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Journal of Chromatography B, 748 (2000) 269–279

JOURNAL OF CHROMATOGRAPHY B

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Application of directly coupled LC–NMR–MS to the structural elucidation of metabolites of the HIV-1 reverse-transcriptase inhibitor BW935U83

John P. Shockcor[a,b,*], Steve E. Unger[a], Paul Savina[b], Jeremy K. Nicholson[c], John C. Lindon[c]

[a]*Drug Metabolism and Pharmacokinetics, DuPont Pharmaceuticals Company, P.O. Box 30 Elkton Road, Newark, DE, 19714 USA*
[b]*Bioanalysis and Drug Metabolism, GlaxoWellcome Inc., 5 Moore Drive, Research Triangle Park, NC 27709 USA*
[c]*Biological Chemistry, Biomedical Sciences Division, Sir Alexander Flemming Building, Imperial College School of Science, Technology and Medicine, South Kensington, London SW7 2AZ UK*

Received 20 January 2000; received in revised form 25 May 2000; accepted 8 June 2000

Abstract

The human in vivo metabolism of the HIV-1 reverse transcriptase inhibitor 5-chloro-1-(2′,3′-dideoxy-3′-fluoro-*erythro*-pentofuranosyl)uracil (BW935U83) was studied using ^{19}F NMR spectroscopy, directly coupled LC–NMR and LC–NMR–MS. The number and relative proportions of the drug metabolites were obtained from ^{19}F NMR spectra of whole human urine. The novel use of the continuous-flow ^{19}F detected LC–NMR experiment yielded chromatographic retention times and ^{19}F chemical shifts for the parent drug, the glucuronide conjugate of the parent and an early eluting polar metabolite. The parent drug and its glucuronide conjugate were easily characterised by directly coupled ^{1}H LC–NMR spectroscopy and two-dimensional TOCSY experiments. The identification of the second metabolite was achieved using ^{19}F NMR and directly coupled ^{1}H LC–NMR–MS which furnished the molecular weight, and through the use of MS–MS techniques, information on the fragment ions. This species was identified as 3-fluoro-ribolactone.

Keywords: Structural elucidation; BW935U83; HIV-1 reverse-transcriptase inhibitor

1. Introduction

NMR and mass spectrometry are the principal techniques used for characterisation of xenobiotic metabolites. Typically the characterisation of xenobiotic metabolites begins with analysis a biological fluid or in vitro incubation broth using LC–MS, followed, if necessary, by isolation of metabolites using HPLC for further characterisation by NMR. Technical improvements in console electronics and probe design, higher magnetic field strengths, multidimensional NMR pulse sequences, and improved solvent suppression methods have allowed NMR to have an impact in this process at a much earlier stage by vastly improving sensitivity and lowering the absolute amount of sample needed for analysis. However, isolation of even the relatively small quantities (1–10 μg) of a metabolite needed for NMR analysis usually represents the rate-determining step in the process of metabolite structure elucidation. Following the path taken by mass spectrometry and infrared spectroscopy a separation

*Corresponding author.

0378-4347/00/$ – see front matter
PII: S0378-4347(00)00360-1

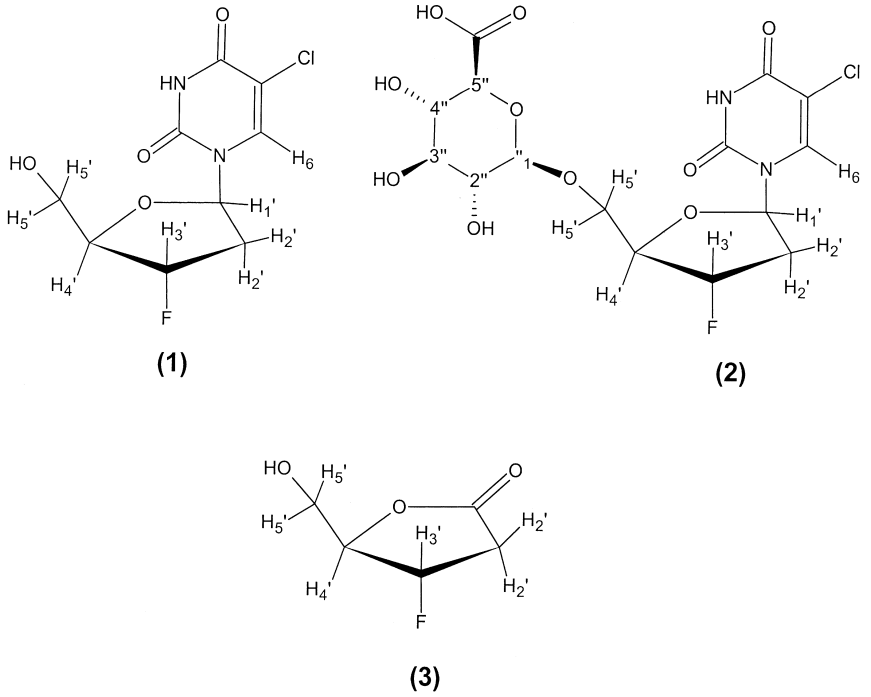

Fig. 1. Structures and numbering systems for BW935U83 (1), its glucuronide conjugate (2) and 3-fluoro-ribolactone (3).

technique, HPLC, was hyphenated to NMR, with the first commercially available LC–NMR hardware being introduced and utilised in the early 1990s [1–4] followed by development of directly coupled LC–NMR–MS in 1996 [5,6]. Using these novel new tools it is often possible to elucidate the structures of metabolites directly out of a biological matrix with little or no sample preparation. The hyphenated methods ^{1}H, and ^{19}F LC–NMR along with directly coupled LC–NMR–MS have been employed in the study of the in vivo human metabolism of anti-viral compound 5-chloro-1-(2′,3′-dideoxy-3′-fluoro-*erythro*-pentofuranosyl)uracil (BW935U83) (1) shown in Fig. 1.

2. Experimental

2.1. Chemicals and reagents

Deuterium Oxide (D_20) and trifluoroacetic acid-d_3 were obtained from Cambridge Isotopes Laboratories (Andover, MA, USA). Acetonitrile was obtained from Riedel-deHaen (Seelze, Germany).

2.2. Sample preparation

Samples of human urine were obtained from HIV-positive volunteers, who had received a single oral dose of 1000 mg, 0–4 h after dosing as part of a clinical trial. The samples were deactivated by heating at 58°C for 5 h and stored at −80°C while awaiting analysis. For ^{19}F NMR spectra on whole urine, the samples were pipetted into 5 mm NMR tubes and 10% (v/v) D_2O was added for field-frequency stabilisation. Several samples of urine were analysed by ^{19}F NMR without the precaution of heat deactivation.

2.3. Chromatography

The chromatography employed a Hewlett-Packard 1050 chromatogragh with a Hewlett-Packard Model

486 UV detector operating at 280 nm (Palo Alto, CA, USA). Typically 50–100 μl of urine was injected on to a 250×4.6 mm Waters Spherisorb ODS-2 column (Milford, MA, USA). The flow-rate was 0.8 ml/min with isocratic elution for 5 min of 100% D_2O containing 0.1% trifluoroacetic acid-d_3 followed by a linear gradient to 50% D_2O, 50% acetonitrile over 50 min.

2.4. NMR

All NMR spectra were measured at 298 K using a Bruker DMX-500 spectrometer (Billerica, MA, USA) operating at 500.13 MHz for ^1H and 470.59 MHz for ^{19}F detection. ^{19}F NMR spectra were measured on whole urine samples using a Nalorac 5-mm dual $^{19}F/^1H$ probe with broadband ^1H decoupling by the Waltz method. The spectral width was 11364 Hz and, typically, 128 transients were collected using 60° pulses acquiring data into 32 768 points giving an acquisition time of 1.44 s. A relaxation delay of 1.5 s was added to minimise saturation effects. The data were multiplied by a line-broadening function of 1 Hz to improve the signal-to-noise ratio and zero-filled by a factor of 2 before Fourier transformation. ^{19}F NMR chemical shifts were referenced to trifluoroacetic acid at −78.5 ppm.

Continuous-flow ^{19}F LC–NMR spectra were acquired using a Bruker 3-mm dual $^{19}F/^1H$ flow probe with a 60 μl active volume. The spectra were acquired for 16 transients using 60° pulses into 8192 data points over a spectral width of 11 364 Hz giving an acquisition time of 0.36 s. A relaxation delay of 0.64 s was added to give a total acquisition time for each spectrum of 16 s. The data were multiplied by a line-broadening function of 3 Hz to improve the signal-to-noise ratio and zero-filled by a factor of 2 before Fourier transformation in the F2 domain. The results are presented as a contour plot of intensity with ^{19}F NMR chemical shift on the horizontal axis and chromatographic retention time on the vertical axis.

Stopped-flow ^1H LC–NMR spectra were measured at retention times obtained from the continuous-flow ^{19}F LC–NMR data using a Bruker 4-mm dual $^1H/^{13}C$ flow probe with an active volume of 120 μl. The spectra were acquired for 8–12 000 transients with double presaturation of residual water and acetonitrile using the F1 and F2 channels of the spectrometer. A composite 90° observe pulse $(\pi/2)_y-(\pi/2)_{-x}-(\pi/2)_{-y}-(\pi/2)_x$ was employed to compensate for anomalies due to inhomogeneity of the radio-frequency field across the sample volume. Spectra were collected into 32 768 data points over a width of 12 019 Hz giving an acquisition time of 1.36 s, with an additional relaxation delay of 1.5 s. The data were multiplied by a line-broadening function of 1 Hz to improve the signal-to-noise ratio and zero-filled by a factor of 2 before Fourier transformation. ^1H NMR spectra of selected whole urine samples were also measured using the same conditions. On selected HPLC peaks, $^1H-^1H$ stopped-flow total correlation (TOCSY) two-dimensional NMR spectra were measured using an acquisition time of 211 ms with a spectral width of 4854 Hz and the same saturation method as described above. The data table was 2K complex points in the acquisition domain (F_2) with 256 increments in the orthogonal domain (F_1). The data were acquired in the phase-sensitive mode with TPPI (time proportional phase incrementation). The spin-lock period was 50 ms and presaturation was applied for 1.5 s. The data were zero-filled to 1K points in F_1 and apodised using a cosine squared function in F_1 and F_2 prior to the double Fourier transformation.

2.5. LC–NMR–MS

LC–NMR–MS studies were carried out using a Finnigan LCQ mass spectrometer (San Jose, CA, USA) directly coupled to the LC–NMR system. This was achieved by splitting the HPLC eluent in a ratio of 20:1 with splitter from LC Packings (San Francisco, CA, USA) and directing the major fraction to the NMR flow probe and the minor portion to the MS system. The transfer lines were calibrated to have the sample reach the NMR and the mass spectrometer at the same time, 31 s after reaching the UV detector. Mass spectra were obtained using electrospray ionisation in positive ion mode scanning a range from 50 to 150 Da. In addition, MS–MS experiments were performed on selected ions scanning a range from 40 to 150 Da.

3. Results and discussion

Urine samples from HIV-infected clinical trial volunteers dosed with BW935U83 (1) were analysed by ^{19}F NMR spectroscopy in order to study its metabolic profile and to provide confirmation of quantitative results obtained by HPLC methods. These studies are in agreement with HPLC analyses, which indicated that BW935U83 was eliminated as parent drug and a glucuronide conjugate (2) as a significant metabolite (Fig. 1). However, ^{19}F NMR spectroscopy also showed the presence of a third fluorinated peak. The identity and quantity of this metabolite was of particular interest because it helped to account for a significant fraction of the drug dose.

Elucidation of the unknown metabolite's structure was greatly simplified by taking advantage of the presence of fluorine in the molecule. The ^{1}H NMR spectrum of urine from HIV infected clinical trial volunteers after a single oral 1000 mg dose of BW935U83(1) is shown in Fig. 2a, and illustrates the difficulty of detecting drug metabolite resonances in the presence of the many endogenous species in human urine. However, a remarkable degree of simplification is possible by measuring the ^{19}F NMR spectrum of the same urine, as shown in Fig. 2a where only compounds containing fluorine appear. Since there are no endogenous fluorine containing compounds in the control urines examined these peaks must be from drug-related substances.

The two large peaks in the ^{19}F NMR spectrum are the parent drug at −178.10 ppm and its major metabolite the glucuronide conjugate at −176.90 ppm. The third peak observed in the urine at −191.25 ppm represents a metabolite which had not been detected previously. The average relative abundance of this metabolite, determined from integration of the ^{19}F NMR spectra is 12%. This metabolite was also observed at this level in samples which had not undergone heat inactivation and is therefore not an artefact of sample processing.

The pseudo-two dimensional contour plot of the continuous-flow ^{19}F NMR spectra (Fig. 3) shows

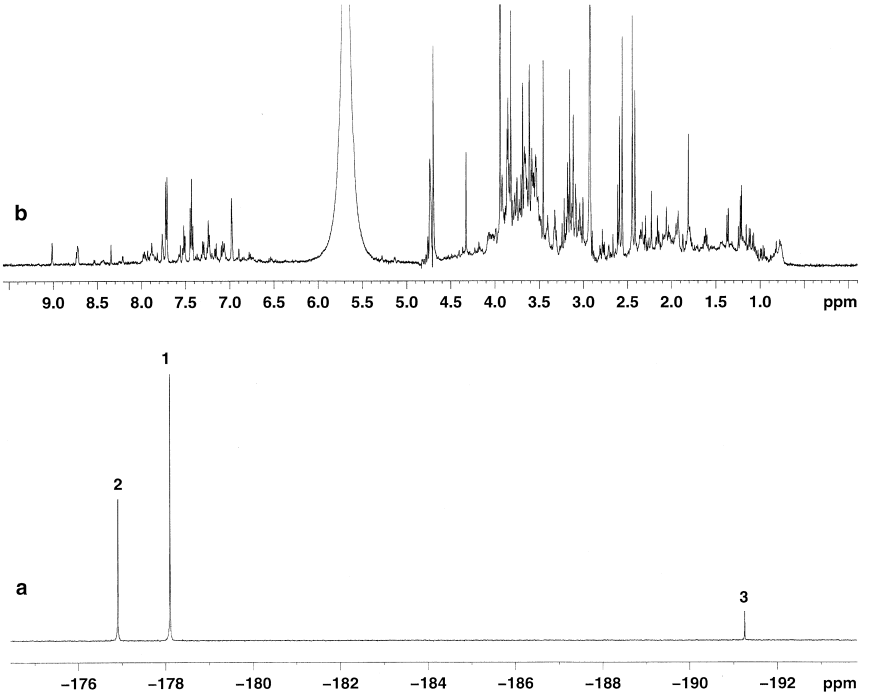

Fig. 2. (a) The ^{19}F NMR spectrum of a sample of human urine collected from 0 to 4 h after dosing with 1000 mg BW935U83. (b) The corresponding ^{1}H NMR spectrum of the same sample.

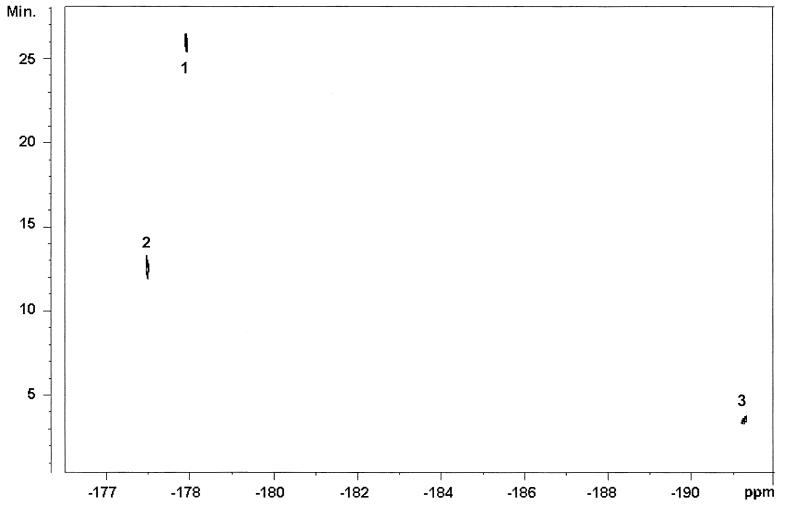

Fig. 3. Pseudo-two dimensional contour plot of the continuous-flow ^{19}F NMR experiment obtained on a 50-μl injection of human urine collected from 0 to 4 h after a 1000-mg dose of BW935U83. The horizontal axis is ^{19}F chemical shift and the vertical axis is HPLC retention time.

responses for the three resonances and provides their retention times on the vertical axis. The retention times for BW935U83, its glucuronide conjugate, and the unknown metabolite are 27.5, 12.2, and 3.5 min, respectively. Fig. 4 shows the UV chromatogram obtained during this experiment.

Knowing the retention times for the components of interest it was then possible to obtain ^{1}H LC–NMR spectra in stop-flow mode. The assigned ^{1}H LC–NMR spectrum of the peak at 27.5 min is shown in Fig. 5a and is consistent with that of BW935U83 (1). The 3′ proton is assigned to the doublet centered at 5.31 ppm and displays a characteristic 53.2 Hz $^{2}J_{HF}$ coupling. The chromatographic peak at 12.2 min gives rise to the stopped-flow ^{1}H LC–NMR spectrum shown in Fig. 5b and is consistent with the glucuronide conjugate of BW935U83 (2). The resonances arising from the BW935U83 moiety and the

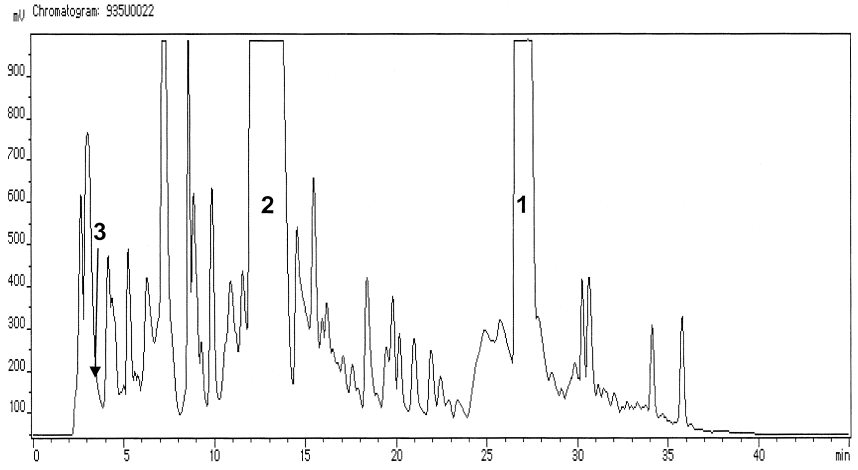

Fig. 4. HPLC chromatogram obtained on 50 μl injection of human urine collected from 0 to 4 h after a 1000-mg dose of BW935U83. The location for BW935U83 (1), its glucuronide conjugate (2), and 3-fluoro-ribolactone (3) are indicated.

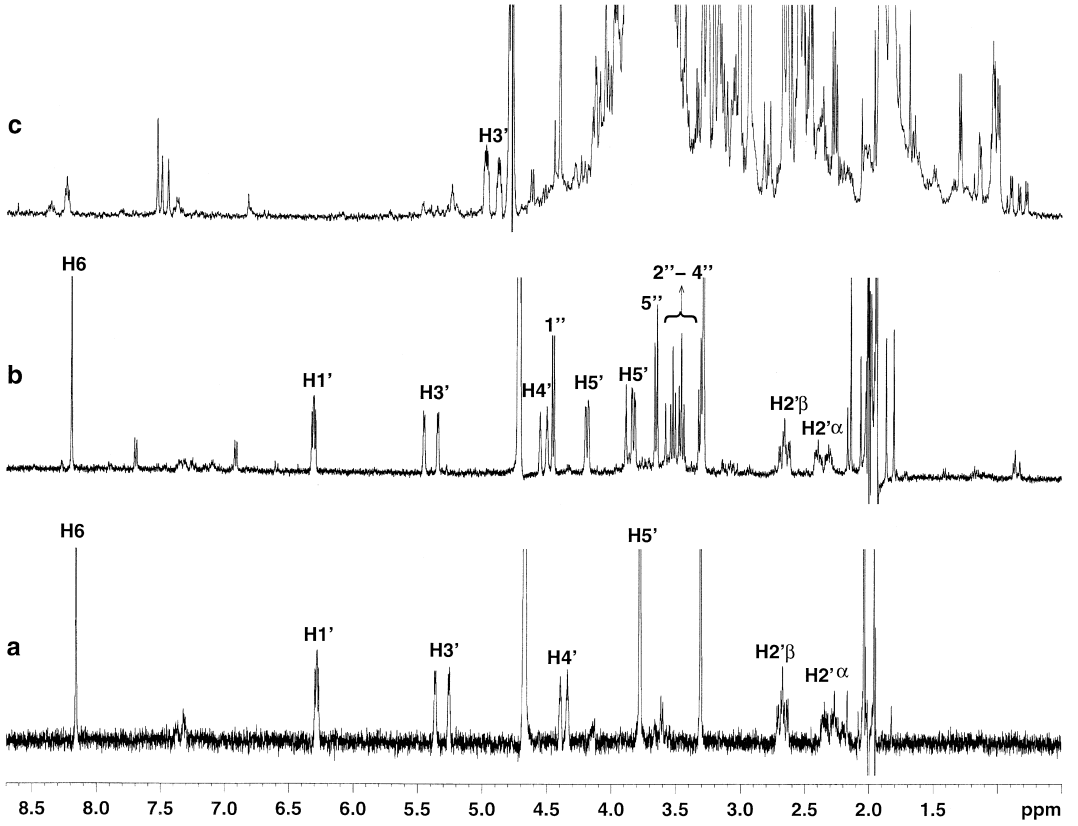

Fig. 5. The stopped-flow ^{1}H LC–NMR–MS spectra of (a) BW935U93, (b) its glucuronide conjugate and (c) the metabolite eluting at 3.5 min, obtained from a 100-μl injection of human urine collected from 0 to 4 h after dosing with 1000 mg BW935U83. The spectrum of (1) was collected in eight scans, (2) in 32 scans, and (3) in 12 000 scans illustrating the relative amounts of the three metabolites.

additional resonances consistent with ether glucuronidation are clearly seen. The chemical shift of the 1″ anomeric proton of the glucuronide conjugate at 4.45 ppm and the altered chemical shifts of the H5′ methylene protons of the furanosyl moiety support glucuronidation at the 5′ position. Confirmation of these assignments is facilitated by the ^{1}H–^{1}H TOCSY spectrum shown in Fig. 6a. The 3′ proton of this major metabolite centred at 5.39 ppm shows a $^{2}J_{HF}$ coupling of 52.8 Hz. The full NMR assignments for both BW935U83 and its glucuronide metabolite are given in Table 1.

The ^{1}H LC–NMR spectrum of the peak, which has a retention time of 3.5 min, is shown in Fig. 5c. The complexity of this spectrum arises from the numerous endogenous polar molecules that co-elute with the metabolite. However, by comparison of this spectrum with the spectra of BW935U83 and the glucuronide conjugate shows the loss of the H6 proton and the presence of a doublet at 4.91 ppm with a 47.3 Hz coupling similar to the coupling observed for $^{2}J_{HF}$ in both the parent and glucuronide (Fig. 5a,b). The ^{1}H–^{1}H TOCSY spectrum of this peak is shown in Fig. 6b for comparison with that from the glucuronide metabolite. There is a similarity in the coupling patterns of the spectra from the H3′ protons to the H2′ protons but no further insight into the structure of the unknown could be extracted from the data because of interference from resonances arising from the co-eluting endogenous material in the urine. Measurement of an ^{1}H-coupled ^{19}F NMR spectrum of the urine allowed the coupling

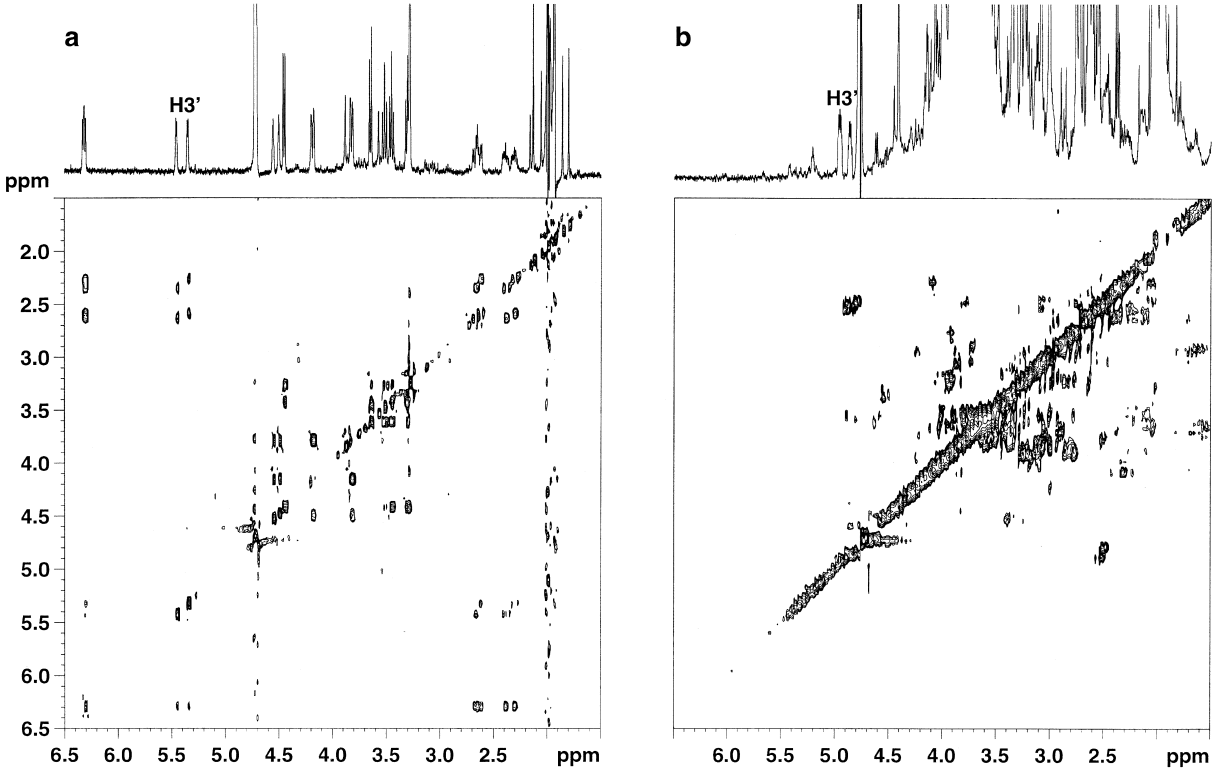

Fig. 6. The stopped-flow ^{1}H–^{1}H TOCSY LC–NMR–MS spectra of (a) the glucuronide conjugate and (b) the early eluting metabolite obtained from a 100-μl injection of human urine collected from 0 to 4 h after dosing with 1000 mg BW935U83. The spectrum of (2) was collected in 3 h and (3) in 16 h.

constants from the ^{19}F nucleus to various protons in the molecules to be determined. The largest coupling observed for this minor metabolite is assigned as $^{2}J_{FH,}$ with an observed coupling of 47.3 Hz. This is identical to the $^{2}J_{HF}$ coupling observed in the ^{1}H spectrum. These observations support the assignment of the doublet at 4.91 ppm as the H3′ proton of the minor metabolite. Analysis of all the observed couplings for the minor metabolite showed coupling constants similar to those observed in the parent and glucuronide conjugates and suggests that the CH–CHF–CH_2 fragment is intact (Table 1).

With the NMR data obtained thus far, it can be concluded that this early eluting substance was the furanosyl portion of the nucleoside analogue cleaved from the base or a derivative of it. In order to obtain additional structural information on this substance, a mass spectrometer was directly coupled to the LC–NMR system. This LC–NMR–MS configuration allowed the acquisition of mass spectral data on the chromatographic peak concurrent with collection of the NMR data. Because the metabolite had no UV chromophore, the the LC–NMR–MS was configured with transfer lines calibrated to allow the peaks to enter the NMR and mass spectrometer at the same time. With the NMR configured to observe ^{19}F, it was possible to observe a free induction decay (FID) as the metabolite entered the NMR, thus ensuring that the mass spectral data was being collected on the correct component.

The mass spectrum of the metabolite shows a molecular ion $(M+D)^{+}$ at $m/z=137$ Da (C5H6O3FD) for the fully deuterated species since D_2O was used in the HPLC eluent (Fig. 7a). This ion lost D_2O and exchanged zero, one or two hydrogens with deuterium to give 117, 118 and 119 fragments.

Table 1
NMR parameters for BW935U83 and its metabolites[a]

Parameter	BW935U83 (1)	(2)	(3)
δ(H6)	8.16	8.19	–
δ(H1′)	6.28	6.31	–
δ(H2α)	2.30	2.35	2.4?
δ(H2′β)	2.67	2.65	2.4?
δ(H3′)	5.31	5.39	4.91
δ(H4′)	4.36	4.53	–
δ(H5′a)	3.77	3.82	–
δ(H5′b)	3.77	4.19	–
δ(H1″)	–	4.45	–
δ(H2″)	–	3.30	–
δ(H3″)	–	3.52	–
δ(H4″)	–	3.45	–
δ(H5″)	–	3.65	–
δ(F)	–178.1	−176.90	−191.25
$^2J_{F',H3'}$ $^2J_{H3',F'}$	53.2	52.8	47.3
$^3J_{F,H2'\alpha}$	38.7	40.5	40.0
$^3J_{F,H2'\beta}$	22.2	22.4	16.4
$^3J_{F,H4'}$ $^3J_{H4',F}$	27.2	27.3	16.4

[a] ^{1}H NMR chemical shifts are relative to TMS at 0.0 ppm. ^{19}F NMR chemical shifts are relative to trifluoroacetic acid at −78.5 ppm. Coupling constants are in Hertz.

The MS–MS spectrum of the $m/z=119$ ion (Fig. 7b) shows the loss of CO to give the 91 fragment with further fragmentation to C_2D_2F at $m/z=47$.

The NMR and mass spectral data are consistent with the proposed, 3-fluoro-ribolactone (3), as the structure of the early eluting peak. The presence of the resonance arising from this component in ^{19}F NMR spectra obtained on both heated-inactivated and untreated urine would suggest it is a metabolite and not a degradation product. A proposed mechanism for formation of this metabolite is shown in Fig. 8. The initial step is cytochrome P450 mediated oxidation at the 1′ position, with subsequent cleavage of the nucleoside–sugar bond to yield the proposed metabolite. The nucleoside portion of the molecule is difficult to detect because it undergoes classic pyrimidine base catabolism to chloro-β-alanine, CO_2 and NH_3 [7].

4. Conclusions

In a conventional metabolism study, extensive work involving radiolabeled drug with labels in both the base and furanosyl portions of the molecule would be required to identify and quantify this unknown metabolite. This work demonstrates the utility and efficiency of the direct coupling of both NMR and MS to an HPLC separation stage. The use of ^{19}F NMR spectroscopy highlighted the presence of a significant metabolite which could not be detected by radiolabelling (because of loss of the label) or by conventional LC–MS because it has no UV chromophore and there was significant chemical noise in the early portion of the chromatogram. In this application of directly coupled LC–NMR–MS the NMR provided the information critical to the timing of the experiment by exploiting ^{19}F NMR detection. However, by configuring the system to have the sample reach the mass spectrometer before it reaches the NMR flow cell the mass spectrometer can be exploited as an experimental control device for analysing complex mixtures. Mass spectrometry is an ideal detector, provided the molecules of interest are ionisable. It provides data rapidly and can thus yield valuable information on parent or daughter ion mass prior to initiating time consuming stop-flow LC–NMR experiments. This synergy is not possible when the instruments are not directly coupled.

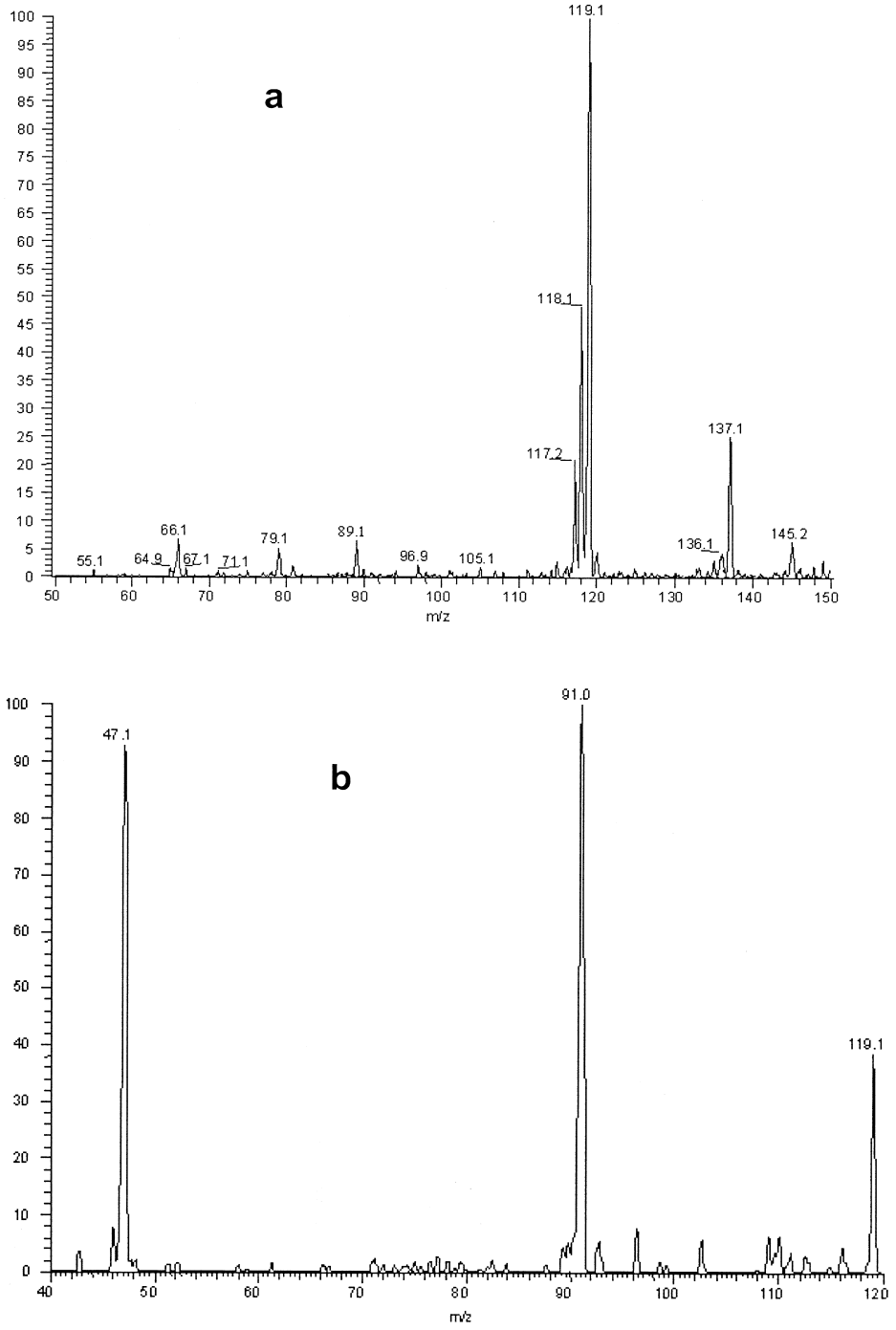

Fig. 7. Directly coupled LC–NMR–MS results on the early eluting metabolite (3). (a) Positive ion electrospray mass spectrum, (b) MS–MS of the $m/z = 119$ ion.

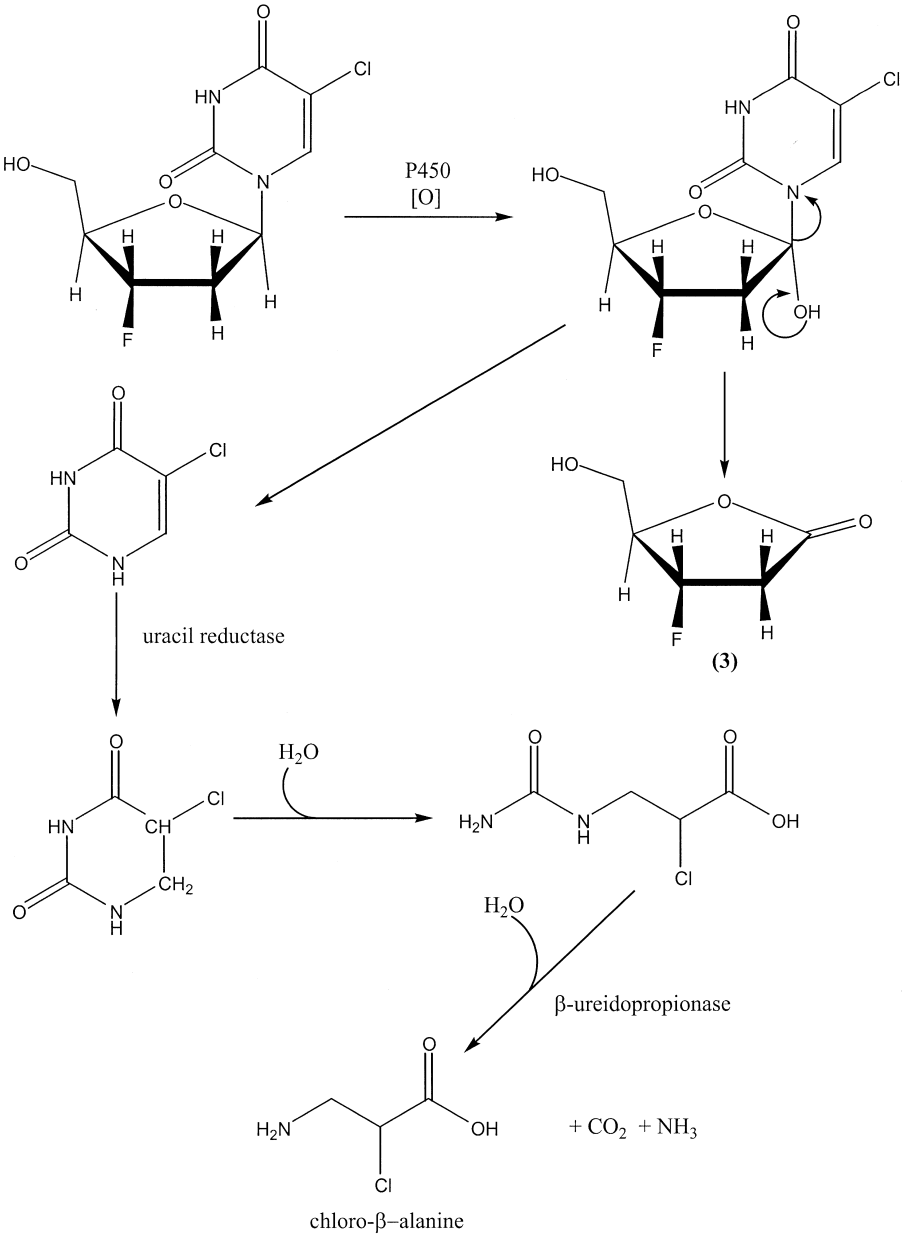

Fig. 8. Proposed mechanism for the formation of the 3-fluoro-ribolactone (3) and catabolism chlorouracil to chloro-β-alanine.

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