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Use of directly coupled ion-exchange liquid chromatography–mass spectrometry and liquid chromatography–nuclear magnetic resonance spectroscopy as a strategy for polar metabolite identification

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Abstract

Ion-exchange LC–MS and LC–NMR have been successfully used to identify a novel *N*-acetyl metabolite of a highly polar drug candidate [2-(ethanimidoylamino)ethyl]sulfonyl alanine (GW273629) under development as a therapeutic agent. This has been achieved using a simple HPLC method without the need for complicated and time consuming pre- or post-column derivatisation. Ion-exchange chromatography using simple ionic strength buffer and organic solvent mobile phases, as applied here, should be suitable for the analysis of other charged polar species. Optimisation of the system described could result in the development of a rational generic HPLC approach specifically designed for the characterisation of polar drug molecules and their metabolites. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

Investigation of the metabolism of potential drug candidate molecules has been an essential part of the development process for many years. It has been required to provide evidence that the similarity of metabolism in humans and in the animal species used for toxicological evaluation results in exposure to similar chemical entities. However, identification of drug metabolites earlier in the discovery process is becoming increasingly important, since this information can facilitate a metabolism-guided approach to structural modification. This should enable the selection of new drug candidates, which are more potent, less toxic and which have better pharmacokinetic profiles. Success in the early discovery stage requires metabolite identification methods to be rapid because of this increased focus on accelerated drug candidate selection and therefore generic analytical approaches are desirable in order to handle the significant increase in sample numbers [1].

Traditionally drug metabolite identification has been based on studies using radiolabelled and unlabelled drug both in vivo and in vitro and often

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relied on the comparison of the ultraviolet (UV) spectral data and high-performance liquid chromatography (HPLC) retention times of isolated ''unknown'' metabolites with those of synthetic standards. Such methods of detecting drug metabolites and subsequently characterising them were time-consuming and afforded very limited structural information [2]. In the last decade this approach has been superseded by structurally more informative methods such as liquid chromatography–mass spectrometry (LC–MS), LC–MS–MS [3], LC–nuclear magnetic resonance spectroscopy (LC-NMR) [4] and more recently LC–NMR–MS [5,6].

Reversed-phase HPLC is usually the chromatographic mode of choice for bioanalysis in drug metabolism studies because of its ability to separate compounds with a broad range of polarities and its compatibility with aqueous based biological samples. Analysis based on reversed-phase LC–MS–MS has significantly increased the throughput in drug metabolism and pharmacokinetic studies [7], and has been adopted by many laboratories for their generic systems [1]. Consequently metabolite identification applications using LC–MS, LC–NMR and LC–NMR–MS have used reversed-phase HPLC as the ''front end'' separation method. However, reversed-phase HPLC is not universally suitable for drug metabolism studies since highly polar compounds and their metabolites will not be retained and therefore elute with the solvent front, together with all of the polar endogenous components found in biological fluids. This makes the isolation and characterisation of these species a difficult and time-consuming process.

The analysis of polar compounds has been achieved using other chromatographic techniques such as ion-pair [8], normal-phase [9] and ion-exchange [10] chromatography, but these techniques have significant limitations in their application to the structural elucidation of drug metabolites in biological matrices. These limitations are particularly severe when coupling techniques such as MS and NMR to provide unequivocal structural elucidation.

Ion-pair HPLC involves the addition of a counter ion, such as octane sulfonic acid to the mobile phase, which forms an ionic bond with the analyte ion thus permitting the complex to chromatograph as a neutral species. However the presence of relatively large quantities of protonated ion-pairing agent in the mobile phase would present a significant amount of background signal in the NMR spectrum thereby adversely affecting the NMR sensitivity and possibly masking signals from potential metabolites.

Normal-phase HPLC requires the use of organic proton-rich solvents that may give rise to multiple signals in the NMR spectrum. Furthermore, there may be other problems associated with the physical nature of the metabolites. The solubility of polar drug metabolites in the mobile phase is likely to be very low, which will limit the column loading, but injecting solutions of the polarity which would be required to solubilise them would significantly affect the chromatographic performance.

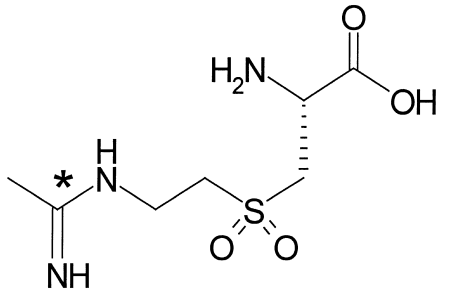
Ion-exchange chromatography depends on an interaction between the analyte and the stationary phase that is ionic rather than lipophilic. The presence of a readily ionisable group within the molecule may permit its separation in either of two modes, anionic mode (where the stationary phase interacts with negatively charged groups on the analyte, e.g., $-COO^-$) or cationic mode (where the stationary phase interacts with positively charged groups on the analyte, e.g., $-NH_4^+$). The mobile phase may be a simple ionic buffer, the nature of which may be selected to avoid complications in the NMR spectrum.

Here we describe a successful application of coupling of ion-exchange HPLC directly with MS and NMR for the identification of a polar drug metabolite. This application of ion-exchange chromatography coupled to MS and NMR using on-line radiochemical detection is exemplified in the study of GW273629 ([2-(ethanimidoylamino)ethyl]sulfonyl alanine) (Fig. 1).

2. Experimental

2.1. Chemicals

HPLC-grade acetonitrile and methanol were purchased from Rathburn (Walkerburn, UK), whilst HPLC-grade water and formic acid (AR grade) were obtained from Fisons (Loughborough, UK). Deuterium oxide and Riedel-de Haen ''PESTANAL'' -grade acetonitrile were purchased from Fluorochem



GW273629

* ^{14}C

Fig. 1. The structure of GW273629.

(Glossop, UK). Ammonium formate and uracil were purchased from Sigma–Aldrich (Poole, UK). GW273629, ^{14}C-GW273629 (see Fig. 1) and its *N*-acetyl metabolite (*N*-acetyl-3-{[2-(ethanimidoylamino)ethyl]sulfonyl}alanine) were synthesised by GlaxoWellcome Research and Development, Stevenage, UK.

2.2. Samples

Urine was collected from a Wistar Han rat for a 0–6 h period following a single intravenous dose of 10 mg/kg ^{14}C-GW273629 (free base) and stored frozen at −20°C prior to analysis.

2.3. Sample extraction

A 1-ml aliquot of 0–6 h rat urine was extracted on a Varian 1 g C_{18} Mega-Bond Elut cartridge (Varian, Palo Alto, CA, USA) following priming of the cartridge with 5 ml methanol followed by 5 ml 0.1% (v/v) aqueous formic acid. The eluate together with a further 1 ml 0.1% aqueous formic acid wash were pooled and taken to dryness under a stream of dry nitrogen. The residue was reconstituted in water in preparation for analysis. Both the eluted sample and the neat urine were analysed by liquid scintillation counting (LSC) and HPLC with both radiochemical and UV (215 nm) detection

2.4. Chromatography

All HPLC separations were performed on either one of two systems:

2.4.1. (i) Reversed-phase chromatography

A 150×2.1 mm RxC8 (Hewlett-Packard, CA, USA), packed with 5 μm Zorbax stationary phase using a mobile phase consisting of 0.1% (v/v) aqueous formic acid (A) and 0.1% (v/v) formic acid in acetonitrile (B). The proportion of acetonitrile was programmed to increase linearly from 0% to 100% in 30 min, with a flow-rate of 0.25 ml/min.

2.4.2. (ii) Ion-exchange chromatography

A 250×4 mm Dionex PCX-100 (Dionex, CA, USA) packed with OmniPac PCX-100 stationary phase using a mobile phase consisting of 5% aqueous acetonitrile (A), 50 m*M* aqueous formic acid (B), 100 m*M* aqueous ammonium formate (C) and 95% aqueous acetonitrile (D). The gradient system used is described in the table below, with a flow-rate of 1.0 ml/min

Time (min)	Solvent (%)			
	A	B	C	D
0	80	10	10	0
5	80	10	10	0
25	0	10	40	50
30	0	10	40	50

2.5. Radiochemical detection

In the absence of any significant UV chromophore, the presence of drug-related material was monitored using radiochemical detection both off-line and on-line. The estimation of radioactivity in fractions from solid-phase extractions (SPEs) was achieved using LSC. Aliquots of the fractions were added to 10 ml Starscint (Packard Bioscience, Groningen, The Netherlands) and counted on a Packard Tricarb LSC 2700TR scintillation counter (Canberra Packard,

Pangbourne, UK). Radioactivity monitoring on-line was achieved by coupling the HPLC systems to a Berthold LB 507B radioactivity monitor using a heterogeneous (i.e., solid scintillant) 150 μl solid yttrium glass flow cell (EG&G, Milton Keynes, UK).

2.6. Reversed-phase analysis of GW273629

Initial reversed-phase analysis of GW273629 was conducted on a Hewlett-Packard HP1100 binary pumping liquid chromatograph (Waldbronn, Germany) coupled to a Bruker Esquire ion-trap mass spectrometer (Bruker-Daltonics, Bremen, Germany), fitted with an atmospheric pressure ionisation (API) source. The column eluent was split 1 in 5, directing approximately 50 μl/min to a pneumatically-assisted electrospray interface. 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 mode with a scan rate of 13 000 u per second and a range of 50–600 m/z. Chromatographic separations were performed using system (i) as described.

2.7. Ion-exchange LC–MS

All LC–MS experiments were conducted on a Hewlett-Packard Model 1050 quaternary pumping system liquid chromatograph coupled in series to a Berthold radioactivity monitor (as above) and a Finnigan-MAT TSQ-700 triple quadrupole mass spectrometer (San Jose, CA, USA), with an API source. The API source heated metal capillary was maintained at 220°C for all analyses. Separations were performed using system (ii), described above. The effluent from the on-line radiochemical detector was directed to a pneumatically-assisted electrospray interface, following a 1 in 10 flow split, and the ^{14}C radioactivity signal was connected to an analog input channel on the TSQ-700 data system, allowing simultaneous display of ^{14}C and MS or MS–MS data. Full-scan positive ion mass spectra were acquired over a mass range of 100–300 u and analysed on a DEC station 5000/33 computer using Finnigan-MAT ICIS software. MS–MS experiments were based on collision-induced dissociations (CIDs) occurring in the radio frequency (RF)-only collision cell of the triple quadrupole, with a collision energy of −20 eV and Argon collision gas at a pressure of approximately 1.5 mTorr (1 Torr=133.322 Pa).

2.8. Ion-exchange LC–NMR

Ion-exchange ^{1}H-LC–NMR was carried out using a Bruker DRX-600 spectrometer operating at 600.13 MHz equipped with a dual ^{1}H-^{19}F LC-flow probe (cell 4 mm I.D with an active volume of approximately 120 μl), coupled to a Bruker LC-22 quaternary pump together with a Berthold radiomonitor (see above). Chromatographic separations for LC–NMR were performed under the control of Bruker HyStar (v. 1.0) software operating in loop storage mode and/or stopped-flow mode. The ^{14}C-radioactivity signal, from the on-line radiochemical detector, was connected to an analog input channel on the Bruker Hystar computer. Analytical chromatography was performed using system (ii), see above. The column eluent was monitored using the Berthold radiomonitor and eluting peaks of interest were stored in the loops of the Bruker BPSU-36/BSFU-0 peak sampling unit prior to transfer to the NMR probe for analysis, or were transferred directly into the probe in stopped flow operation. A schematic diagram of the configuration of this system is shown in Fig. 2. The samples were recovered after analysis by LC–NMR to permit further analysis and additional spectra were recorded using a standard dual ^{1}H-^{19}F 5 mm NMR probe.

^{1}H-NMR and ^{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 between 256 and 2048 transients were acquired depending on sample concentration into 64K data points over a spectral width of 12 019 Hz (20 ppm) giving a pulse repetition time of 4.0 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. The ^{1}H chemical shifts were referenced to acetonitrile at δ2.0.

Inverse gradient-selected hetero-nuclear multiple bond correlation (HMBC) for ^{1}H–^{13}C experiments was performed on a Varian Inova 600, operating at 599.98 MHz for ^{1}H and 149.998 MHz for ^{13}C observation. Typically 32 transients were acquired into 4 or 8K data points over a spectral width of

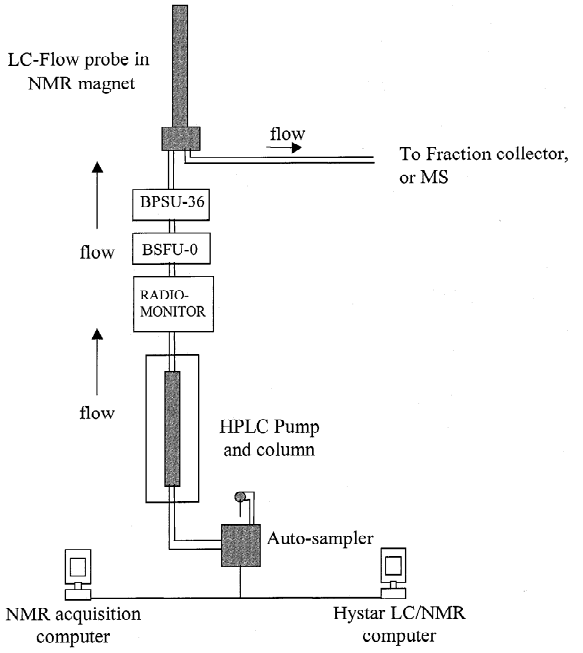


Fig. 2. Schematic diagram of LC–NMR configuration, with on-line radiochemical detection.

6948 Hz in the acquisition domain (F2). A total of 128 complex increments were acquired to define the second hetero-nuclear dimension (F1), 200 ppm for ^{13}C. The data was processed by application of a non-shifted cosine window apodisation function prior to Fourier transformation in both dimensions. Reference one-dimensional ^{1}H NOESYPRESAT experiments were acquired into 98 304 data points over a spectral width of 15 009 Hz (25 ppm). A varying number of transients, 32 to 64 were acquired with a mixing time of 100 ms and a total pulse repetition of 4.5 s. All proton chemical shifts were referenced to acetonitrile peak at 2.00 ppm.

3. Results and discussion

3.1. Selection of a chromatographic system for analysis of GW273629

3.1.1. Reversed-phase HPLC

In the absence of any significant UV chromophore, GW273629 standard was first analysed using a generic reversed-phase HPLC method (system (i)), coupled to an ion trap spectrometer, operating with positive ion electrospray ionisation. This HPLC system had a programmed linear increase in the organic component of the mobile phase from 0 to 100% over 30 min, thereby covering the entire range of analyte hydrophobicity.

The extracted ion chromatogram obtained from this generic reversed-phase LC–MS analysis is shown in Fig. 3, compared with an extracted ion chromatogram of uracil acquired under identical conditions. GW273629 eluted at 1.6 min and was detected as its MH^+ ion at m/z 238, whilst uracil eluted at 3.6 min and was observed as its MH^+ ion at m/z 113. This comparison was made because uracil is an extremely polar compound routinely used as an unretained marker for t_0 in reversed-phase HPLC analyses [11]. GW273629 eluted from this reversed-phase HPLC system with the solvent front, exhibiting even less chromatographic retention than uracil. Clearly GW273629, which has a log P of approximately -4, is not suitable for this type of generic reversed-phase analysis due to its highly hydrophilic nature.

3.1.2. Ion-exchange HPLC

Cation-exchange chromatography was performed on an OmniPac PCX-100 analytical column, which has been developed to specifically allow the use of organic solvents in eluents for polymer-based ion-exchange separations. Since the stationary phase is polymeric, ionic eluents in the pH range of 0–14 can be used to alter the selectivity and convert molecular species into ionic compounds. In addition, conventional reversed-phase solvents can be used in the solvent systems to elute hydrophobic species, to dissolve certain sample matrices and to clean columns contaminated with organic contaminants.

In the system used here, linear gradients of both the organic solvent (acetonitrile) and the ionic eluent (ammonium formate and formic acid) were achieved through the use of a quaternary pumping system. Traditionally, cation-exchange eluents have consisted of non-volatile buffer solutions (e.g., phosphate), but here more volatile components such as ammonium formate and formic acid, were employed, allowing connection to the API interface of the mass spectrometer without any deleterious effects (e.g., build up of salts within the API interface).

The presence of a ^{14}C-radiolabel in GW273629 in this study enabled a radiomonitor with a heterogen-

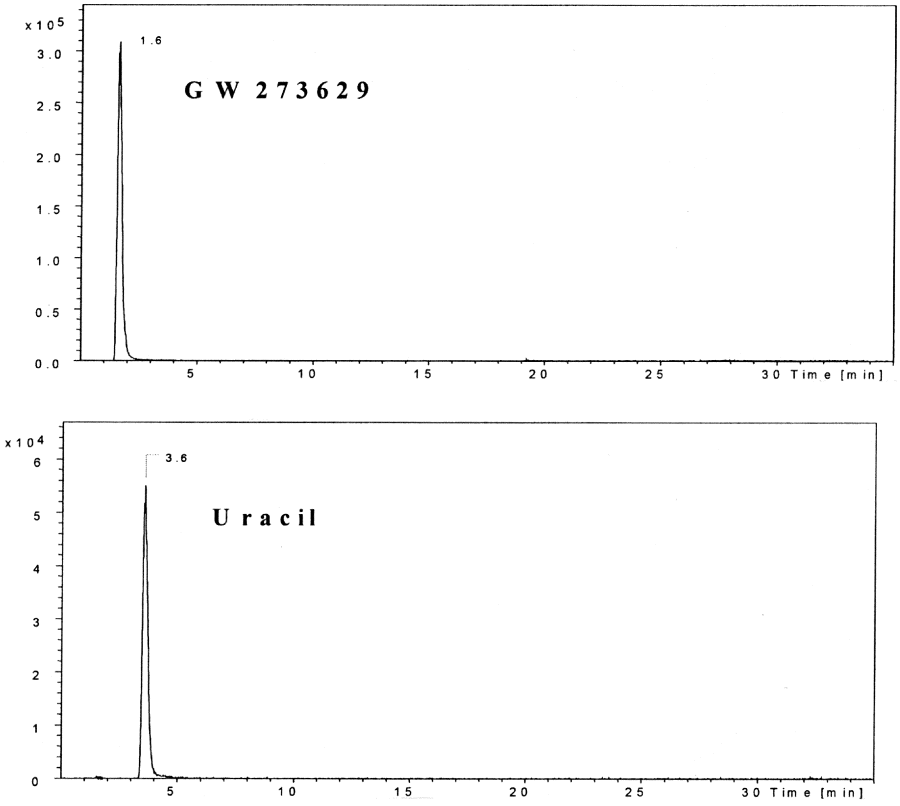


Fig. 3. Reversed-phase UV chromatograms of GW273629 and uracil acquired using the generic system described as (i).

ous flow cell to be used as a primary means of peak detection. The radiochromatogram obtained using the cation-exchange system, following analysis of standard ^{14}C-GW273629 is shown in Fig. 4. GW273629 is clearly observed within this radiochromatogram, eluting after approximately 17.5 min. This cation-exchange system therefore gives adequate chromatographic retention of the parent compound relative to the reversed-phase HPLC and offers the possibility of direct coupling of ion-exchange HPLC to NMR and MS.

3.2. Sample clean-up using solid-phase extraction

SPE is a widely used method in bioanalysis and drug metabolism studies as a means of sample clean-up and pre-concentration, prior to analysis by techniques such as LC–MS and LC–NMR. The most common phase used is C_{18} bonded silica, since this is able to retain all but the most polar of compounds, which pass directly through the column, the retained analytes being eluted in a small volume of organic solvent. Other bonded phases such as C_8, C_2 and CN are also available, each has its own selectivity, but none was able to retain GW273629. However, GW273629 was retained on a cation-exchange (SCX) type phase but, since this mode of chromatography had been chosen for the analytical processing of urine samples, it was considered likely that any components which co-elute on the analytical HPLC column would also be retained together on the SCX cartridge. Therefore advantage was taken of the ''catch all'' nature of C_{18} SPE by applying the sample urine to a C_{18} cartridge, and allowing GW273629 and any associated metabolites to pass through unretained whilst retaining the lipophilic endogenous components. This effected a partial sample clean-up prior to concentration and analysis by ion-exchange LC–NMR and LC–MS.

The recovery of radioactive material using this

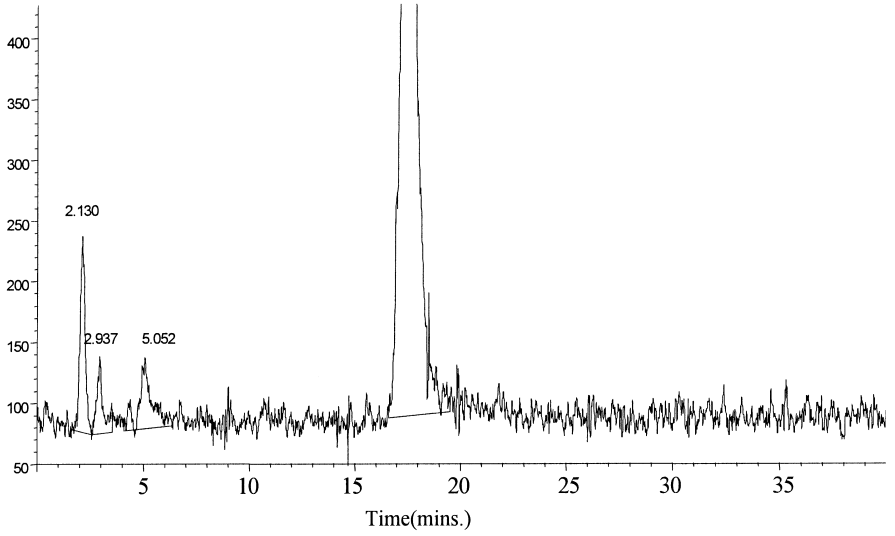


Fig. 4. Ion-exchange radiochromatogram of ^{14}C-GW273629 acquired using system (ii).

procedure was determined to be approximately 80%. Subsequent ion-exchange HPLC radiochromatograms showed that there were no significant qualitative differences between the extracted and unextracted urine. The associated UV chromatograms are compared in Fig. 5, and highlight the level of sample clean-up obtained using this extraction protocol.

3.3. Ion-exchange LC–MS and LC–MS–MS

The on-line radiochromatogram obtained following injection of 10 μl of rat urine extract onto the ion-exchange HPLC system is shown in Fig. 6. The peak eluting at 19.2 min was assigned as unchanged GW273629, although this result differed from the 17.5 min retention time observed for GW273629 when injected as a standard solution (Fig. 4). This difference was attributed to the buffering effect of the sample matrix. The presence of a second major radioactive peak at 5.9 min was assigned as a rat urine metabolite (metabolite **I**). This peak exhibited an MH^+ ion at m/z 280 which was 42 mass units higher than the MH^+ ion observed for standard GW272629.

A comparison of the MS spectra of unchanged GW273629 and that of metabolite **I**, obtained directly from rat urine extract are shown in Fig. 7. The MH^+ ions identified above are clearly discernible as the base peak in each spectrum. The observed mass difference of 42 between GW273629 and metabolite **I** is consistent with the incorporation of an acetyl group ($–CO-CH_3$) into GW273629.

MS–MS spectra for GW273629 and metabolite **I** were similarly acquired, using the respective MH^+ ion as the precursor ion in each case (data not shown). Although the spectra exhibit product ions, which are qualitatively identical, slight differences in the relative intensities were observed. However, these minor differences in the acquired MS–MS spectra of GW273629 and metabolite **I** did not enable confirmation of the incorporation of an acetyl group.

3.4. Directly coupled ion-exchange ^{1}H-LC–NMR

In contrast to LC–MS, LC–NMR is a relatively insensitive technique and requires microgram quantities of analyte to be transferred into the probe for analysis, in order to achieve a suitable signal to noise in the spectrum within a reasonable time. It was therefore essential to scale up the amount of metabolite **I** that could be isolated by this chromatographic method in one single injection. However, it was noted that when making a large injection of sample urine (>50 μl) onto the cation-exchange column the

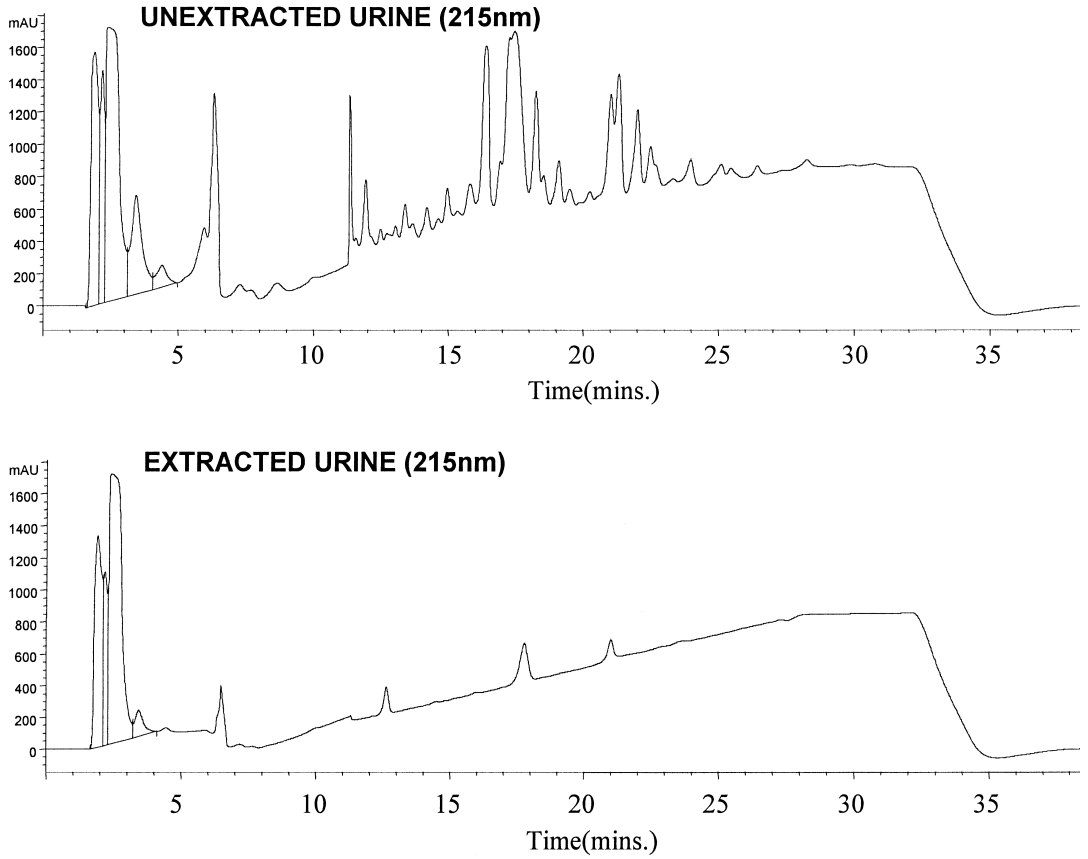


Fig. 5. UV chromatograms (215 nm) of (a) unextracted 0–6 h rat urine and (b) extracted 0–6 h rat urine, acquired using system (ii).

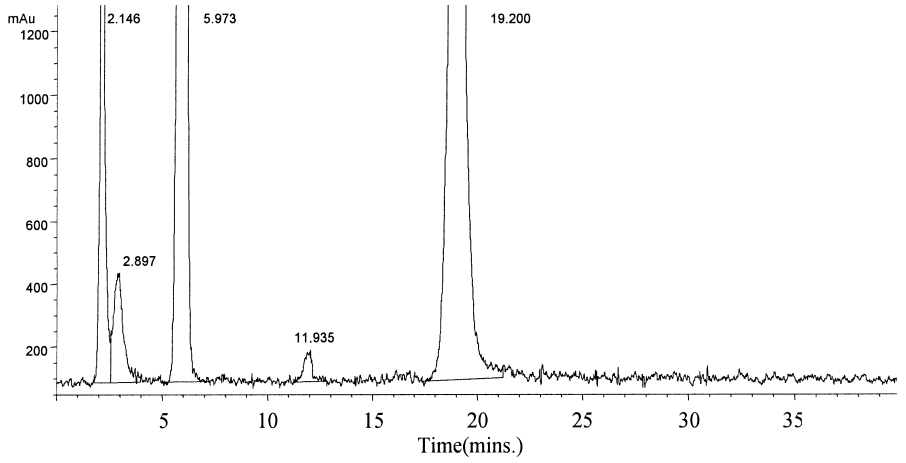


Fig. 6. Ion-exchange radiochromatogram of extracted 0–6 h rat urine, acquired using system (ii).

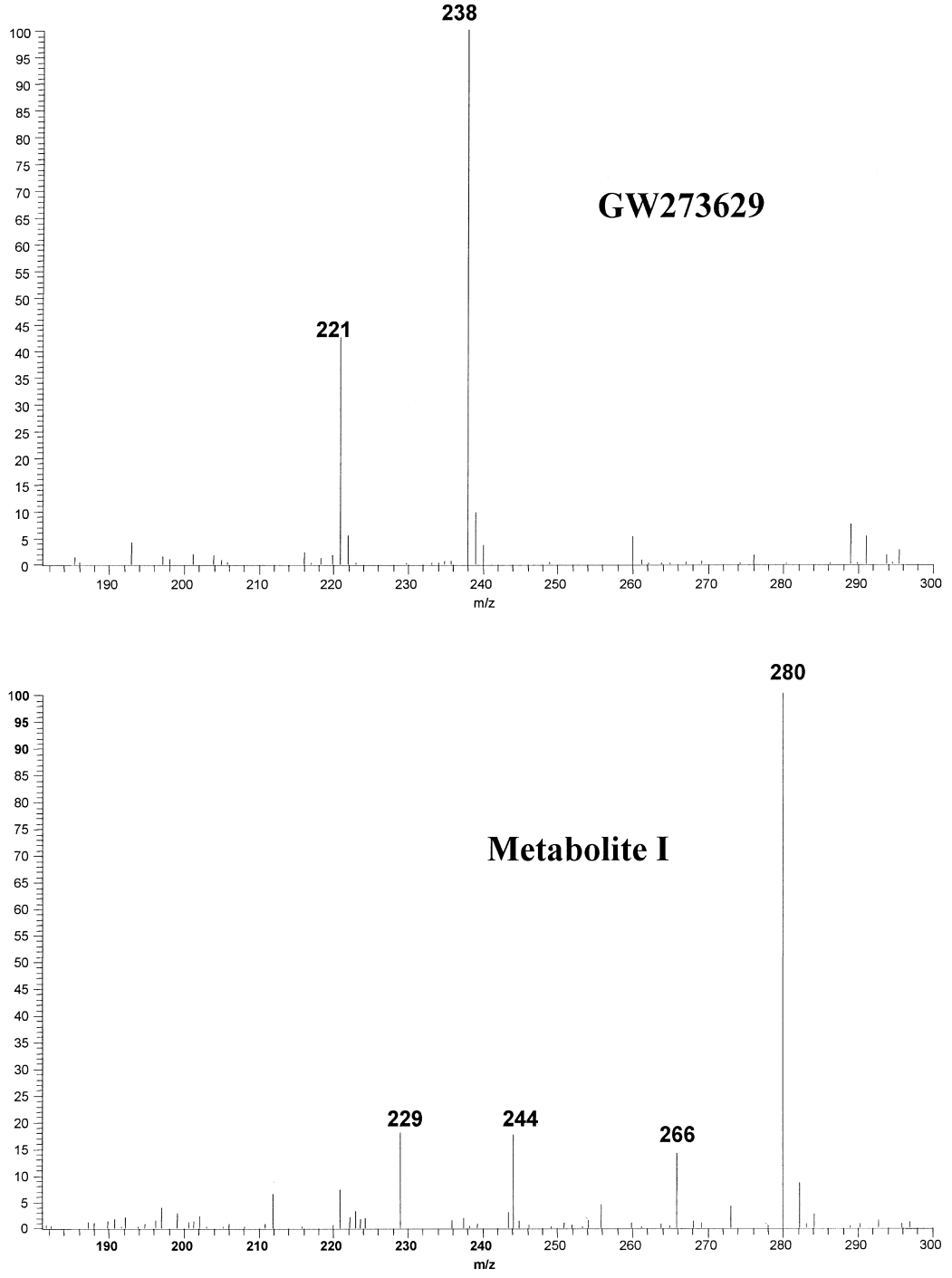


Fig. 7. Positive ion electrospray spectra of GW273629 and metabolite **I**, identified in rat 0–6 h urine.

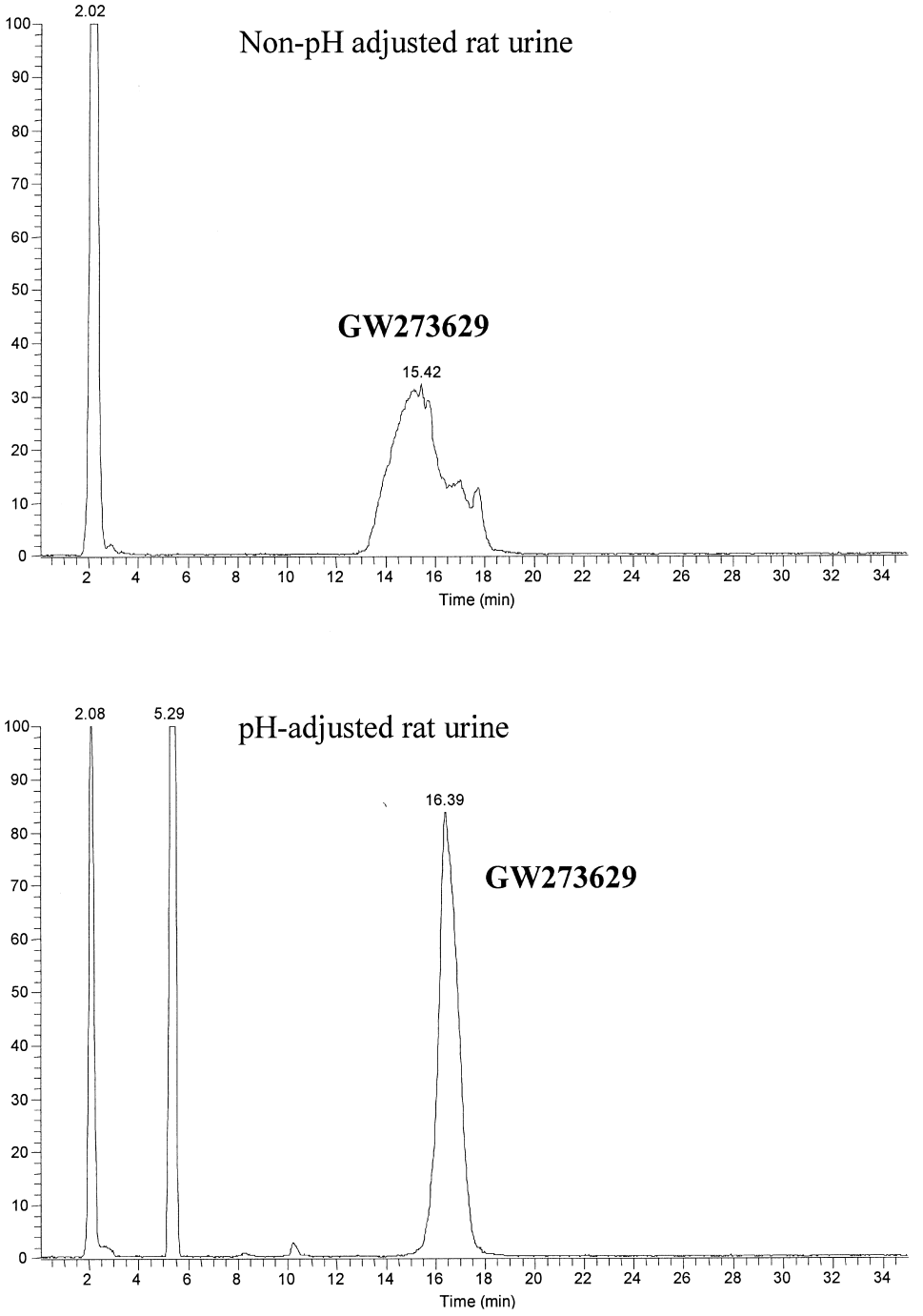


Fig. 8. Ion-exchange radiochromatogram of a non-pH adjusted and a pH adjusted urine extract, acquired using system (ii).

chromatographic performance decreased and the peak shape deteriorated. Adjustment of the pH of the injected sample by the addition of approximately 1% (v/v) formic acid was found to maintain chromatographic performance and retain good peak shape.

The radiochromatograms of a non-pH adjusted and a pH adjusted injection of urine are shown in Fig. 8, and clearly the peak shape of the non-pH adjusted sample is much broader than that of the adjusted sample. This observation may be attributable to the fact that when a large injection volume (>50 μl) is made, the sample can account for greater than 4% of the column volume. As a result, for a small period of time, the sample becomes its own mobile phase, causing significant chromatographic band broadening.

The ion-exchange LC–NMR system shown in Fig. 2 relied entirely on the on-line radiochemical detection to operate the software and stop the peak of interest in the NMR probe. Control of the chromatographic system in LC–NMR usually requires the analytes to have an UV response in order to initiate actions by the controlling software. Unfortunately and perhaps unusually, neither GW273629 or its metabolite (**I**), possess a chromophore and hence the on-line radiomonitor was used for this purpose. It would have equally been possible to utilise the MS or MS–MS response for this purpose, as we have shown in the other paper in this edition, but the presence of a radiolabel in GW273629 offered an attractive alternative.

The annotated ^{1}H-NMR spectrum resulting from the stopped flow ion-exchange LC–NMR experiment following injection of 100 μl of rat urine extract is shown in Fig. 9. The presence of drug-related material was readily discernible and confirmed that the molecule was intact and without any substitution into the molecule. The incorporation of an *N*-acetyl function into the molecule could not be confirmed directly from the ^{1}H-NMR spectrum since the region of the spectrum where the resonance from the *N*-acetyl methyl protons appear are masked by the

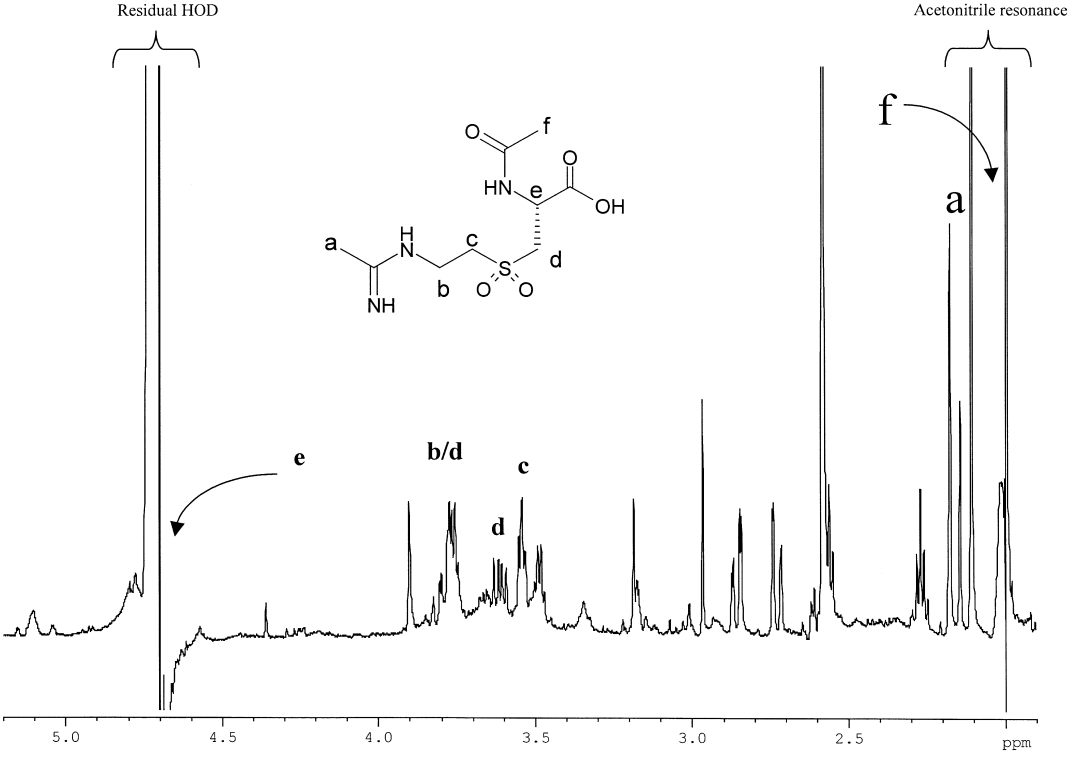


Fig. 9. ^{1}H-LC–NMR spectrum of metabolite **I** in rat 0–6 h urine, acquired in stop-flow mode.

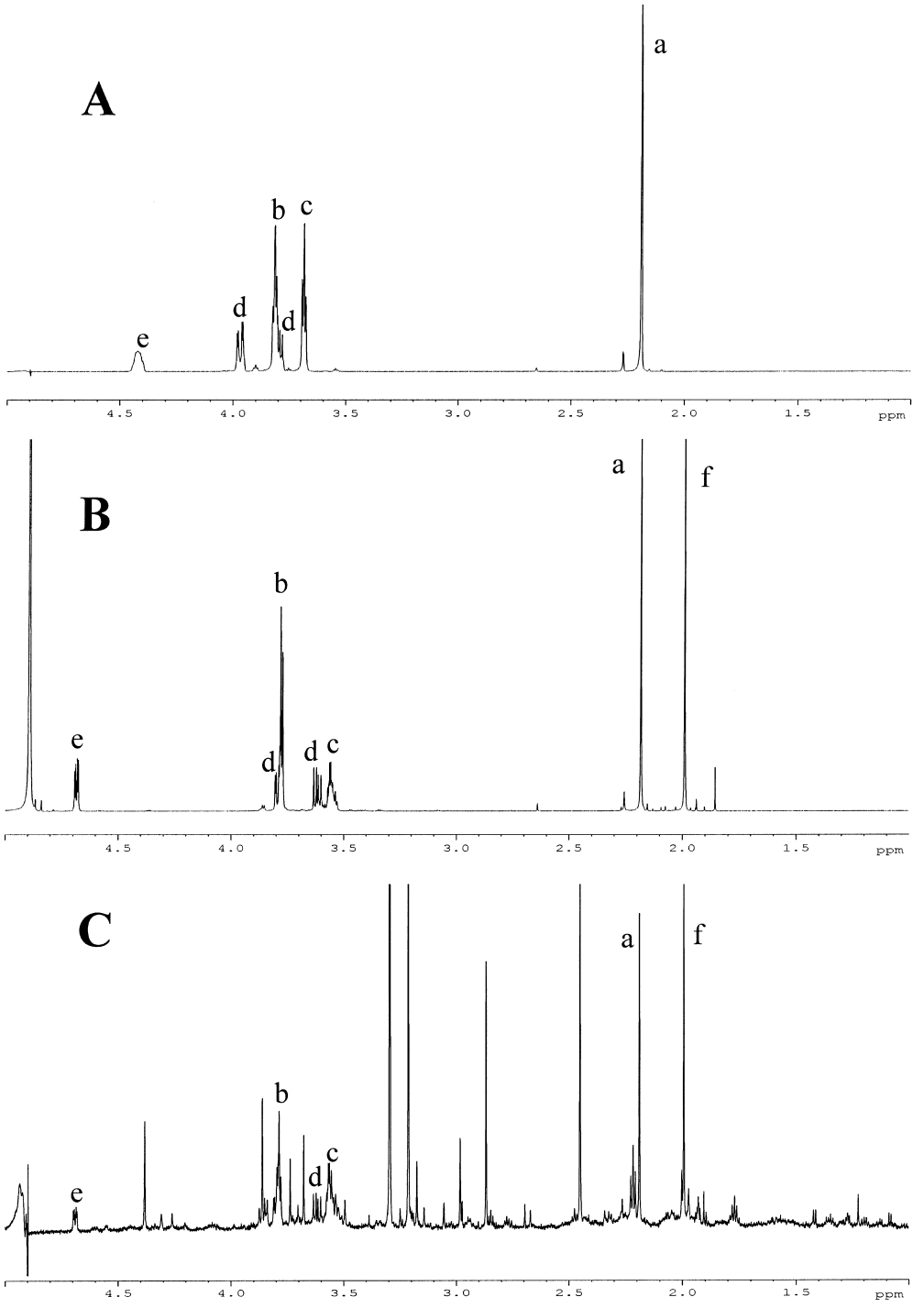


Fig. 10. ^{1}H-NMR spectra (in $^{2}H_2O$) of (a) authentic GW273629, (b) the authentic *N*-acetyl analogue and (c) metabolite **I**, as isolated from rat 0–6 h urine by LC–NMR.

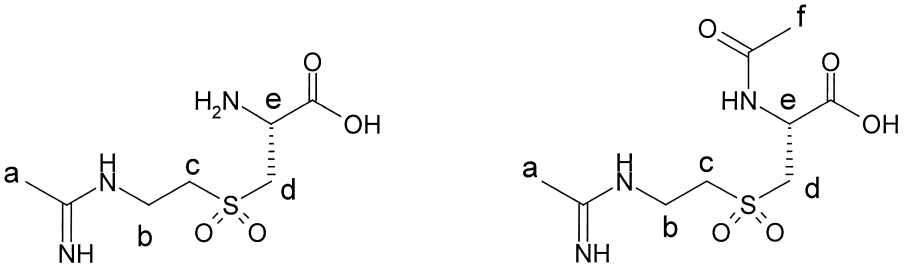


Fig. 11. Assignment of the NMR proton signals of GW273629 and metabolite **I** (see Fig. 10).

acetonitrile peak at δ2.0, arising from the HPLC mobile phase. This limitation to the application of ion-exchange LC–NMR, peculiar to this specific metabolic outcome may have been overcome with the development of an alternative methanol-based separation system, where the residual signals would have left the acetyl region free of background signals.

A simpler more rapid solution to the problem was to recover the peak trapped in the LC–NMR probe, remove the solvent under a stream of dry nitrogen and re-suspend the residue in pure deuterium oxide. The resulting ^{1}H-NMR spectrum, free of masking signals in the acetyl region, is shown in Fig. 10c together with the ^{1}H-NMR spectra of authentic parent drug, Fig. 10a, and synthetic *N*-acetyl analogue Fig. 10b, acquired in the same solvent under identical conditions.

A comparison of the ^{1}H-NMR spectrum of GW273629 with that obtained for the isolated metabolite **I** (Fig. 10a and c) immediately highlights the presence of the additional methyl peak of the *N*-acetyl group at δ2.0 which is not present in the spectrum of GW273629 confirming the MS evidence.

Additionally the methine CH (proton e) observed at δ4.42 in GW273629 has shifted to δ4.70 in metabolite **I**. This suggests that acetylation has occurred at the α-amine as opposed to the amidine group. From the combined MS and ^{1}H-NMR data it is possible to deduce the structure of metabolite **I** (see Fig. 11).

Final structural confirmation was provided by comparison of the ^{1}H-NMR spectrum of metabolite **I** with that of authentic *N*-acetyl standard, where there was good agreement (Fig. 10b and c). The chemical shifts and assignments for parent drug, metabolite **I** and the authentic *N*-acetyl analogue are given in Table 1. Confirmation of the structure of the *N*-acetyl authentic standard was obtained by a ^{1}H–^{13}C inverse HMBC experiment (see Fig. 12). In this experiment the methine CH proton (e) at δ4.70 and the methyl proton (f) at δ2.0 gave a long-range correlation to the carbonyl at δ175.2 in the ^{13}C spectrum confirming the structure of the chemically synthesised metabolite. In the metabolism of primary amines and

Table 1
Chemical shifts and assignments for GW273629, metabolite **I** and the authentic *N*-acetyl analogue

	a	b	c	d	e	f
GW273629	2.2 s	3.82 J=6.0 Hz	3.69 J=6.0 Hz	3.80&3.98 J=16.2, 3.6 and 8.4 Hz	4.42	–
Metabolite M1	2.2 s	3.79 m	3.58 m	3.61&3.8 J=15.9, 3.6 and 9.2 Hz	4.70 J=3.6 and 9.2 Hz	2.0
Authentic *N*-acetyl standard	2.2 s	3.79 m	3.58 m	3.61&3.8 J=15.9, 3.6 and 9.2 Hz	4.70 J=3.6 and 9.2 Hz	2.0

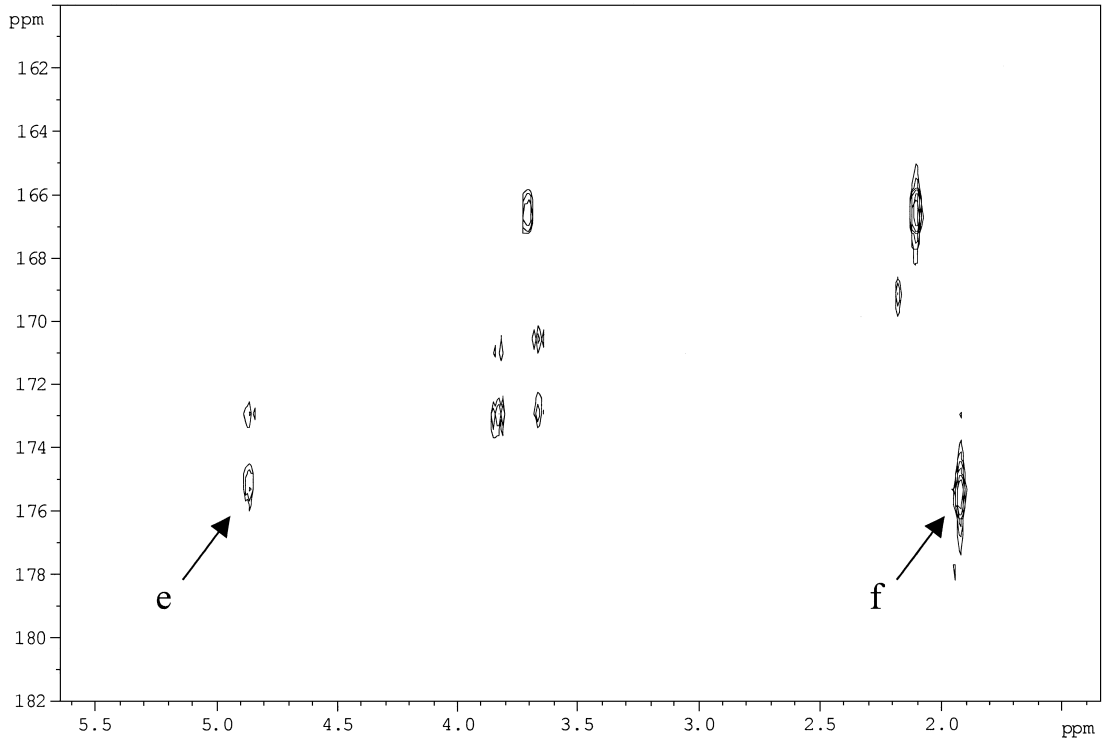


Fig. 12. $^{1}H–^{13}C$ inverse hetero-nuclear multiple bond correlation (HMBC) spectrum of authentic *N*-acetyl analogue.

secondary amines acetylation is a well-documented metabolic transformation [12]. In particular *N*-acetylation of *S*-substituted cysteines, such as *S*-benzyl-L-cysteine, has been shown by rat liver and kidney microsomal *N*-acetyltransferase preparation [13], and therefore as GW273629 is an *S*-substituted cysteine the formation of an *N*-acetyl metabolite is consistent with the literature data.

4. Conclusions

Ion-exchange LC–MS and LC–NMR have been successfully used to identify a novel *N*-acetyl metabolite of a highly polar drug candidate molecule. The use of a simple volatile ionic strength buffer like ammonium formate/formic acid together with an organic solvent, as employed here, should be suitable for the analysis of other charged polar species. Optimisation of the system described could result in the development of a high-throughput generic HPLC approach specifically designed for ionic polar drugs and their metabolites.

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References

[1] M.S. Lee, E.H. Kerns, M.E. Hali, J. Liu, L.J. Volk, LC·GC Int. September (1997) p. 586.
[2] M. Mitchard, in: J.W. Gorrod, A.H. Beckett (Eds.), Drug Metabolism in Man, Taylor and Francis, London, 1978, p. 175.
[3] T. Baille, in: Proceedings of the 42nd ASMS Conference on Mass Spectrometry and Applied Topics, Chicago, IL, 29 May–3 June 1994.
[4] J.C. Lindon, J.K. Nicholson, U.G. Sidelmann, I.D. Wilson, Drug Metab. Rev. 29 (3) (1997) 705.

[5] J.P. Shocker, S.E. Unger, I.D. Wilson, P.J. Foxall, J.K. Nicholson, J.C. Lindon, Anal. Chem. 68 (1997) 4431.
[6] G.J. Dear, J. Ayrton, R. Plumb, B.C. Sweatman, I. Ismail, I.J. Fraser, P.J. Mutch, Rapid Commun. Mass Spectrom. 12 (1998) 2023.
[7] J. Ayrton, G.J. Dear, W.J. Leavens, D.N. Mallet, R.S. Plumb, J. Chromatogr. B 709 (1998) 243.
[8] Y. Yokoyama, T. Amaki, S. Horikoshi, H. Sato, Anal. Sci. 13 (6) (1997) 963.
[9] R. Schwarzenbach, J. Liq. Chromatogr. 2 (1979) 205.
[10] A.A. Reilly, R. Bellisario, K.A. Pass, Clin. Chem. 42 (2) (1998) 317.
[11] G. Guansajonz, G. Guiochon, J. Chromatogr. 743 (1996) 247.
[12] G.G. Gibson, P. Skett, Introduction to Drug Metabolism, Chapman and Hall, London, New York, 1986.
[13] R.M. Green, J.S. Elce, Biochem. J. 147 (1975) 283.