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Analysis of the hypoxia-activated dinitrobenzamide mustard phosphate pre-prodrug PR-104 and its alcohol metabolite PR-104A in plasma and tissues by liquid chromatography–mass spectrometry

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Abstract

PR-104 is a dinitrobenzamide mustard pre-prodrug that is activated by reduction to a cytotoxic hydroxylamine metabolite in hypoxic tumour cells; it has recently commenced Phase I clinical trial. Here, we report two validated methods for the determination of PR-104 and its alcohol hydrolysis product, PR-104A in plasma and tissues across species. A high pH LC/MS/MS method was optimised for rapid and sensitive analysis of both analytes in rat, dog and human plasma. This assay was linear over the concentration range 0.005–2.5 μg/ml for PR-104 and 0.05–25 μg/ml for PR-104A (0.005–2.5 μg/ml for rat). A second method, using a low pH LC separation, was designed to provide higher chromatographic resolution, facilitating identification of metabolites. Both methods were successfully applied to the plasma pharmacokinetics of PR-104 and PR-104A in rats. In addition, cytotoxic reduced metabolites of PR-104A were identified in human tumour xenografts by the higher chromatographic resolution method.

Keywords: Nitrogen mustards; Prodrugs; PR-104; PR-104A; Metabolite identification

1. Introduction

Regions of very low oxygen levels (hypoxia) are unique features of solid tumours [1–3] and confer resistance to radiotherapy [4–6] and chemotherapy [7–9]. This has led to the development of strategies to exploit hypoxia as a basis for tumour selectivity, particularly the development of drugs that are toxic only under hypoxic conditions [10]. The dinitrobenzamide mustard (DNBM) prodrugs provide an opportunity to explore this strategy. DNBM prodrugs contain a latent nitrogen mustard moiety which becomes activated when either of the nitro groups is reduced to the corresponding hydroxylamine or amine [11]. This provides an "electronic switch" to activate the nitrogen mustard selectively in hypoxic cells, resulting in hypoxia-selective cytotoxicity [12,13]. PR-104 is a water-soluble phosphate "pre-prodrug" that is converted efficiently to the more lipophilic DNBM alcohol PR-104A (Fig. 1A), which is a hypoxia-selective DNA cross-linking agent and cytotoxin [14]. PR-104 is currently in phase I clinical trial, which commenced in January 2006.

In order to assist pharmacokinetic evaluation of this new drug during toxicological and clinical studies, we developed two methods for simultaneous bioanalysis of both PR-104 and PR-104A. One method was optimised for sensitive and rapid quantitation of PR-104 and PR-104A, using a high pH, low chromatographic resolution HPLC method with triple quadrupole (LC/MS/MS) tandem mass spectrometry. The second method was optimised for separation of metabolites using a high chromatographic resolution HPLC method which can be interfaced with a variety of detectors (photodiode array, single stage or tandem MS). We report validated assays for both methods here, together with their application for pharmacokinetic analysis.

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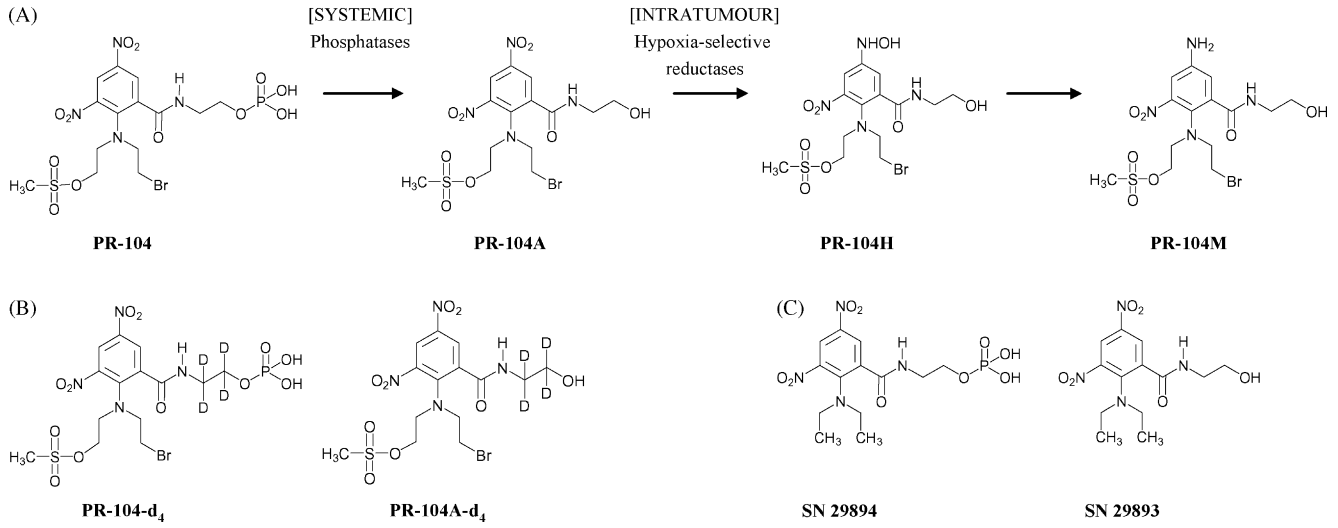

Fig. 1. Chemical structures of the water-soluble phosphate pre-prodrug PR-104, its more lipophilic alcohol metabolite PR-104A, and hydroxylamine (PR-104H) and amine (PR-104M) cytotoxins resulting from its metabolic reduction (A), tetra-deuterated stable isotope standards, PR-104-d_4 and PR-104A-d_4 (B) and diethyl analogue internal standards, SN 29894 and SN 29893 (C).

2. Experimental

2.1. Chemicals and reagents

PR-104 (2-((2-bromoethyl)-2-{[(2-hydroxyethyl) amino] carbonyl}-4, 6-dinitroanalino) ethyl methanesulfonate phosphate ester, was synthesized as the free acid from PR-104A [15]. The batches used in this study varied in purity from 96 to 98% for PR-104 and 96–100% for PR-104A, based on HPLC with absorbance detection (254 nm). The analogue internal standards SN 29894 (99% purity) and SN 29893 (100% purity) were synthesized in this laboratory. Tetra-deuterated stable isotope standards of PR-104 (PR-104-d_4) and PR-104A (PR-104A-d_4) were prepared as described elsewhere [16]. The chemical structures of PR-104, PR-104A and all internal standard compounds are shown in Fig. 1. Unless otherwise stated, all other chemicals were commercially available and of analytical grade. Acetonitrile and formic acid were purchased from BDH (Poole, UK). Glacial acetic acid, ammonium hydroxide and phosphoric acid (85%, *v/v*) were obtained from EM Science (Gibbstown, New Jersey, USA). Methanol was purchased from Burdick & Jackson (Muskegon, Michigan, USA) and ammonium acetate from Mallinckrodt (Paris, Kentucky, USA). EDTA anticoagulant from BioChemed (Winchester, Virginia, USA) was used for studies with whole blood and for preparation of plasma.

2.2. Preparation of standards and quality control samples

Stock solutions of PR-104 (0.1 mg/ml), PR-104A (1 mg/ml) and internal standards (10–100 μg/ml) were prepared by dissolving individually weighed samples (corrected for purity) in methanol. Duplicate stock solutions were compared and considered to be of acceptable accuracy where the difference in concentrations was ≤3.0%. All stock solutions in methanol were stable for at least 336 days at −20 °C, and were stored at this temperature until use. Working standard solutions were prepared by diluting mixed individual aliquots of PR-104 and PR-104A stock solutions in methanol to 0.05, 0.1, 0.25, 0.5, 1.0, 2.5, 5.0, 10, 25, 50, 100 and 250 μg/ml of each analyte. Each of these (0.50 ml) was spiked into 4.5 ml precipitated pooled blank rat, dog or human plasma, to obtain calibration curve standards ranging from 0.005 to 25 μg/ml for LC/MS/MS analysis. For the LC/MS assay, working standard solutions were prepared as described above to 1.7, 5.8, 17.3, 57.9, 173 and 579 μg/ml (PR-104) and 1.5, 5.0, 15.0, 49.9, 150 and 499 μg/ml (PR-104A). Calibration curve standards for PR-104 (0.17–57.9 μg/ml) and PR-104A (0.15–49.9 μg/ml) were obtained by spiking 100 μl of working stock solutions into 900 μl of pooled blank plasma or tissue homogenate. All calibration curve standards were prepared on ice and in triplicate on each day of analysis. Quality control (QC) samples were prepared as a single batch in the same way as described above and stored at −70 °C. Rat, dog and human plasma QC samples contained PR-104 at 0.015, 0.075 and 2.00 μg/ml, and PR-104A at 0.15, 0.75 and 20.0 μg/ml (0.015, 0.075 and 2.00 μg/ml for rat). Mouse plasma and tissue QC samples were prepared at 0.58, 5.79 and 57.9 μg/ml (PR-104) and 0.50, 4.99 and 49.9 μg/ml (PR-104A). All QC samples contained >98% biological content.

2.3. Extraction procedures and sample preparation

2.3.1. Plasma extraction

Blood was collected by cardiac puncture under terminal CO_2 anaesthesia in mice; serially from the jugular vein in rats and dogs; and by standard phlebotomy procedures in humans. All samples (0.5–2.5 ml) were placed into chilled tubes containing EDTA as the anticoagulant, and plasma immediately prepared by centrifugation (2500–3000 × *g* for 3 min). The solvent used for PR-104 and PR-104A extraction from plasma consisted of methanol: ammonium acetate: acetic acid (1000: 3.5: 0.2,

v/w/v). At the time of collection plasma samples (100 μl) were precipitated immediately on ice with 900 μl cold extraction solvent. Samples were then vortex mixed for 30 s and cooled on ice. For human plasma, tetra-deuterated isotope standards (10 μl; 10 μg/ml stock) were added into 200 μl of each deproteinized sample, to final concentrations of 500 ng/ml PR-104-d_4 and 5000 ng/ml PR-104A-d_4. Diethyl analogue internal standards (2 μl; 10 μg/ml stock) were added to the same volume of precipitated rat and dog plasma, to final concentrations of 100 ng/ml SN 29894 and 100 ng/ml SN 29893. Deproteinized plasma samples were then filtered through a Millipore hydrophobic PFTE plate for analysis by LC/MS/MS. For the mouse studies and one rat study (Fig. 4B), 10 μl plasma was extracted with 30 μl methanol containing 2.3 μg/ml (PR-104-d_4) and 2.0 μg/ml (PR-104A-d_4). For these samples, particulates were removed from the final extracts by centrifugation rather than filtration and analyzed by LC/MS.

2.3.2. Tumour, liver and brain extraction

Mouse tumour, liver and brain tissue samples were obtained by dissection from untreated specific pathogen-free homozygous *nu/nu* (CD-1) mice, and immediately frozen in liquid nitrogen. These were then pulverized at liquid nitrogen temperature using a BioPulverizer™ (BioSpec Products, USA). The resulting tissue powder was transferred to tared 1.5 ml microcentrifuge tubes and weighed. An equal volume of cold phosphate buffered saline (PBS) was added to each tube and vortex mixed to prepare tissue homogenates. Aliquots (100 μl) of each tissue homogenate were deproteinized with three volumes of cold methanol containing PR-104-d_4 (2.3 μg/ml) and PR-104A-d_4 (2.0 μg/ml) internal standards. Samples were then vortex mixed (1 min), stored at −80 °C (60 min), and centrifuged at 13,000 *g* (10 min, 4 °C). A 35 μl aliquot of each precipitated supernatant was then diluted in 75 μl of 45 mM ammonium formate buffer (pH 4.5) for analysis.

2.4. Instrumentation

2.4.1. Rapid LC separation for tandem MS

Chromatographic analysis was performed using an Agilent 1100 HPLC system with a LEAP CTC autosampler. Separation was achieved by automated injection of 20 μl samples onto a reversed phase analytical column (Waters XTerra Phenyl column, 150 mm × 2.1 mm 5 μm packing). The isocratic mobile phase (0.3 ml/min) was a mixture of ammonium acetate solution (26.0 mM) pH 9.5: methanol (48:52, v/v). Total run time was 7.5 min. The HPLC was connected to a Micromass Ultima MS/MS system operating in the electrospray negative ionization mode. Source temperature was 130 °C. Nitrogen was used as the nebuliser gas, cone gas with argon as collision gas. Multiple reaction monitoring (MRM) was used to monitor the following ion transitions: PR-104 (578 > 400), PR-104-d_4 (582 > 404), PR-104A (498 > 95), PR-104A-d_4 (502 > 95), SN29893 (324 > 86), SN29894 (404 > 97). Data were collected using an IBM operating system, and analyzed using Masslynx software.

2.4.2. Higher chromatographic resolution method for metabolite separation

The high pH mobile phase LC/MS/MS method described above for quantitation of PR-104 and PR-104A intentionally uses a short cycle time (7.5 min) and provides little chromatographic resolution of metabolites. A low pH LC method (also suitable for quantitation of PR-104 and PR-104A) was therefore developed to optimise metabolite resolution. Chromatographic separation was achieved on an Alltima C8 HPLC analytical column (150 mm × 2.1 mm I.D; Alltech Associates Inc., Chicago, IL) with 5 μm particles) at 25 °C. Mobile phase (A) consisted of acetonitrile in water (80:20, v/v); mobile phase (B) was 45 mM ammonium formate buffer (pH 4.5). Linear gradient elution was employed with a 40 min run-time at a flow rate of 0.3 ml/min. The gradient was: 20% of (A) initially (4 min), then increasing linearly to 90% (A) over 21 min, which was held for a further 1 min, with return to the initial conditions over 4 min. This was maintained for 10 min before the next injection. Absorbance detection was at 370 nm (bandwidth 4 nm).

For LC/MS analysis, the single stage quadrupole MS system was an Agilent MSD model interfaced with an Agilent 1100 LC having a refrigerated autosampler. The electrospray ionisation (ESI) source was set at positive ionization mode, and $[M–H]^+$ ions of PR-104 (*m/z* 579), PR-104A (*m/z* 499), PR-104-d_4 (*m/z* 585) and PR-104A-d_4 (*m/z* 505) were detected using selected ion monitoring (SIM). The MS operating conditions were optimised as follows: capillary voltage 3.5 kV, nebuliser pressure 45 psi, fragmentation voltage 100 V, gas temperature 350 °C, drying gas flow 12 l/min. Data was collected and analysed with HP Chemstation Software, version B.01.01.

For identification of metabolites, a single quadrupole LC/MS as described above and Agilent LC/MSD Trap-SL equipped with Agilent capillary HPLC were used. Chromatographic separation was achieved on a Zorbax SB C18 capillary column (150 mm × 0.5 mm, 5 μ) at a flow rate of 15 μl/min. Mobile phase (A) consisted of acetonitrile in water (80:20, v/v); mobile phase (B) was 5 mM ammonium formate buffer (pH 4.5). The gradient was: 20% of (A) initially (4 min), then increasing linearly to 90% (A) over 19 min, which was held for a further 4 min, with return to the initial conditions over 3 min. This was maintained for 10 min before the next injection. The electrospray ionisation source was set at positive ionization mode and Auto MS(n), other parameters were as follows: capillary voltage 4.5 kV, nebuliser pressure 12 psi, gas temperature 325 °C, drying gas flow 4.4 l/min.

2.4.3. Calibration curves

For analysis by each method, the ratio of peak height (LC/MS/MS) and peak area (LC/MS) of PR-104 or PR-104A analyte to that of the respective internal standard was calculated. Calibration curves were then obtained by plotting calculated ratios against spiked analyte concentrations. A power regression analysis was employed to determine the slope and intercept. These parameters were then used to compute concentrations for QC and unknown samples by interpolation.

2.5. Validation procedures

Analytical specificity was tested by inspection of chromatograms of extracted drug-free plasma samples for interfering peaks. Recovery was assessed by comparing back-calculated concentrations of PR-104 and PR-104A from extracted plasma and tissue QC samples, to standards prepared in blank matrix extract. All recovery studies were performed at three different concentrations and in triplicate. To determine intra-day reproducibility 5–6 replicate QC samples were analysed at four different concentrations, including at the lower limit of quantitation (LLOQ). Inter-day reproducibility was calculated from QC samples analysed on three or more different days. At each concentration, precision was calculated as the relative standard deviation (RSD) and accuracy as percent deviation (DEV). Acceptable precision and accuracy was defined by a RSD within 15.0% and DEV within ±15.0%. The LLOQ was defined to be the lowest concentration within the calibration curve linear range that had a DEV within ±20.0% and RSD within 20.0%. Matrix effects on each analyte were assessed in triplicate by spiking different batches of extracted plasma at the LLOQ (mouse, dog and human), and at an intermediate concentration (0.25 μg/ml) in rat. The matrix effect was acceptable where DEV within ±15.0% were obtained. Each analytical run consisted of a single calibration curve, triplicate QC samples at three concentration levels, one reagent blank, one plasma or tissue blank and one zero-level standard.

The stability of PR-104 and PR-104A was measured in duplicate at 37 °C over 60 min in blood containing EDTA as the anticoagulant. At each timepoint, blood samples, spiked with PR-104 (57.9 μg/ml) or PR-104A (49.9 μg/ml) were removed, chilled and centrifuged immediately (3000 × *g* for 3 min) to prepare plasma. These were then extracted as described above. In addition, the stability of PR-104 and PR-104A in plasma was assessed on ice at three different concentrations in triplicate on the benchtop over 3–24 h. Autosampler stability was determined at three different concentrations in triplicate in plasma and tissues over 36–120 h at 5 °C. Three freeze–thaw cycles at −70 °C were used to test the stability of PR-104 and PR-104A in plasma. Long-term plasma stability was assessed at −70 °C over 27–95 days. For short-term, long-term and freeze–thaw stability, mean concentrations of triplicate samples were compared to that of the initial values.

2.6. Application to pharmacokinetic evaluation

Two intravenous pharmacokinetic studies of PR-104 were undertaken in female Sprague–Dawley rats. In the first study, PR-104 was administered at 225 mg/kg intravenously via the tail vein as a fast bolus injection, using animals with body weights 201–251 g. Blood samples were collected into EDTA tubes at specified times from three rats per timepoint following dosing of PR-104. EDTA-plasma samples were then analysed for PR-104 and PR-104A by the high pH LC/MS/MS method described above. An independent study was conducted in a different laboratory with intravenous administration of PR-104 at 243 mg/kg to rats (body weights 180–220 g), and subsequent analysis of EDTA-plasma samples by the low pH LC/MS method. Non-compartmental pharmacokinetic parameters were estimated using WINNONLIN version 4.0.1; AUC values were calculated using the log trapezoidal rule with extrapolation of the terminal slope to infinity by linear regression.

2.7. Application to metabolite identification

Female mice (three animals/group, body weight 24–34 g) were dosed with PR-104 (326 mg/kg) intravenously via the tail vein as a fast bolus injection. Blood was collected by cardiac puncture under terminal CO_2 anaesthesia into EDTA tubes and plasma prepared by centrifugation (3000 × g for 3 min). Mouse tumour, liver and brain samples were obtained by dissection and snap-frozen immediately in liquid nitrogen. The low pH LC/MS

Table 1
Calibration curve data showing mean accuracy (DEV) and precision (RSD) calculated at each concentration level in rat, dog and human plasma samples determined over five separate analytical runs by the high pH LC/MS/MS method

Concentration (μg/ml)	PR-104						PR-104A					
	Rat		Dog		Human		Rat		Dog		Human	
	RSD (%)	DEV (%)	RSD (%)	DEV (%)	RSD (%)	DEV (%)	RSD (%)	DEV (%)	RSD (%)	DEV (%)	RSD (%)	DEV (%)
0.005	4.9	3.0	6.7	3.8	5.4	6.0	3.7	−1.4	BLR[a]	BLR	BLR	BLR
0.010	3.5	1.0	4.9	−0.5	4.8	0.0	2.8	−0.3	BLR	BLR	BLR	BLR
0.025	1.9	−1.2	4.1	−2.4	4.3	−2.4	3.3	−1.6	BLR	BLR	BLR	BLR
0.050	2.2	−2.6	4.1	−0.4	4.4	−4.0	3.7	1.2	2.7	−9.6	1.6	1.4
0.10	3.7	0.3	2.3	−0.4	2.7	−2.3	1.3	1.0	3.0	−1.7	2.1	−0.5
0.25	1.6	−2.2	2.3	−2.3	5.3	−2.2	3.6	2.6	2.8	4.0	1.3	2.0
0.50	2.3	−1.5	1.5	−1.4	3.6	1.2	1.6	3.5	1.3	7.2	3.1	−3.7
1.00	2.9	−2.6	1.9	1.6	2.2	3.7	2.1	−0.8	3.5	7.0	1.5	−0.2
2.50	2.7	6.1	3.0	2.3	4.4	0.9	3.9	−3.7	3.7	5.2	0.2	0.2
5.00	ALR[b]	ALR	ALR	ALR	ALR	ALR	ALR	ALR	1.5	−0.8	2.9	−0.5
10.0	ALR	ALR	ALR	ALR	ALR	ALR	ALR	ALR	4.4	−4.2	2.5	1.6
25.0	ALR	ALR	ALR	ALR	ALR	ALR	ALR	ALR	2.9	−5.3	2.2	0.1

[a] Below linear range.
[b] Above linear range.

method was then used for quantitative analysis of PR-104 and PR-104A, and for qualitative identification of metabolites in mouse plasma and tissue samples.

3. Results

3.1. Validation of the two assays

3.1.1. Linearity

For the high pH LC/MS/MS method, calibration curves obtained by plotting peak height ratios (d_0/d_4) against nominal (spiked) concentrations were linear for rat, dog and human plasma over the range 0.005–2.5 μg/ml for PR-104 and 0.05–25 μg/ml for PR-104A (0.005–2.5 μg/ml for rat). Mean accuracy and precision values at each concentration within these ranges gave RSD values within 15.0 % and DEV within ±15.0% (Table 1). Analysis of mouse and rat plasma, and mouse tissues by the less sensitive LC/MS method were linear over the ranges 0.17–57.9 μg/ml for PR-104 and 0.15–49.9 μg/ml for PR-104A. In mouse plasma and tissues mean accuracy over these concentration ranges varied from −4.4 to 4.1% for PR-104, and −2.2 to 7.9% for PR-104A. The corresponding mean precision values ranged from 0.2 to 10.8% for PR-104 and from 0.1 to 7.8% for PR-104A (data not shown). For all calibration curves, linear regression provided r^2 values > 0.99 (range = 0.9915–0.9999).

3.1.2. Specificity

To assess interference from endogenous compounds, six different batches of drug-free control rat, dog and human plasma, and two batches of blank mouse plasma and tissues were investigated. Typical mass chromatograms obtained from analysis of blank rat, dog and human plasma by LC/MS/MS are shown in Fig. 2. No chromatographic interferences (peaks >20% of the LLOQ response) were detected at the retention times of PR-104

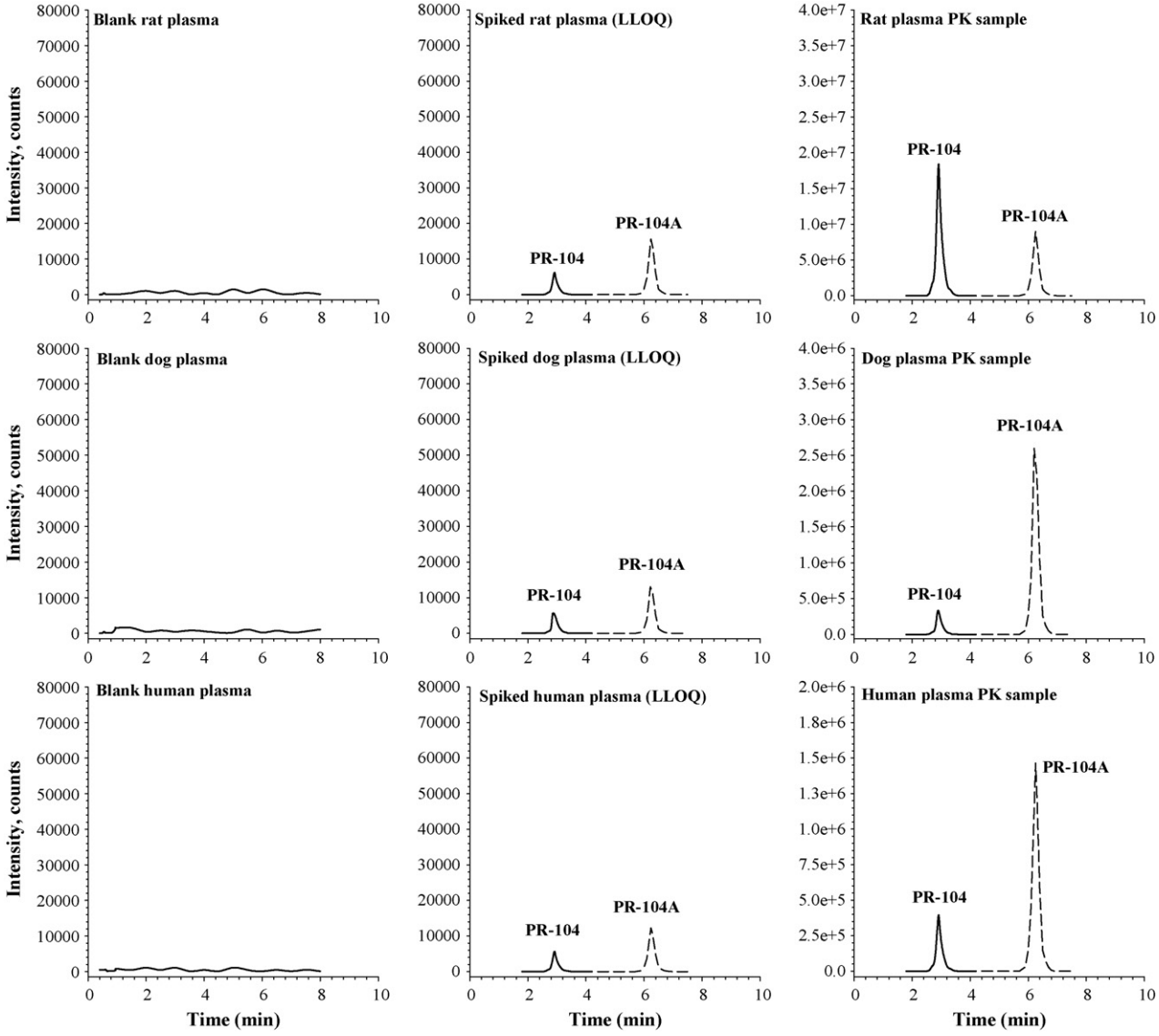

Fig. 2. Representative chromatograms from LC/MS/MS analysis of rat, dog and human plasma extracts obtained from a blank sample (left), a sample spiked at the LLOQ of PR-104 and PR-104A (middle) and select samples obtained 10 min (5 min in dogs) after intravenous administration of PR-104 (right) in rats (388 mg/kg), dogs (259 mg/kg) and humans (3.65 mg/kg). Solid lines are PR-104 (MRM: 578 > 400) and dashed lines are PR-104A (MRM: 498 > 95).

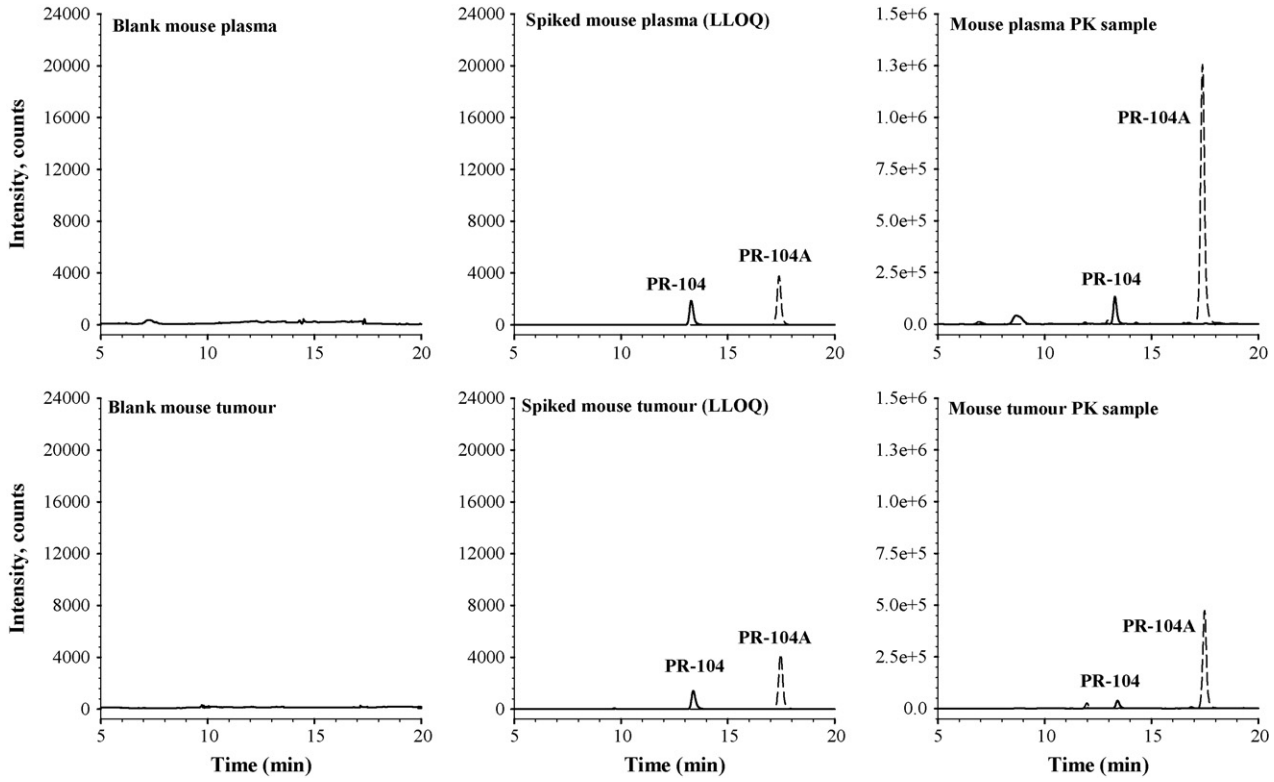

Fig. 3. Representative chromatograms from LC/MS analysis of mouse plasma and tumour extracts obtained from a blank sample (left), a sample spiked at the LLOQ of PR-104 and PR-104A (middle) and select samples obtained 15 min after intravenous administration of 326 mg/kg of PR-104 (right) in mice bearing SiHa tumours. Solid lines are PR-104 (*m/z* 579) and dashed lines are PR-104A (*m/z* 499).

and PR-104A. This was also the case for blank mouse plasma and tissues (Fig. 3) analysed by the low pH LC/MS method. Using the high pH LC/MS/MS method, the diethyl analogue internal standard SN 29894 co-eluted with PR-104 (retention time, 3.2 min). The corresponding alcohol internal standard SN 29893 eluted with PR-104A (retention time, 6.3 min). Analysis of zero-level standards gave no peaks >20% of the LLOQ response for the d_0 analytes, indicating lack of chromatographic interferences due to residual parent compound (from IS synthesis) and/or cross talk.

3.1.3. Recovery

To determine recovery, concentrations of PR-104 and PR-104A in extracted plasma and tissue QC samples were compared to standards prepared in blank matrix extract. The recovery was evaluated at three different concentrations in triplicate, in all

Table 2
Mean recoveries on extraction of PR-104 and PR-104A from triplicate mouse plasma and tissues analysed using the low pH LC/MS method

Matrix[a]	PR-104		PR-104A	
	Concentration (μg/ml)	Recovery (%)[b]	Concentration (μg/ml)	Recovery (%)
Plasma	0.579	100.6 ± 0.1	0.499	95.4 ± 2.5
	5.79	104.3 ± 0.8	4.99	94.2 ± 1.9
	57.9	101.9 ± 1.0	49.9	94.9 ± 1.8
Tumour	0.579	95.8 ± 0.4	0.499	102.7 ± 1.5
	5.79	98.3 ± 3.8	4.99	95.3 ± 3.9
	57.9	103.4 ± 3.3	49.9	94.8 ± 2.1
Liver	0.579	95.1 ± 0.8	0.499	93.1 ± 0.8
	5.79	93.1 ± 0.4	4.99	91.4 ± 1.7
	57.9	101.4 ± 4.2	49.9	101.8 ± 3.2
Brain	0.579	100.5 ± 1.6	0.499	92.2 ± 0.3
	5.79	99.1 ± 1.0	4.99	99.1 ± 1.7
	57.9	97.2 ± 3.6	49.9	94.8 ± 1.8

[a] Samples precipitated using three volumes of cold methanol.
[b] Values are mean ± SEM.

Table 3
Intra- and inter-day precision (RSD) and accuracy (DEV) calculated from replicate rat, dog and human plasma samples analysed using the high pH LC/MS/MS method

Species	PR-104					PR-104A				
	Concentration (μg/ml)	Intra-day[a]		Inter-day[b]		Concentration (μg/ml)	Intra-day		Inter-day	
		RSD (%)	DEV (%)	RSD (%)	DEV (%)		RSD (%)	DEV (%)	RSD (%)	DEV (%)
Rat	0.015	3.4	−2.7	6.5	−2.0	0.015	4.0	−6.0	2.8	−4.0
	0.075	2.3	−7.9	4.8	−5.7	0.075	4.0	−4.5	1.4	−3.9
	2.000	4.2	−4.5	5.1	−2.6	2.000	4.1	−8.1	4.5	−4.7
Dog	0.015	3.1	−7.3	6.0	−6.0	0.150	1.7	−4.0	2.6	−8.2
	0.075	6.1	−2.5	4.5	−4.3	0.750	5.4	4.3	2.8	0.9
	2.000	3.4	−11.2	6.3	−2.2	20.000	1.6	−7.7	3.5	−7.8
Human	0.015	6.2	−3.3	5.1	−3.3	0.150	3.3	−3.3	2.5	−3.5
	0.075	3.2	−2.4	3.5	−3.7	0.750	1.4	−4.0	2.5	−4.5
	2.000	2.1	−1.6	2.8	0.7	20.000	3.5	−0.5	2.6	−0.2

[a] Determined in five replicate samples at each concentration level for rat and dog and six replicate samples at each concentration for human.
[b] Determined in samples ($n = 5$) on 5 different days for rat and dog and in samples ($n = 6$) on 3 different days for human.

Table 4
Intra- and inter-day precision (RSD) and accuracy (DEV) calculated from replicate mouse plasma and tissue samples analysed using the low pH LC/MS method

Matrix	PR-104					PR-104A				
	Concentration (μg/ml)	Intra-day[a]		Inter-day[b]		Concentration (μg/ml)	Intra-day		Inter-day	
		RSD (%)	DEV (%)	RSD (%)	DEV (%)		RSD (%)	DEV (%)	RSD (%)	DEV (%)
Plasma	0.579	3.9	−7.0	3.2	−8.0	0.499	1.3	−10.0	6.2	−4.0
	5.79	0.5	−3.1	6.7	−8.9	4.99	0.6	1.1	3.5	−2.2
	57.9	0.8	1.0	3.1	1.0	49.9	0.9	−5.0	2.5	−7.0
Tumour	0.579	1.5	8.0	5.5	3.0	0.499	2.0	9.0	5.3	4.0
	5.79	1.0	−2.1	2.1	−0.9	4.99	3.5	8.7	2.2	7.5
	57.9	1.6	−8.0	1.6	−7.0	49.9	0.7	−5.0	0.7	−5.0
Liver	0.579	6.8	−1.0	6.5	3.0	0.499	3.4	4.0	4.6	7.0
	5.79	2.6	−8.8	2.3	−7.8	4.99	1.0	−1.8	1.1	−2.3
	57.9	1.5	−8.0	1.4	−7.0	49.9	0.5	−3.0	0.5	−3.0
Brain	0.579	7.0	12.0	4.9	11.0	0.499	7.1	13.0	6.6	10.0
	5.79	3.4	8.6	3.3	6.7	4.99	2.7	9.1	2.8	−8.1
	57.9	0.4	5.0	1.5	−6.0	49.9	1.7	8.0	2.0	9.0

[a] Determined in five replicate samples at each concentration level.
[b] Determined in samples ($n = 5$) on 3 different days.

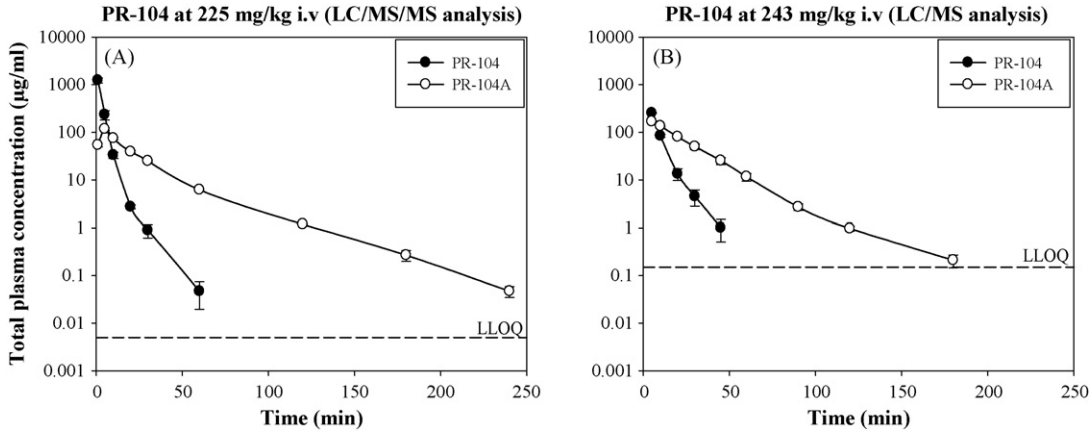

Fig. 4. Pharmacokinetic profiles of PR-104 (●) and PR-104A (○) following intravenous administration of PR-104 at similar dose levels in rats ($n = 3$ per time point). Plasma samples from rats dosed at 225 mg/kg were analysed by the rapid, high pH LC/MS/MS method (A). In a different laboratory, the low pH LC/MS method was used to analyse plasma samples from rats dosed at 243 mg/kg (B). Data are presented as mean ± SEM.

mouse matrices, and ranged from 93.1 to 104.3% for PR-104 and 91.4 to 102.7% for PR-104A (Table 2).

3.1.4. Matrix effect

The spiked blank matrix samples (mouse plasma, tumour, liver and brain) used to evaluate recovery (Table 2) were compared with PR-104 and PR-104A at the same nominal concentrations in extraction solvent. This demonstrated absolute ion counts in the matrix in the range 88–106% of that in the extraction solvent for PR-104, and 89–105% for PR-104A, indicating a lack of ion suppression by matrix components. Comparison of six different batches of rat, dog and human plasma gave mean DEV values for each batch from −6.5 to 4.8% for PR-104 and −9.7 to 2.6% for PR-104A (data not shown). This indicates a lack of significant variation in matrix effects.

3.1.5. Lower limit of quantification

The LLOQ in rat, dog and human plasma samples analysed by the high pH LC/MS/MS was 0.005 μg/ml for PR-104 and 0.05 μg/ml for PR-104A (0.005 μg/ml for rat). By LC/MS analysis, a LLOQ of 0.17 μg/ml was determined for PR-104 and 0.15 μg/ml for PR-104A in mouse plasma and tissues.

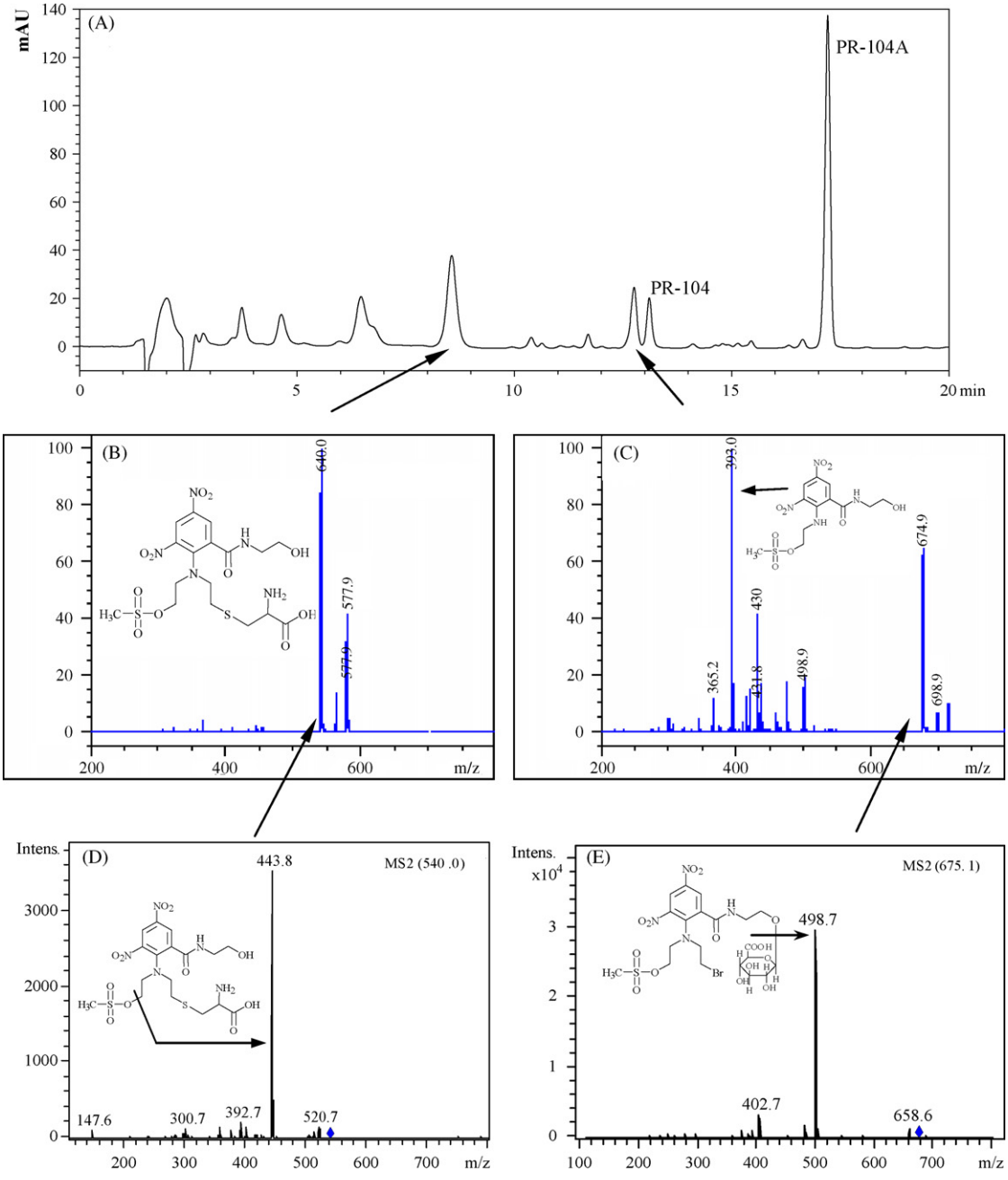

Fig. 5. Representative HPLC chromatogram of mouse plasma obtained 15 min following intravenous administration of PR-104 (326 mg/kg), and Ion Trap mass spectra of key metabolites. Samples were analysed using the low pH high chromatographic resolution method optimised for metabolite identification. The peak at retention time 12.8 min contains both a half mustard metabolite (C) and *O*-glucuronide (E), while the peak at 8.6 min is a cysteine adduct of PR-104A (B and D).

3.1.6. Precision and accuracy

To determine precision and accuracy of the assays, the RSD and DEV of the intra- and inter-assay variations were calculated at three concentrations. The inter- and intra-day accuracies and precisions were within the required limits for both PR-104 and PR-104A at all three concentrations, for rat, dog and human plasma using the high pH LC/MS/MS method (Table 3), although there appeared to be a bias towards negative values for DEV. The low pH LC/MS method also provided acceptable accuracy and precision for mouse plasma and tissues (Table 4).

3.1.7. Stability

Investigation of stability of PR-104 in whole blood-EDTA at 37 °C, using the low pH LC/MS method, demonstrated substantial loss over 30 min with accompanying formation of PR-104A. The first order half-lives were 76.3 min for mouse blood, 87.2 min for rat blood, 161 min for dog blood and 188 min for human blood. Stability was acceptable for up to 5 min, with <5% loss of PR-104 at this time. PR-104A was also unstable in whole blood at 37 °C, with similar first order kinetics for all species (half lives in the range 98.9–115 min) but stability was acceptable (<5% loss) at 3 min. In contrast, PR104 and PR104A were stable when plasma QC samples were prepared on ice and extracted at intervals over 3–24 h (data not shown). Post-preparative stability was confirmed when plasma and tissue QC samples were processed, maintained in the autosampler at 4 °C and injected over 36–120 h. Both analytes were also stable when extracted plasma QC samples were stored at −70 °C (24 h) and then analysed after three freeze–thaw cycles. Long-term storage of extracted plasma at −70 °C for 30 days (mouse), 27 days (rat), 44 days (dog) or 95 days (human) showed acceptable stability of both PR-104 and PR-104A. In each of the above cases, mean values for the QC samples at all concentration levels remained within 15% of theoretical values for both analytes.

3.2. Application to pharmacokinetic evaluation

Two independent studies were conducted in two different laboratories, using animals from separate colonies, to determine the pharmacokinetics of bolus intravenous PR-104 in rats. In one study, the dose was 225 mg/kg and plasma concentrations were determined using the high pH LC/MS/MS method (Fig. 4A). Plasma concentrations of PR-104 following single intravenous bolus administration were maximal at the first timepoint (1 min after the injection); declining rapidly with an initial half life ($t_{1/2}$) of approximately 3.3 min. PR-104A was readily detectable as early as 1 min, with a T_{max} of 5 min and a terminal $t_{1/2}$ of 23.7 min. After 5 min, the concentration of PR-104A exceeded that of PR-104. The C_{max} and AUC (0-inf) values for PR-104 were 1014 μg/ml and 84 μg h/ml. The corresponding values for PR-104A were 99 μg/ml and 41 μg h/ml. In the second study, animals were dosed at 243 mg/kg and plasma was analysed using

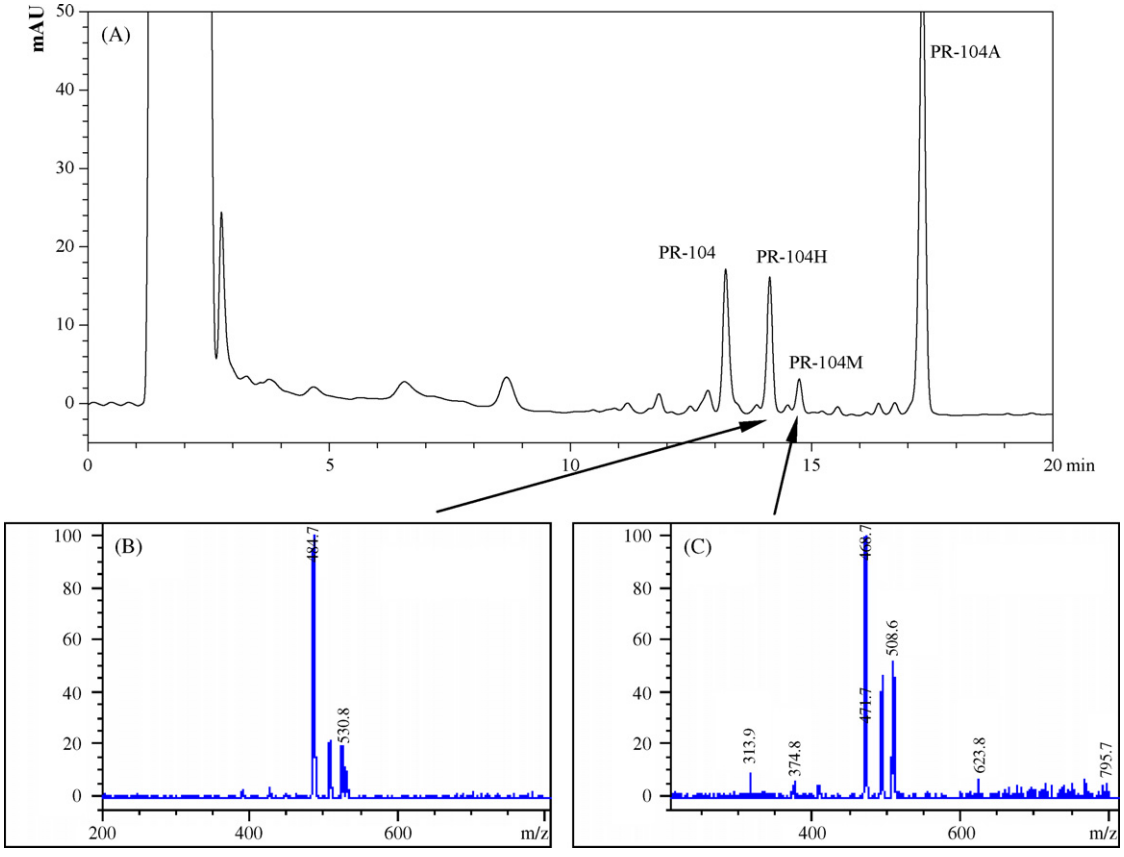

Fig. 6. Representative HPLC chromatogram of SiHa tumour tissue obtained 15 min following intravenous administration of PR-104 (326 mg/kg) to mice, and mass spectra of key reduced metabolites (hydroxylamine PR-104H and amine PR-104M). Samples were analysed using the low pH high chromatographic resolution (LC/MS) method optimised for metabolite identification.

the low pH LC/MS method. This demonstrated similar pharmacokinetics to the first study, with C_{max} and AUC (0-inf) values for PR-104 of 756 μg/ml and 72 μg h/ml. The corresponding values for PR-104A were 168 μg/ml and 93 μg h/ml.

3.3. Application to metabolite identification

Improved metabolite resolution was achieved using the low pH LC method optimised for this purpose with a longer cycle time of 40 min. An illustration is provided in Fig. 5, which shows the metabolite profile in mouse plasma 15 min after PR-104 administration, and compares this with the profile in a SiHa tumour (Fig. 6) in the same animal. Several metabolites in plasma were resolved by absorbance, each representing a single species as judged by the uniform absorbance spectrum and single quadrupole and ion trap mass spectrum across the peak. The exception was the peak at a retention time of 12.8 min, for which the mass spectrum (Fig. 5C) suggested the presence of two metabolites. One (*m*/*z* 393) was consistent with a half-mustard metabolite of PR-104A resulting from oxidative debromoalkylation; the other (*m*/*z* 675) was consistent with the *O*-glucuronide of PR-104A. The latter assignment was supported by a neutral loss of 176 in the fragment mass spectrum (Fig. 5E). The most prominent metabolite in plasma had a mass spectrum consistent with a cysteine conjugate of PR-104A (Fig. 5B) and the assignment is also supported by its fragment mass spectrum (Fig. 5D). The metabolite profile in SiHa tumours (Fig. 6) was different from plasma, with the reduced metabolites PR-104H and PR-104M (see Fig. 1A) being prominent.

4. Discussion and conclusion

The objective of this study was to validate two assays developed for the determination of PR-104 and PR-104A, and identification of their metabolites, in plasma and tissues from different species. The two assays were developed with different primary objectives. The high pH LC/MS/MS method is optimised for rapid and sensitive analysis of PR-104 and PR-104A, and uses either stable isotope (tetra-deuterated) internal standards or analogue standards (which co-elute in this system which has low chromatographic resolution). With the stable isotope standards, we used the Br-81 peak for the d_4 compounds and the Br-79 peak for the d_0 analytes to achieve a six-unit *m*/*z* separation, thus minimising signal overlap. For both analytes, sensitive quantitation (with a LLOQ of 0.005 μg/ml for PR-104, but a higher value of 0.05 μg/ml for PR-104A) was achieved with this method.

The second method was designed to provide higher chromatographic resolution, facilitating identification of metabolites (with use of photodiode array detection as well as single stage quadrupole or ion trap MS). This method was also suitable for quantitation of PR-104 and PR-104A, using stable isotope rather than analogue internal standards to avoid additional peaks in the diode array chromatogram. With a single stage quadrupole MS, this gave a LLOQ of 0.17 μg/ml for PR-104 and 0.15 μg/ml for PR-104A. The two methods gave similar plasma pharmacokinetics in rats (Fig. 4) when applied to different samples from two independent in-life studies at different institutions, although the methods have not been cross validated. The utility of the low pH LC/MS method for metabolite identification was also demonstrated (Figs. 5 and 6).

Sufficient specificity with no significant matrix effects was observed in all matrices with both assays. In plasma and tissues, high recoveries (>90%) for both PR-104 and PR-104A in all mouse matrices were achieved by extraction in cold methanol. The RSD and DEV calculated from the different repeatability assays demonstrate that the precision and accuracy of the two methods is reliable for routine analysis. To ensure minimal loss (<5%) of both PR-104 and PR-104A at 37 °C, blood samples obtained during pharmacokinetic evaluation were chilled and centrifuged within 3 min following collection; this is an important requirement because of the instability of both analytes in whole blood at 37 °C. Furthermore, all plasma and tissue homogenate samples were prepared on ice and precipitated immediately with cold extraction solvent to minimize PR-104 degradation, although this was much slower in cold plasma than in warm whole blood. The resulting methanolic extracts showed acceptable stability for up to 24 h at 4 °C and good freeze/thaw stability. Overall, we have developed reliable analytical methods for quantitative and qualitative analysis of this new hypoxia-activated anti-tumour drug for Phase I clinical trials.

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