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Rapid and sensitive ultra-high-pressure liquid chromatography–tandem mass spectrometry analysis of the novel anticancer agent PR-104 and its major metabolites in human plasma: Application to a pharmacokinetic study

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ABSTRACT

PR-104 is a dinitrobenzamide mustard currently in clinical trial as a hypoxia-activated prodrug. It is converted systemically to the corresponding alcohol, PR-104A, which is activated by nitroreduction to the hydroxylamine (PR-104H) and amine (PR-104M). PR-104A is also metabolised to the *O*-glucuronide (PR-104G), and by oxidative debromoethylation to the semi-mustard PR-104S. We now report a validated ultra-high-pressure liquid chromatography and tandem mass spectrometry (UHPLC–MS/MS) method for the determination of these metabolites in human plasma. Plasma proteins were precipitated with acidified methanol and the supernatant diluted into water. Aliquots were analysed by UHPLC–MS/MS using a Zorbax Eclipse XDB-C18 Rapid Resolution HT (50 mm × 2.1 mm, 1.8 μm) column and gradient of acetonitrile and 0.01% formic acid with a 6 min run time. The method had a linear range of 0.1–50 μM for PR-104, PR-104A and PR-104G, 0.05–5 μM for PR-104H, 0.025–2.5 μM for PR-104M and 0.01–1 μM for PR-104S. The intra-day and inter-day precision and accuracy were within 14%. The extraction recovery of all analytes was over 87%. The validated method was illustrated by using it to study the pharmacokinetics of PR-104 and its metabolites in a human patient.

1. Introduction

Hypoxia is a characteristic of solid tumours and an important therapeutic target because of the resistance of hypoxic cells to radiation therapy and chemotherapy [1,2]. This has led to the development of hypoxia-activated prodrugs (also called bioreductive drugs) that are metabolised to active cytotoxins by pathways that are inhibited by oxygen [3–7]. One of these is the dinitrobenzamide nitrogen mustard PR-104 (see Fig. 1 for chemical structures), a phosphate ester pre-prodrug currently in phase II clinical trial which is converted systemically to the corresponding alcohol PR-104A; the latter is metabolised selectively under hypoxia by reduction of the nitro group *para* to the mustard moiety, which activates the nitrogen mustard moiety. The resulting hydroxylamine (PR-104H) and amine (PR-104M) are responsible for the cytotoxicity of PR-104A through the formation of DNA crosslinks [8–10].

Validated analytical methods for quantifying PR-104 and PR-104A in plasma and tissue extracts by LC/MS and LC-MS/MS have been reported [11], and have been used to evaluate the pharmacokinetics of PR-104 in preclinical and clinical studies [8,12]. The LC/MS method was used in full scan mode to identify (but not quantify) metabolites of PR-104A in mice, including the *O*-glucuronide PR-104G and the semi-mustard PR-104S (resulting from oxidative debromoalkylation of the nitrogen mustard moiety) although these were not chromatographically resolved. This LC/MS method also detected the active metabolites PR-104H and PR-104M in extracts from SiHa tumour xenografts, but not in mouse plasma. Subsequently it has been shown that PR-104A is reduced to PR-104H not only by one-electron reductases such as NADPH:cytochrome P450 oxidoreductase under hypoxia [10,13], but also by aldo–keto reductase 1C3 (AKR1C3) in the presence of oxygen [14]. AKR1C3 is highly expressed in some tumours, and appears to contribute to the antitumour activity of PR-104A [14], but its expression in normal tissues increases the importance of monitoring PR-104H in humans. The original LC-MS/MS method [11] for PR-104 and PR-104A used a high pH mobile phase which is unsuitable for the unstable and reactive [8,18] reduced metabolites. Low pH LC-MS/MS methods have been recently used to quantify PR-104H and PR-104M in *in vitro* metabolism studies [9,10], but have not yet been validated for the assay of plasma. In addition, in metabolite profiling studies we have found that the glucuronide PR-104G is a much more prominent metabolite in humans than in rodents. Glucuronidation could be of pharmacological significance in humans if its rate of formation influences the clearance of PR-104A or if biliary excre-

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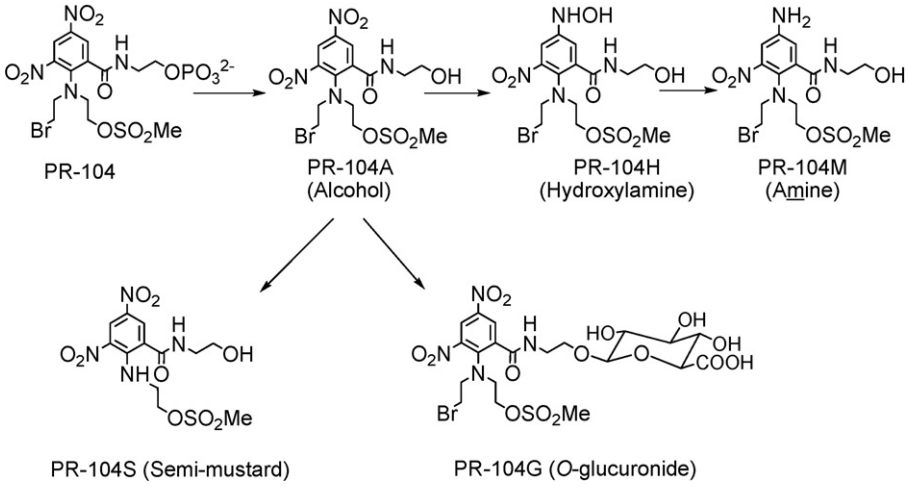

Fig. 1. Structure of the dinitrobenzamide mustard PR-104 and metabolites representing its major routes of biotransformation (reduction to hydroxylamine PR-104H and amine PR-104M, oxidative dealkylation to the semi-mustard PR-104S and *O*-glucuronidation to PR-104G).

tion results in the regeneration of PR-104A in the gastrointestinal tract.

Here, we report a modified LC–MS/MS method that provides for sensitive and rapid ultra-high-pressure liquid chromatography (UHPLC) quantitation of PR-104 and all of its key metabolites (PR-104A, PR-104H, PR-104M, PR-104G and PR-104S) in human plasma in a single analytical run. The method uses stable isotope internal standards for PR-104, PR-104A, PR-104H and PR-104M and shows excellent performance in terms of selectivity, sensitivity and throughput (6 min per sample). The method is currently being utilised in on-going clinical trials of PR-104.

2. Experimental

2.1. Chemicals and materials

PR-104, PR-104A [15], PR-104H [8], PR-104M, PR-104S [10] and the tetra-deuterated stable isotope internal standards of PR-104 (PR-104-d4) and PR-104A (PR-104A-d4) [16] were synthesised as previously described. Stable isotope standards of PR-104H (PR-104H-d4), and PR-104M (PR-104M-d4) were prepared using the same methods as the d0 compounds. PR-104G was synthesised from PR-104A by reacting with methyl 2,3,4-tri-*O*-acetyl-1-bromo-1-deoxy-α-D-glucuronate in the presence of silver triflate and 4A molecular sieves, deblocking under basic conditions ($LiOH/H_2O/MeOH/THF$) and treating with Amberlite IR-120 (H^+); full characterisation will be reported elsewhere. All compounds had a purity of at least 95% by HPLC, and PR-104H and M (including d4 internal standards) were stored in acetonitrile as stock solutions at −80 °C. Methanol (HPLC grade), acetonitrile (LC-MS grade) and formic acid, were obtained from Merck (Darmstadt, Germany). Water was purified using a Milli-Q Water Purification System (Millipore, Bedford, MA, USA). Other chemicals were all of analytical grade. Drug-free (blank) pooled human plasma from healthy donors originated from the Regional Blood Transfusion Centre, Auckland Healthcare, NZ.

2.2. Liquid chromatography and mass spectrometric conditions

The LC–MS/MS system was an Agilent 1200 Rapid Resolution HPLC and Agilent 6410 triple quadrupole mass spectrometer equipped with a multimode ionisation source (Agilent Technologies, USA). Chromatographic separation was achieved on a Zorbax Eclipse XDB-C18 Rapid Resolution HT (50 mm × 2.1 mm, 1.8 μm) column with a 0.2 μm in-line filter and was maintained at 35 °C. The mobile phase consisted of acetonitrile (A) and 0.01% formic acid (B) with fast gradient elution at a flow rate of 0.5 ml/min and run time of 6 min. The following gradient was applied: 0 min, 80% B; 3 min, 60% B; 4.5 min, 20% B; 4.9 min, 20% B; and 5 min, 80% B. The column was equilibrated for 1 min between injections. The eluent flow was led into the mass spectrometer starting 0.6 min after injection by switching the MS inlet valve. The sample volume injected was 25 μl and the autosampler was set at 4 °C. The mass spectrometer was run in positive ion ESI-APCI combined mode using multiple reaction monitoring (MRM) to monitor the mass transitions. Compound-dependent parameters are reported in Table 1. The mass resolutions were set at 0.7 u FWHM (unit mass resolution) for both quadrupoles. Other parameters of the mass spectrometer were: gas flow 5.5 L/min; gas temperature 350 °C; vaporizer temperature 225 °C; nebulizer 55 psi; capillary 3000 V, corona current positive 3 μA, charging voltage 1500 V. Data were acquired and analysed with Agilent MassHunter software.

2.3. Stock solutions, calibration curves and quality control samples

Stock solutions were prepared in acetonitrile at the following concentrations: PR-104H (1 mM), PR-104M (1 mM), PR-104A (10 mM), PR-104G (10 mM), PR-104 (10 mM) and PR-104S (0.2 mM), PR-104H-d4 (30 μM), PR-104M-d4 (30 μM), PR-104A-d4 (100 μM) and PR-104A-d4 (100 μM). All stock solutions were stored at −80 °C. A mixed (combined) working solution was prepared each time by appropriate dilution of stock solutions in acetonitrile to achieve the following concentrations: PR-104H (0.1 mM), PR-104M (0.05 mM), PR-104A (1 mM), PR-104G (1 mM), PR-104 (1 mM) and PR-104S (0.02 mM). The calibration curves of PR-104 and its metabolites were prepared at the concentration levels of 0.5, 1.5, 5, 15, 50 μM for PR-104, PR-104A and PR-104G; 0.05, 0.15, 0.5, 1.5, 5 μM for PR-104H; 0.025, 0.075, 0.25, 0.75, 2.5 for PR-104M; 0.01, 0.03, 0.1, 0.3, 1 3, 1 μM for PR-104S by spiking appropriate amount of the working solution in blank human plasma followed by serial dilution with blank plasma. Quality control (QC) samples were prepared in blank human plasma so that each contained 1 μM PR-104H, 0.5 μM PR-104M, 10 μM PR-104, PR-104A and PR-104G, and 0.2 μM PR-104S, and a second set of QC samples were prepared by 10-fold dilution in plasma. All samples were stored at −80 °C.

Table 1
Compound-dependent mass spectrometry parameters.

Compound	Fragmentation voltage (V)	Precursor ion, *m/z*	Collision energy (V)	Product ion, *m/z*
PR-104H	120	487	15	391
PR-104H-d4	120	491	15	395
PR-104M	120	469	15	373
PR-104M-d4	120	475	15	379
PR-104	120	579	7	481
PR-104-d4	120	585	7	487
PR-104A	100	499	10	403
PR-104A-d4	100	505	10	409
PR-104-G	120	675	10	499
PR-104S	120	393	7	297

2.4. Sample preparation

Plasma samples (100 μl) were precipitated immediately on ice with 900 μl cold solvent comprising methanol:ammonium acetate:acetic acid (1000:3.5:0.2, v/w/v). Samples were then vortex mixed for 30 s, cooled on ice and centrifuged at 13,000 × *g* (5 min, 4 °C). A 25 μl aliquot of each precipitated supernatant was then diluted in 25 μl of water containing each of the PR-104-d4 (0.5 μM), PR-104A-d4 (0.5 μM), PR-104H-d4 (0.15 μM) and PR-104M-d4 (0.075 μM) internal standards for analysis.

2.5. Method validation

Assay selectivity was tested by analysing six individual drug-free (pre-dose) human plasma samples from a PR-104 Phase I clinical trial to investigate potential interferences of endogenous compounds co-eluting with analytes and internal standards. The chromatogram of blank plasma samples was compared with those of plasma samples spiked with analytes at LLOQ and internal standards and plasma sample after intravenous infusion of PR-104. Chromatographic peaks of analytes and internal standards were identified on the basis of their retention times and MRM responses. Linearity of calibration curves based on peak area ratios (target/internal standard) was assessed by weighted ($1/x$) least squares regression analysis using tetra-deuterated internal standards of PR-104, PR-104A, PR-104H and PR-104M. PR-104-d4 was used as internal standard for PR-104G due to its partial co-elution. External standards were used for quantification of PR-104S. Intra- and inter-day precision (expressed as relative standard deviation (RSD) and accuracy (expressed as percentage of the nominal value) were determined by analysis of replicates ($n = 6$) of low and high QC samples on three different days. The lower limit of quantitation (LLOQ) was defined as the lowest concentration providing RSD and accuracy <20%. Recovery from plasma was tested at the two QC levels. The recoveries were evaluated by comparing extracted plasma samples with blank plasma extracted solution spiked with analytes.

The matrix effect was defined as the change in response due to the presence of other substances in the samples. It was evaluated by comparing the analytes diluted into the blank plasma extracted solution with that of the same stock solutions diluted in water. Two different concentration levels (low and high QC) were evaluated by analysing three samples at each level.

Although the stable isotope internal standards are expected to compensate, the instability of the reactive nitrogen mustards PR-104H and PR-104M could lead to significant loss of signal intensity. Therefore stability experiments were performed for those two analytes to evaluate post-preparative plasma extracts in the autosampler at 4 °C. 20 μM of PR-104H and PR-104M were spiked into blank plasma extracted solution and aliquoted. Samples were analysed using photodiode array detection at 254 nm with same HPLC conditions as described in 2.2. Both methanol and the acidified methanol solvent (above) were investigated. In addition, the stability of all analytes in human plasma was evaluated using QC samples under following conditions: short-term stability in protein precipitated plasma on ice for 1 h, long-term stability in protein precipitated plasma stored at −80 °C for 30 days, and post-preparative stability in the autosampler up to 8 h at 4 °C.

2.6. Application for pharmacokinetic study

The validated method was applied to evaluate the pharmacokinetics of PR-104 and its metabolites in a patient from a PR-104 Phase I clinical trial. PR-104 solution was intravenously infused over 1 h and blood samples were collected for up to 5 h. The samples were transferred to EDTA coated tubes and centrifuged at 4000 × *g* for 10 min. Plasma was precipitated immediately on ice with 9 vol of cold acidified methanol solvent and stored at −80 °C until analysis. The pharmacokinetic parameters of PR-104 and its metabolites were estimated using non-compartmental methods by WinNonlin v5.0 (Pharsight Corp.).

2.7. Comparison of two methods for PR-104 and PR-104A measurement

Concentrations of PR-104 and PR-104A were determined in stored plasma samples from PR-104 Phase I clinical trials (50 patients) using the present method ("New method") and compared with previous determinations for the same samples using the high pH LC–MS/MS method of Patel et al. [11] ("Old method) at Microconstants Inc., San Diego. Agreement between the two methods was evaluated by Bland–Altman analysis [17] using Prism v5.02 software (GraphPad Software Inc.). To achieve homoscedasticity, the difference between methods was normalised by dividing by the mean for both methods. Multiple linear regression was used to evaluate the effect of differences in storage time using SigmaStat v3.5 (Systat Software Inc.), using log-transformed concentrations to achieve equal variance.

3. Results and discussion

3.1. Selectivity

Assay selectivity tested the ability of the method to distinguish and determine the analytes in the presence of other endogenous compounds in the sample. Representative chromatograms of blank plasma, spiked plasma at LLOQ and plasma after administration of PR-104 are shown in Fig. 2. The retention times were 1.79, 3.01, 2.03, 1.87, 2.07 and 1.59 min for PR-104, PR-104A, PR-104G, PR-104H, PR-104M and PR-104S, respectively. As shown in the figures, there was no significant endogenous substance interfering with the analytes and internal standards (peaks >20% of the LLOQ responses). No interference was seen from the internal standards,

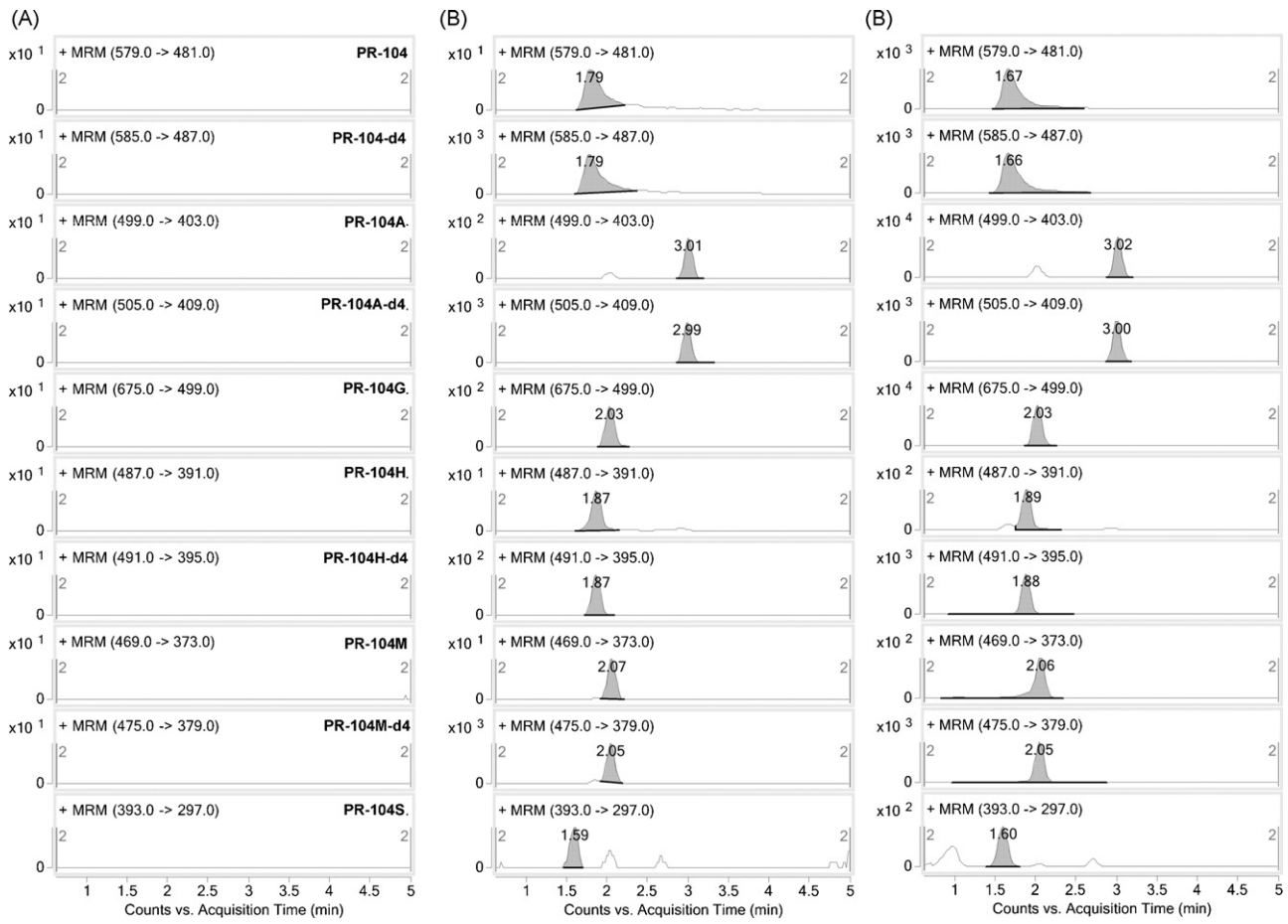

Fig. 2. Typical chromatograms of (A) blank plasma; (B) PR-104 and its metabolites spiked into human plasma at LLOQ (0.5 μM for PR-104, PR-104A and PR-104G; 0.05 μM for PR-104H; 0.025 μM for PR-104M and 0.01 μM for PR-104S; and (C) plasma from a patient 10 min after the end of a 60 min intravenous infusion of PR-104 at 770 mg/m^2.

which was checked using the zero levels in the calibration curves in each run.

3.2. Linearity, accuracy and precision

The calibration curves obtained by plotting peak area ratios (d0/d4), or peak area in the case of PR-104S, against nominal (spiked) concentrations were linear for human plasma over the range 0.1–50 μM for PR-104, PR-104A and PR-104G and 0.05–5 μM for PR-104H, 0.025–2.5 μM for PR-104M and 0.01–1 μM for PR-104S; R^2 values were all >0.99. Mean accuracy and precision values at each concentration within these ranges gave RSD values and accuracies within 14.0% (Table 2). Representative chromatograms for spiked plasma at the LLOQ are shown in Fig. 2B.

3.3. Recovery and matrix effect

To determine recovery, concentrations of PR-104 and its metabolites in extracted plasma QC samples were compared to standards prepared in blank matrix extract. Recoveries, evaluated at two different concentrations in triplicate, ranged from 87.6% to 99.6% (Table 2). Matrix effects on analyte quantification with respect to consistency in signal suppression were tested by comparing standards in blank plasma extracts with that in extraction

Table 2
Intra- and inter-day precision and accuracy for PR-104 and its metabolites in human plasma.

	Concentration added (μM)	Intra-day			Inter-day			Recovery (%)
		Measured (μM)	Accuracy (%)	RSD (%)	Measured (μM)	Accuracy (%)	RSD (%)	
PR-104	1	0.93	92.8	5.3	0.93	93.4	5.9	90.1
	10	9.60	96.0	5.7	9.91	99.1	7.8	92.4
PR-104A	1	0.90	89.9	3.1	0.95	94.5	7.2	95.5
	10	10.4	103.5	8.3	9.85	98.5	9.1	99.6
PR-104H	0.1	0.11	106.4	5.5	0.11	107.4	7.5	97.1
	1	1.03	102.6	5.1	1.03	102.7	6.6	90.3
PR-104M	0.05	0.050	100.8	13.8	0.052	103.7	11.2	95.9
	0.5	0.49	97.0	10.6	0.49	97.2	8.3	89.2
PR-104G	1	0.94	93.9	4.3	0.92	91.5	4.2	91.6
	10	10.2	102.2	11.5	10.1	101.0	9.1	91.1
PR-104S	0.02	0.018	88.2	13.3	0.019	94.1	10.3	87.6
	0.2	0.18	88.1	2.8	0.19	93.2	6.1	95.2

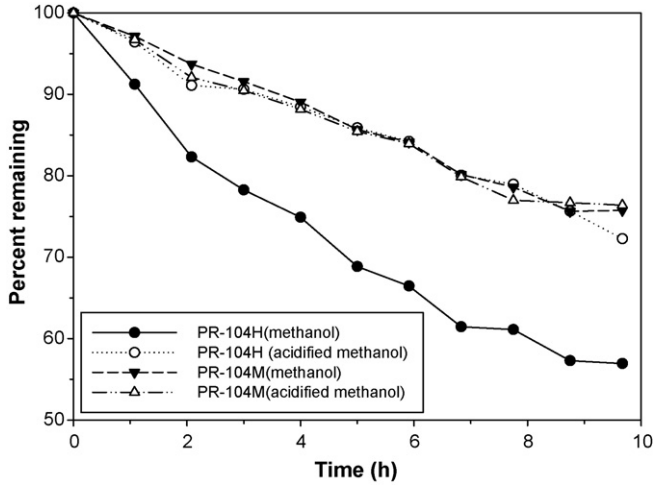

Fig. 3. Stability of PR-104H and PR-104M in autosampler at 4 °C. Human plasma was precipitated with 9 vol methanol or acidified methanol (methanol:ammonium acetate:acetic acid, 1000:3.5:0.5, v/w/v), diluted 1:1 with water, spiked with PR-104H or PR-104M to 20 μM and aliquoted to HPLC vials. Samples were assayed using UHPLC with photodiode array detection at 254 nm, with a single injection per vial.

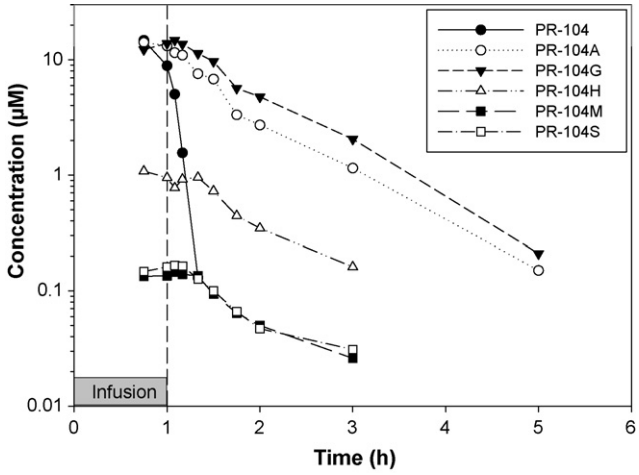

Fig. 4. Plasma concentration–time profiles of PR-104 and its metabolites in a female 70 kg patient after a 1 h intravenous infusion of PR-104 (770 mg/m^2).

solvent. Three replicates, each of two concentration levels (low and high QC), were prepared. The results for extracted plasma ranged from 87.4% to 112.6% of the values in the extraction solvent. Thus there was no significant matrix effect for the target analytes.

3.4. Stability of analytes

No significant degradation of any of the analytes in protein precipitated human plasma occurred during holding on ice for 1 h or during storage at −80 °C for at least 30 days, or on reinjection of samples after 8 h in the refrigerated autosampler, as demonstrated by <15% bias from the added concentrations (Table 3). In the latter case the expected chemical instability of PR-104H and PR-104M [8,18] is presumably compensated by the deuterated internal standards, but if too severe would lead to loss of sensitivity. We therefore undertook a separate study to assess how quickly these metabolites are degraded in the autosampler by monitoring their decay by UV detection in the absence of internal standards (Fig. 3). The data indicated that acidification of the methanol solvent improved the stability of PR-104H, and that in acidified methanol both PR-104H and PR-104M decreased by approximately 25% in 8 h at 4 °C. This minor loss of signal intensity is readily compensated by the stable isotope internal standard making it feasible to run autosampler batches of at least 80 samples (8 h) with little loss of sensitivity.

3.5. Pharmacokinetic studies

In a preliminary investigation, the method was successfully applied to determine the plasma concentrations of PR-104 and its metabolites up to 5 h after a 60 min intravenous infusion of PR-104 to a patient. The concentration-time profile for PR-104 and its metabolite are presented in Fig. 4. The corresponding non-compartmental pharmacokinetic parameters for this patient are shown in Table 4.

3.6. Comparison of the method with previous LC–MS/MS assay for PR-104 and PR-104A

The human plasma pharmacokinetics of PR-104 and PR-104A has been evaluated in a phase I clinical trial [8], and in additional unpublished phase I studies, using the previous LC–MS/MS

Table 4
Pharmacokinetic parameters in a female 70 kg patient with non-small cell lung cancer after a 60 min intravenous infusion of PR-104 at 770 mg/m^2.

	C_{max} (μM)	T_{max} (h)	AUC_{0-t} (μM h)	$AUC_{0-\infty}$ (μM h)	$t_{1/2}$ (h)
PR-104	14.8	0.75	9.51	9.51	0.067
PR-104A	14.2	0.75	18.7	18.8	0.666
PR-104G	14.8	1.08	23.0	23.2	0.654
PR-104H	1.08	0.75	1.60	1.77	0.741
PR-104M	0.145	1.08	0.221	0.253	0.860
PR-104S	0.166	1.08	0.275	0.283	0.923

Table 3
Stability of PR-104 and its metabolites in protein precipitated human plasma.

Concentration (μM)	PR-104		PR-104A		PR-104H		PR-104M		PR-104G		PR-104S	
	1	10	1	10	0.1	1	0.05	0.5	1	10	0.02	0.2
Short-tem stability (1 h on wet ice)												
Measured (μM)	0.92	9.75	0.95	9.74	0.095	1.08	0.053	0.48	0.89	9.66	0.019	0.20
RSD (%)	6.25	9.49	8.07	8.76	9.86	6.85	11.3	8.81	2.40	8.31	5.07	5.14
Bias (%)	−7.55	−2.45	−4.97	−2.60	−5.12	7.53	6.78	−3.48	−11.1	−3.39	−4.38	−2.48
Long-term stability (30 days at −80 °C)												
Measured (μM)	0.97	10.5	0.99	9.38	0.093	0.97	0.044	0.49	0.92	10.5	0.018	0.19
RSD (%)	5.42	6.06	6.69	9.10	12.21	9.36	11.5	5.92	4.16	5.54	10.6	5.03
Bias (%)	−3.24	4.64	−1.36	−6.21	−7.25	−2.97	−11.8	−1.68	−8.11	5.01	−9.29	−6.07
Post-preparative stability (8 h in autosampler at 4 °C)												
Measured (μM)	0.94	10.2	0.99	9.51	0.091	1.03	0.052	0.49	0.93	10.48	0.019	0.19
RSD (%)	3.91	1.32	7.54	9.78	8.88	7.58	11.9	6.83	3.99	6.39	6.23	5.70
Bias (%)	−5.55	1.85	−1.38	−4.94	−8.67	3.29	4.85	−1.82	−7.32	4.83	−6.47	−6.52

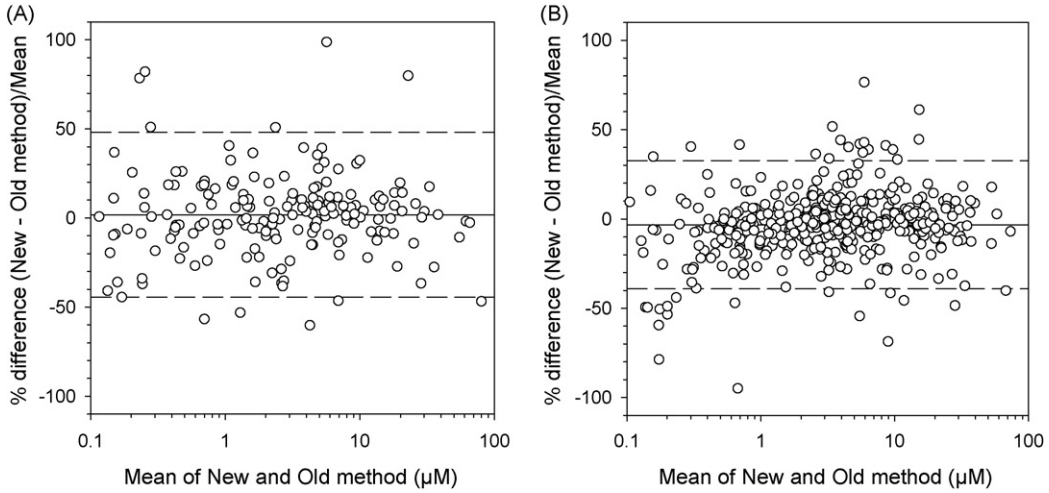

Fig. 5. Comparison of the current UHPLC–MS/MS method ("New method") with the previous LC–MS/MS method of Patel et al. [11] ("Old method") for 184 measurements of PR-104 (A) and 450 measurements for PR-104A (B) from 50 patients in phase I clinical studies. Solid lines are the mean bias, and dashed lines represent the 95% limits of agreement as determined using Bland–Altman analysis.

method [11], at Microconstants Inc., San Diego ("Old method"). We determined PR-104 and PR-104A concentrations in the same plasma samples using the present UHPLC–MS/MS method ("New method"). Using Bland–Altman analysis, the two methods showed excellent agreement, with a mean bias of only +1.6% for PR-104 and −3.3% for PR-104A (Fig. 5). The 95% limits of agreement were −44% and +48% for PR-104, and −39% and +33% for PR-104A. The storage times for the new assay were 3–29 months longer than for the old assay, which exceeds the validated storage time of 95 days [11]. However, multiple linear regression of the concentrations for the two methods, using storage time as a covariate, demonstrated there was no trend with storage time for either analyte ($p = 0.807$ for PR-104 and $p = 0.281$ for PR-104A).

4. Conclusions

A fast, sensitive and selective UHPLC–MS/MS method for the detection of PR-104 and its metabolites in human plasma has been developed. This is the first reported method that quantifies all the major metabolites of PR-104, and represents the first use of ultra-high-pressure LC in this context. The method performs very well in terms of accuracy and within laboratory reproducibility. It also provides results equivalent to the previous validated LC–MS/MS method for PR-104 and PR-104A [11], determined in a different laboratory, The results presented for a single patient demonstrate that *O*-glucuronidation of PR-104A to PR-104G is a major metabolic route in humans, and that the reduced metabolites (especially PR-104H) are also present at relatively high concentrations. The reported method is suitable for monitoring PR-104H, PR-104M and PR-104G routinely and for investigating relationships between these routes of biotransformation and the clearance and toxicity of PR-104A.

Role of the funding sources

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Disclosure statement

Yongchuan Gu has no conflicts of interest to disclose. William R. Wilson is a founding scientist, stockholder and consultant to Proacta, Inc.

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