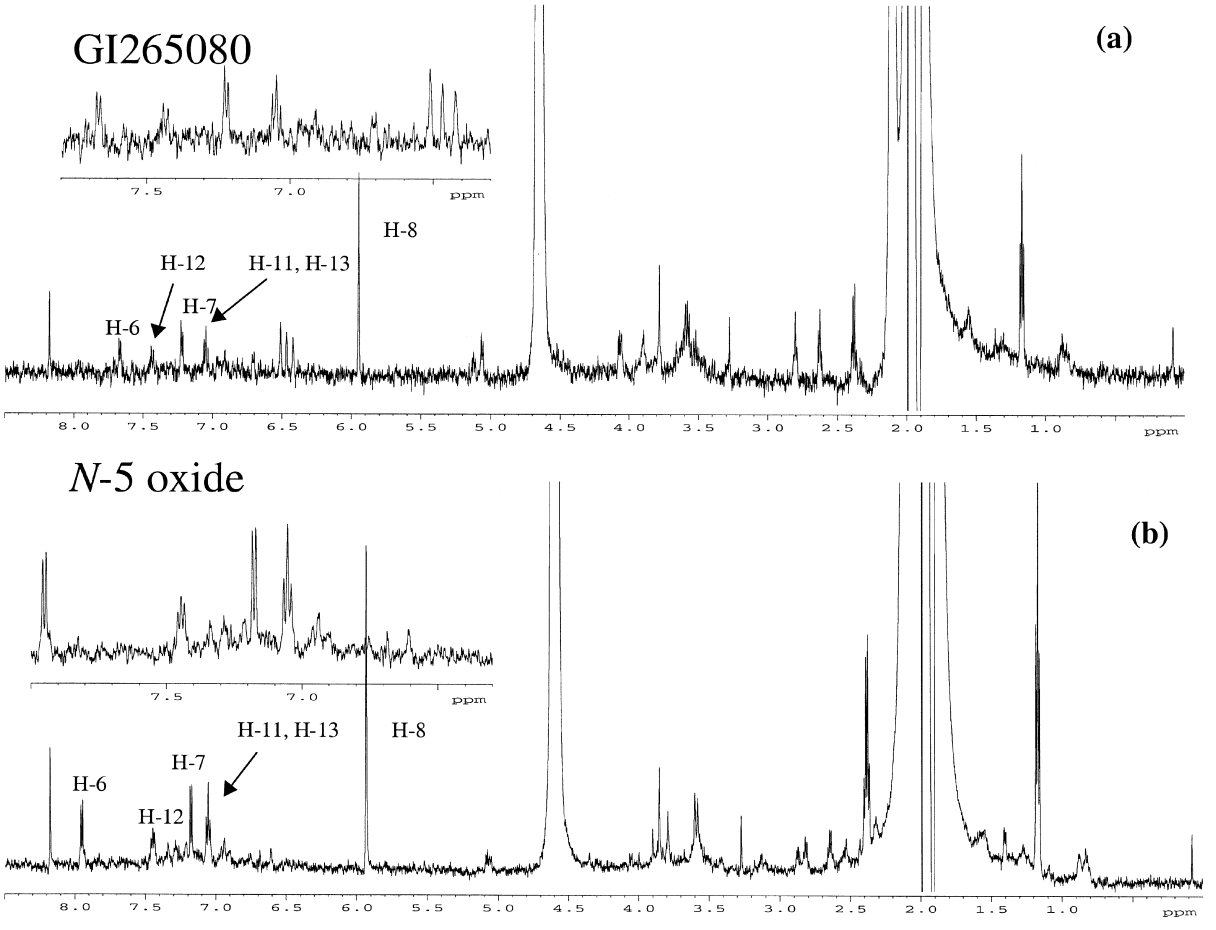

Fig. 7. ^{1}H-NMR spectra of GI265080 and its *N*-5 oxide metabolite acquired using MS directed stopped-flow LC–NMR.

In order to test the system using samples from a typical drug metabolism study, post-dose dog urine was analysed following administration of GW420867, a novel non-nucleoside reverse transcriptase inhibitor (Fig. 8), currently under development for the treatment of HIV infections. The UV chromatogram resulting from the injection (50 μl) of urine sample onto the LC–UV–MS–NMR system is shown in Fig. 9a, together with the extracted ion chromatogram, corresponding to the MD^+ ion of a suspected *O*-glucuronide conjugate, Fig. 9b. The MS and MS–MS spectra of the peak at 22.4 min are shown in Fig. 9c and d, respectively. The product ions resulting from the MD^+ ion at m/z 479 are rationalised in Fig. 10, and are used to confirm the drug-related nature of the peak. The dominant product ions at m/z 186 and 258, indicate that this peak is an aromatic *O*-glucuronide, with these fragments being consistent with equivalent fragments produced from parent drug (data not shown), albeit with a mass difference of 16 u, corresponding to the

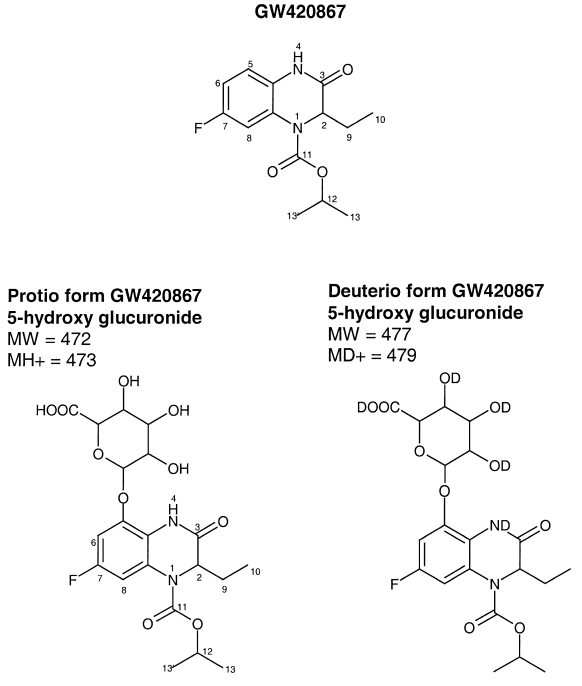

Fig. 8. GW420867 and a novel human urine glucuronide metabolite.

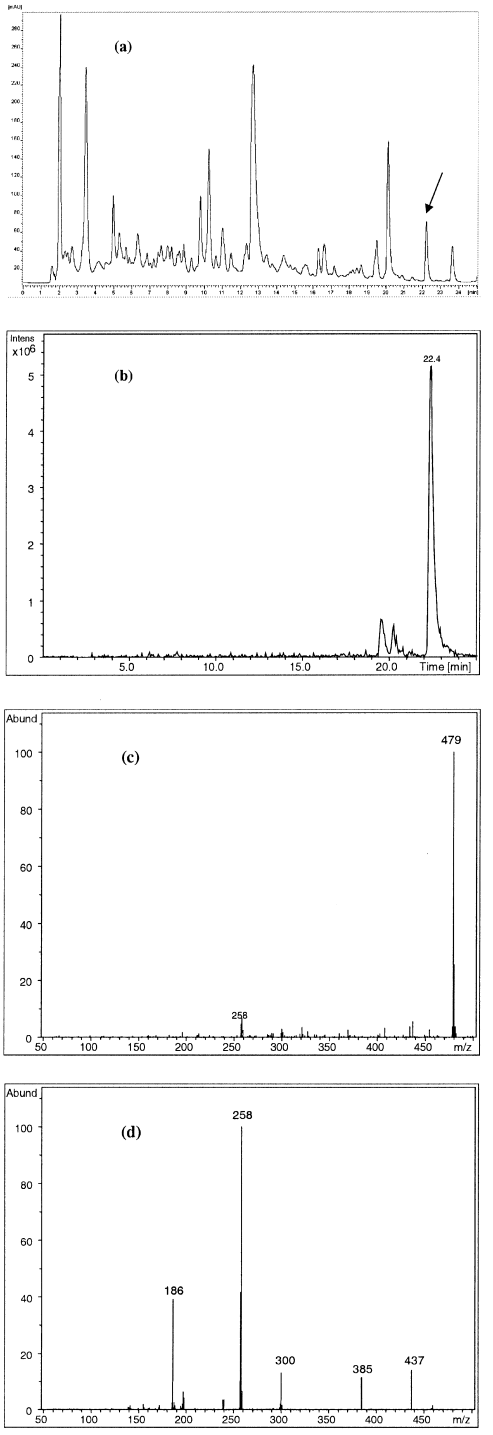

Fig. 9. UV and extracted ion chromatograms following injection of post-dose dog urine following administration of GW420867, onto the LC–UV–MS–NMR system and MS and MS–MS spectra of peak at 22.4 min.

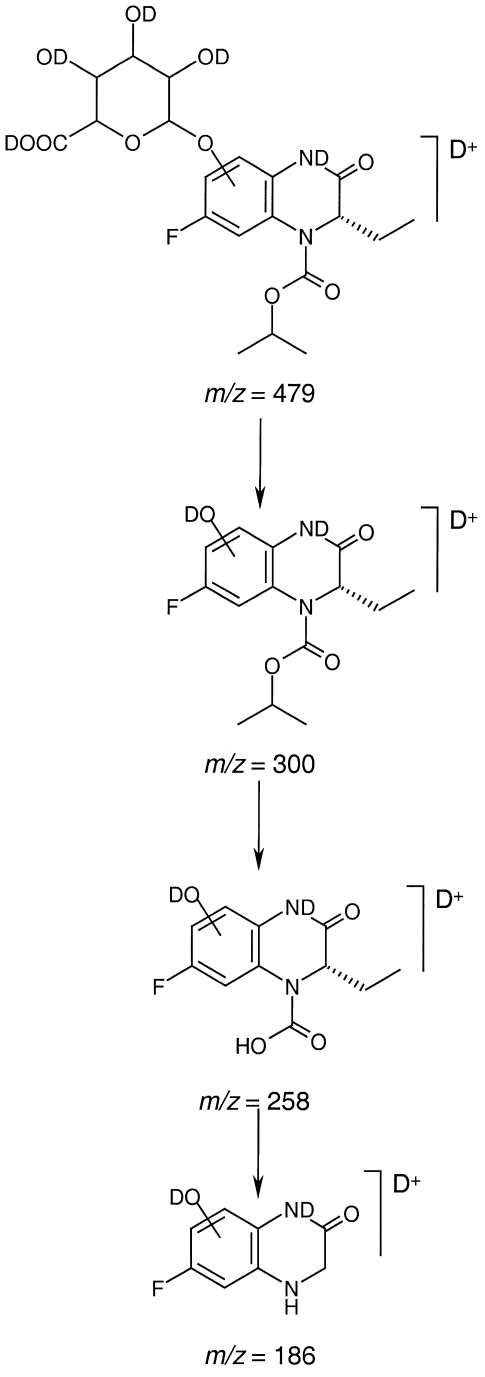

Fig. 10. Product ion scheme of an aromatic hydroxylated glucuronide metabolite of GW420867 prior to LC–NMR.

addition of a hydroxyl moiety. The loss of 179 u from the precursor MD^+ ion is consistent with glucuronidation and compares to the loss of 176 u usually encountered in non-deuterated systems (see Fig. 10). Although the product ions observed in this instance are not common with parent drug (data not shown), as a result of the suspected hydroxylation, they are still diagnostic, as they can be readily interpreted with reference to parent drug.

The subsequently acquired ^{1}H-NMR spectrum of the MS peak at 22.4 min is shown in Fig. 11. Inspection of the NMR spectrum enabled confirmation of the structure of the metabolite as the 5-hydroxy glucuronide conjugate. The loss of an aromatic proton in comparison to the NMR spectrum of the parent material together with rationalisation of the chemical shifts and coupling constants of the remaining protons (δ 6.84, J=9.5 Hz and a broad singlet at δ 7.13) readily enabled confirmation of the position of substitution. The appearance of a diagnostic anomeric H-1 proton at δ 5.05 confirmed the presence of a glucuronyl moiety. Elucidation was aided by comparison with the ^{1}H-NMR spectrum of authentic parent drug and a previous characterisation of this same metabolite using the semi-preparative HPLC approach [16].

The approach demonstrated in this work can be seen to be an alternative to the automated semi-preparative HPLC approach as described previously [15,16]. Where appropriate (e.g., when dealing with $>$5 ml of sample) the semi-preparative approach provides the opportunity to acquire superior NMR spectra in a fraction of the time as a result of the increased concentration of analyte derived from the preparative process. In contrast, LC–MS–NMR on the analytical scale as illustrated in this paper, is best suited to samples that contain a high concentration of analyte(s), since the volume of sample which can be loaded onto the analytical HPLC column is limited if good separations are to be achieved. However, this configuration is ideally suited to occasions in which sample volume is limited (e.g., in vitro hepatocyte incubations, rodent plasma), allowing the whole sample to be utilised analytically. One major benefit of the direct LC–MS–NMR approach is that it lessens ambiguity of whether the NMR and MS are detecting the same peak, since the data acquisition and subsequent interpretation is synchronised.

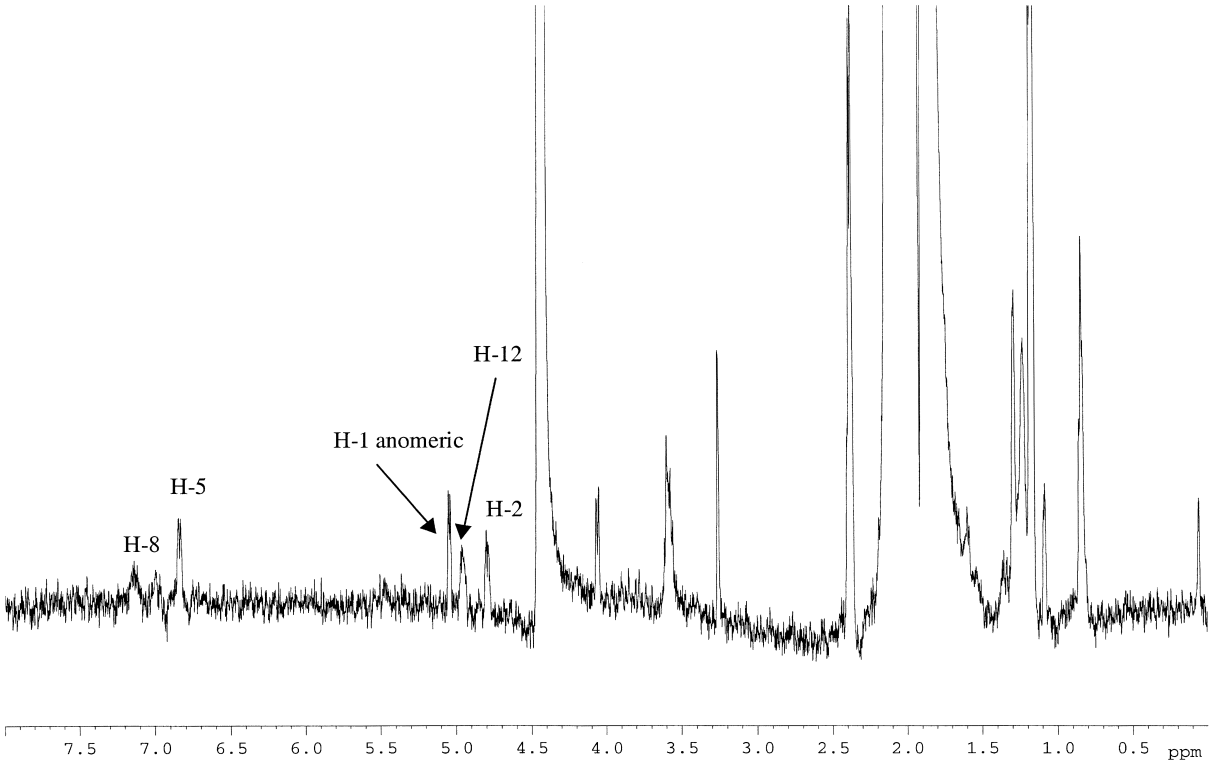

Fig. 11. ^{1}H-NMR spectrum of an aromatic hydroxylated glucuronide conjugate of GW420867 using MS directed stopped-flow LC–NMR.

4. Conclusions

HPLC, MS and NMR have been successfully combined to allow rapid and effective identification of metabolites directly from urine following administration of novel drug candidates. The combination of these techniques has enabled the use of mass spectrometry to initiate acquisition of stop-flow LC–NMR data, in the presence of masking UV signals. The coupling of these techniques in this manner has enabled molecular mass information to be most efficiently utilised in the separation and characterisation of drug metabolites in biological fluids. This work further serves to demonstrate the potential of coupling MS–MS using an ion trap spectrometer with LC–NMR spectroscopy, to provide an extremely powerful tool in structural elucidation.

References

[1] J.C. Lindon, J.K. Nicholson, I.D. Wilson, Adv. Chromatogr. 36 (1996) 315.
[2] J.C. Lindon, J.K. Nicholson, U.G. Sidelmann, I.D. Wilson, Drug Metab. Rev. 29 (1997) 705.
[3] N. Mistry, I.M. Ismail, M.S. Smith, J.K. Nicholson, J.C. Lindon, J. Pharm. Biomed. Anal. 16 (1997) 697.
[4] R.J.P. Cannell, T. Rashid, I.M. Ismail, P.J. Sidebottom, A.R. Knaggs, P.S. Marshall, Xenobiotica 27 (1997) 147.
[5] K. Ndjoko, J.L. Wolfender, E. Roder, K. Hostettman, Planta Med. 65 (1999) 562.
[6] F.S. Pullen, A.G. Swanson, M.J. Newmann, D.S. Richards, Rapid Commun. Mass Spectrom. 9 (1995) 1003.
[7] J.P. Shockcor, S.E. Unger, I.D. Wilson, P.J. Foxall, J.K. Nicholson, J.C. Lindon, Anal. Chem. 68 (1997) 4431.
[8] K.I. Burton, J.R. Everett, M.J. Newmann, F.S. Pullen, D.S. Richards, A.G. Swanson, J. Pharm. Biomed. Anal. 15 (1997) 1903.

[9] G.J. Dear, J. Ayrton, R. Plumb, B.C. Sweatman, I.M. Ismail, I.J. Fraser, P.J. Mutch, Rapid Commun. Mass Spectrom. 12 (1998) 2023.
[10] M. Spraul, M. Hoffman, D. Dvortsak, J.K. Nicholson, I.D. Wilson, Anal. Chem. 65 (1993) 327.
[11] J.P. Shockcor, R.M. Wurm, L.W. Frick, P.N. Sanderson, R.D. Farrant, B.C. Sweatman, J.C. Lindon, Xenobiotica 26 (1996) 189.
[12] M.E. Lacey, R. Subramanian, D.L. Olson, A.G. Webb, J.V. Sweedler, Chem. Rev. 99(10) (1999) 3133.
[13] K. Pusecker, J. Schewitz, P. Gforer, L. Tseng, K. Albert, E. Bayer, Anal. Chem. 70 (1998) 3280.
[14] P. Styles, N.F. Scoffe, C.A. Scott, D.A. Cragg, F. Row, D.J. White, P.C.J. White, J. Magn. Res. 60 (1984) 397.
[15] R.S. Plumb, J. Ayrton, G.J. Dear, B.C. Sweatman, I.M. Ismail, Rapid Commun. Mass Spectrom. 13 (1999) 845.
[16] I.M. Ismail, G.J. Dear, P. J Mutch, L.H. Davies, R.S. Plumb, B.C. Sweatman, Xenobiotica 29 (1999) 957.
[17] G.J. Dear, R.S. Plumb, B.C. Sweatman, I.M. Ismail, J. Ayrton, Rapid Commun. Mass Spectrom. 13 (1999) 886.