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Quantitative analysis of Variolin analog (PM01218) in mouse and rat plasma by high-performance liquid chromatography/electrospray ionization tandem mass spectrometry

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Abstract

PM01218 is a novel marine-derived alkaloid and has shown potent growth inhibitory activity against several human cancer cell lines. A rapid and sensitive high performance liquid chromatography/tandem mass spectrometry (HPLC–MS/MS) method was developed and validated to quantify PM01218 in mouse and rat plasma. The lower limit of quantitation (LLOQ) was 0.05 ng/mL. The calibration curve was linear from 0.05 to 100 ng/mL ($R^2 > 0.999$). The assay was specifically based on the multiple reaction monitoring (MRM) transitions at m/z 278.4 → 184.2, no endogenous material interfaced with the analysis of PM01218 and its internal standard from blank mouse and rat plasma. The mean intra- and inter-day assay accuracy remained below 15 and 8%, respectively, for all calibration standards and QC samples. The intra- and inter-day assay precision was less than 12.8 and 8.5% for all QC levels, respectively. The utility of the assay was demonstrated by pharmacokinetics studies of i.v. (bolus) PM01218 on SD rats.

Keywords: PM01218; HPLC–MS/MS; Pharmacokinetics

1. Introduction

Variolins were isolated from the rare and difficult to access Antarctic sponge, *Kirkpatrickia variolosa* [1]. This new class of alkaloids is interesting from both structural and biological points of view. All the variolins have a common pyridopyrrolopyrimidine core, strictly a pyrido[3′,2′:4,5]pyrrolo[1,2-c]pyrimidine, with either a heterocyclic or methoxycarbonyl group attached at C5, which has no precedents in either terrestrial or marine natural products [2]. The first total synthesis of Variolin B was reported in 2001 [3]. Since then, several other approaches were developed to complete a total synthesis of Variolin B [4–7]. An important feature of variolins is their significant bioactivity. PM01218 (Fig. 1) is one of Variolin B analogs and has shown potent cytotoxic activity against the P388 murine leukemia cells, and also being effective against *Herpes smples* type I [8]. In addition, PM01218 appears to induce apoptosis as assessed by both morphological and biochemical methods in human epithelial cancer cells and in human leukemic cells [9,10]. Further studies to evaluate the in vivo activity and mechanism of action of PM01218 are in progress. To address the pharmacokinetics in preclinical species, it is essential to have a highly sensitive analytical method that can accurately measure trace levels of PM01218 in small volumes of plasma. The use of tandem mass spectrometry (MS/MS) as a highly specific detection technique for PM01218 could allow us to develop such a method. Here, we describe an HPLC–MS/MS method for the quantification of PM01218 in mouse and rat plasma. This method has been validated and can be used to selectively and sensitively determine sub-ng/ml concentration of PM01218 with very small amount of plasma samples in order to facilitate the preclinical pharmacokinetic evaluation.

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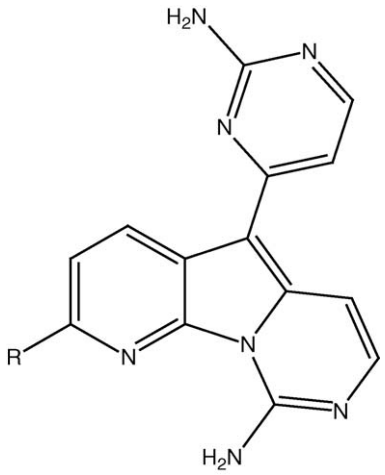

Fig. 1. The chemical structures of PM01218 and its internal standard.

2. Materials and methods

2.1. Materials

PM01218 and its analog (used as the internal standard, Fig. 1) were provided by PharmaMar S.A. (Colmenar Viejo, Madrid, Spain). HPLC grade methanol was obtained from Fisher Scientific (Pittsburgh, PA, USA). Formic acid and ammonium formate were obtained from Sigma (St Louis, MO, USA). Deionized water was purified by an in-house Barnstead E-pure™ System. The drug-free (blank) mouse and rat plasma containing sodium EDTA as anticoagulant was obtained from Bioreclamation Inc. (Hicksville, NY, USA).

2.2. Instrumentation

The HPLC system consisted of a Shimadzu LC-10ADvp solvent delivery unit, on-line degasser, gradient mixer and system controller (Shimadzu Scientific, Columbia, MD, USA). A CTC-PAL autosampler (LEAP Technologies, Carrboro, NC, USA) was used to inject samples. This autosampler allowed the use of two separate wash solvents (water with 0.1% formic acid and methanol with 0.1% formic acid) to eliminate sample carryover. The mass spectrometer was a PE Sciex API 4000 LC/MS/MS system (Toronto, Canada). The API 4000 mass spectrometer was interfaced with Shimadzu HPLC system using a TurboIonSpray (TIS) ion source. A Dell GX400 Optiplex computer with PE Sciex Analyst software (version 1.2) was implemented to control the API 4000 mass spectrometer, the Shimadzu LC-10ADvp solvent delivery unit (via the system controller), and the CTC PAL autosampler. The API 4000, Shimadzu LC-10ADvp, and the CTC PAL autosampler were also connected by contact closures to synchronize time events.

2.3. Chromatographic conditions

The mobile phase flowing through the HPLC column consisted of methanol (0.1% formic acid)–water (10 mM ammonium formate, pH 3.5) (50:50, v/v). The column was run under isocratic condition with a flow rate 400 μl/min. The analytical column was Zorbax Bonus-RP, 5 μm, 2 mm × 50 mm (Agilent, Wilmington, Del, USA). The CTC-PAL Leap cooling unit was set at 4 °C. The sample injection volume was 20 μL. The syringe, injection loop, and the switching valve were postwashed (100 μL) four times by two washing solution sequentially. The first wash solution was water (0.1% formic acid), and the second wash solution was methanol (0.1% formic acid).

2.4. Mass spectrometric conditions

The ion polarity was set to positive mode. The nebulizer, curtain, and collision gas was UHP nitrogen (99.999%). User controlled voltages, gas pressures, and source temperature were optimized for the detection of the parent and product ions of PM01218 and the internal standard. The analytes were infused at 10 μL/min by an infusion pump (Harvard Apparatus, South Natick, MA) teed into the mass spectrometer. The turbo temperature was set to 250 °C. The curtain gas, gas 1 (nebulizer gas), and gas 2 (turbo gas, nitrogen) were 15, 40, and 50 psi, respectively. The nitrogen gas was delivered from a nitrogen Dewar with the gas regulator maintained at 100 psi. The ion spray and entrance potential were 5500 and 10 eV. The declustering potential, collision energy, and collision cell exit potential were optimized at 92, 55, and 14 eV, respectively, for PM01218 and the internal standard. The dwell time per channel was 300 ms with a 5 ms pause between scans. Both Q1 and Q3 were set to unit resolution, with a peak width of 0.7 ± 1 Th at 50% of the maximum peak height.

2.5. Data acquisition and analysis

Data acquisition was performed using multiple reaction monitoring (MRM) of PM01218 with its internal standard (Fig. 2). Transition was monitored at *m/z* 278.4–184.2 for PM01218 and *m/z* 292.4–198.3 for the internal standard. Automated data acquisition and data analysis were performed using PE Sciex Analyst software. Unknown sample concentrations of PM01218 were calculated from the linear regression equation $y = ax + b$ with a weighted factor of $1/x$. The peak area ratios of PM01218 to its internal standard versus corresponding concentrations were used for the linear least-squares regression of the calibration lines.

2.6. Standard solution and sample preparation

PM01218 stock solution was prepared in methanol at a concentration of 1.0 mg/mL. The series dilution led to the working solution at a concentration of 10 μg/mL in methanol. The calibration standards, with PM01218 concentrations of 100, 25, 5, 2.5, 1.25, 0.5, 0.25, 0.125, and 0.05 ng/mL, were prepared from series dilution of the working solution in mouse or rat plasma. Quality control samples, containing 100, 25, 2.5,

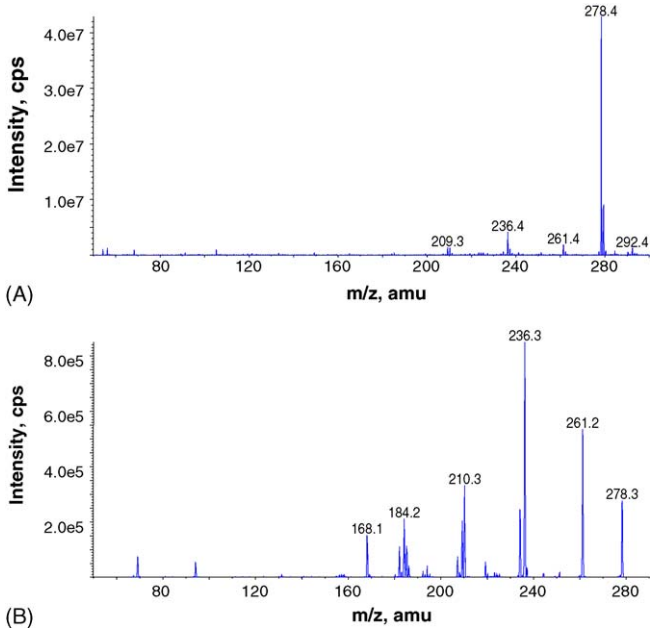

Fig. 2. (A) Mass spectrum (Q1) of PM01218 obtained by positive turbo ion spray ionization. (B) Product ion scan of the MH^+ ion.

0.25, 0.125, and 0.05 ng/mL were prepared in mouse or rat plasma.

The plasma samples were processed using liquid-liquid extraction procedure. To 200 μL of plasma sample, 100 μL of internal standard solution (200 ng/mL in methanol–water (1:9, v/v)) was added, followed by 1000 μL *tert*-butyl methyl ether. After mixing and centrifugation, the organic layer (800 μL) was transferred into a 96 deep well plate. The extracted media were evaporated to dryness under a nitrogen stream at 25 °C, using a TurboVap 96 concentration workstation (Zymark Corporation, Hopkinton, MA, USA). Finally, the dry residues were reconstituted in 100 μL of methanol (0.1% formic acid)–water (10 mM ammonium formate, pH 3.5) (1:1, v/v). An aliquot containing 20 μL of mixture was injected onto the analytical column.

2.7. Method validation

The analytical method was validated to demonstrate the specificity, recovery, limit of quantification (LOQ), accuracy, and precision of measurements. Triplicate sets of calibration standard and quality control (QC) samples were analyzed on 3 different days to determine the intra- and inter-day validation.

Acceptable specificity was defined as the absence of any detectable MRM HPLC–MS/MS ion currents at the retention time regions of PM01218 and the internal standard in six different double blank plasma samples.

The recovery of PM01218 from the extraction procedure from mouse or rat plasma was determined by comparing the pre-extraction spikes to the post-extraction spikes for QC samples. For the post-extraction spike, the QC analytes were spiked into the blank plasma extract and mixed immediately prior to evaporation under a stream of nitrogen.

The calibration range for PM01218 was established using triplicate set of standards from 0.05 to 100 ng/mL PM01218 in blank mouse or rat plasma. The calibration standard concentrations were 0.050, 0.125, 0.25, 0.50, 1.25, 2.50, 5.0, 25.0, and 100.0 ng/mL. The internal ratios (PM01218 peak area versus internal standard peak area) were calculated for each point and standard curves were constructed using least square linear regression analysis of internal ratios over concentration ratios. The linear regression is weighted by $1/x$ (the reciprocal of the concentration ratio). The correlation coefficient is used to evaluate the linearity of the calibration curve.

Twelve replicates at 0.125, 0.250, 2.50, 25.0, and 100.00 ng/mL were used to make the low- to high-range QC concentrations. The target values for intra- and inter-assay mean accuracy and precision of each QC sample should be within ±15% of the expected concentration.

2.8. Pharmacokinetic studies

Male and female SD rats (with four rats per sampling time point) were used to monitor samples for the PM01218 pharmacokinetic studies. Each animal received a single bolus intravenous injection of 1.0 mg PM01218/kg. Blood samples were taken by cardiac puncture at 5, 15, 30 min, and 1, 2, 4, 6, 12, 24, and 48 h after dosing. Blood samples were heparinized and kept on ice. Plasma was harvested from the blood samples following centrifugation for 15 min at 3000 rpm. The plasma was immediately frozen and remained frozen at −80 °C until analyzed.

On the basis of the plasma levels of the test substances, the following noncompartmental analysis parameters were calculated as follows [11]: peak plasma concentration (C_{max}) was obtained from observed data. Plasma $AUC_{0\to t}$ values (t being the time of the last plasma concentration measured) were calculated by the log–linear trapezoidal rule. The terminal phase was determined by visual inspection of the log-transformed concentration–time data. The elimination rate constant, k_{el}, was obtained from linear regression analysis of the terminal log–linear phase of the concentration versus time curve. Plasma $AUC_{0\to\infty}$ values were estimated by the combination of $AUC_{0\to t}$ and $AUC_{t\to\infty}$, where $AUC_{t\to\infty}$ represents the residual area of drug from time t to infinity and were calculated by dividing the last plasma concentration value measured by the elimination rate constant. Plasma clearance (CL_p), terminal half-life ($t_{1/2}$), and apparent volume of distribution (V_d) were estimated based on Eq. (1) through (3) shown below:

$$CL_p = \frac{\text{Dose}}{AUC_{0\to\infty}}, \tag{1}$$

$$t_{1/2} = \frac{\ln 2}{k_{el}}, \tag{2}$$

$$V_d = \frac{\text{Dose}}{AUC_{0\to\infty} k_{el}}. \tag{3}$$

The above parameters were calculated using the WinNonlin™ Professional Version 4.01 (Pharsight Corporation, Mountain View, CA, USA).

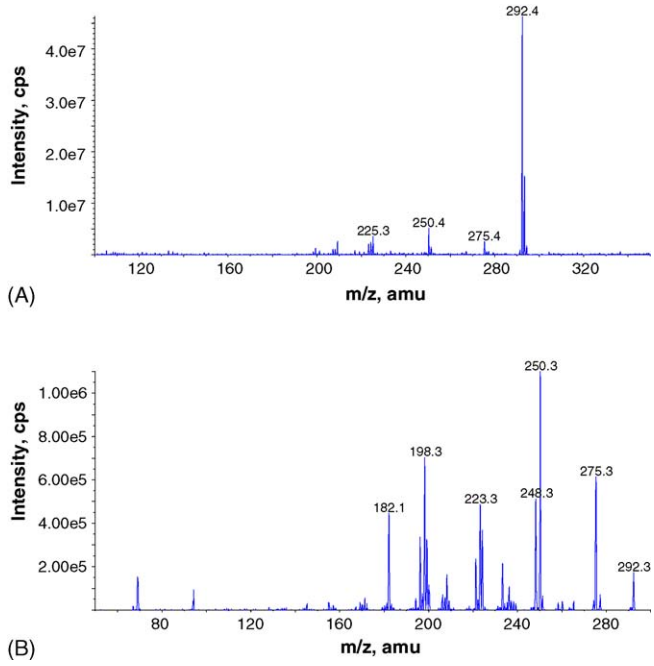

Fig. 3. (A) Mass spectrum (Q1) of the internal standard obtained by positive turbo ion spray ionization. (B) Product ion scan of the MH^+ ion.

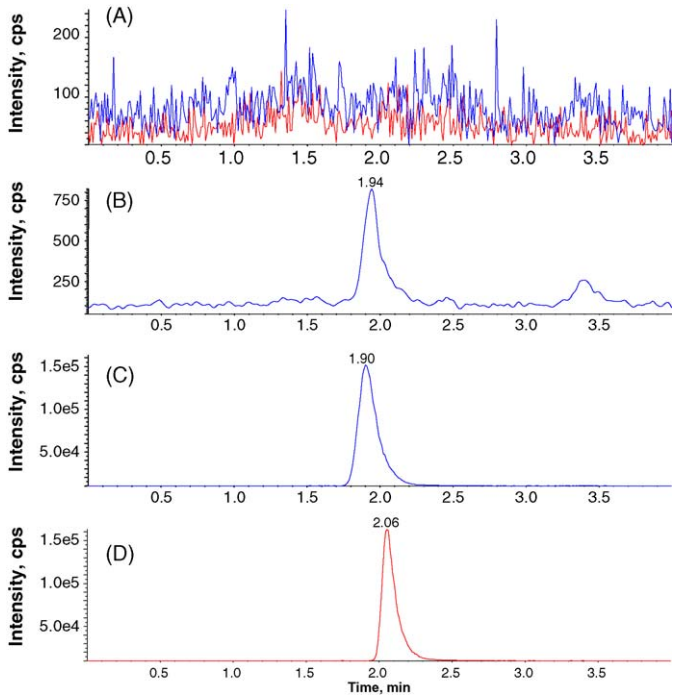

Fig. 4. (A) Chromatogram for the MRM transitions at *m/z* 278.4 → 184.2 (for PM01218) and *m/z* 292.4 → 198.3 (for IS) of double blank (drug-free) plasma. (B) Chromatogram for blank plasma spiked with 0.05 ng/mL PM01218. (C) Chromatogram for blank plasma spiked with 100 ng/mL PM01218 (retention time is 1.9 min). (D) Chromatogram for blank plasma spiked with 100 ng/mL internal standard (retention time is 2.06 min).

3. Results and discussion

3.1. Chromatography and mass spectrometry

PM01218 and the internal standard were too polar for adequate retention and separation on C_{18} reversed-phase columns and eluted at or near the void volume. Several silica supports and stationary phases were investigated for reversed-phase chromatography. We found that Zorbax Bonus-RP from Agilent gave us very good selectivity and excellent peak shape for PM01218 and its internal standard. The Agilent Zorbax Bonus-RP column has a polar amide group embedded in a long alkyl chain, which reduces interactions between basic compounds and the silica support. In addition, the column lifetime at low pH and the peak shape are further improved by unique triple endcapping with sterically protecting isopropyl group.

Meanwhile, several buffer systems had been investigated in order to find a suitable mobile phase for PM01218 chromatography. We determined that the acidification of the mobile phase was essential for retention of PM01218 on reversed-phase HPLC column. In order to be compatible with electrospray mass spectrometry we used ammonium formate and adjusted the buffer solution pH to 3.5 using formic acid.

The ionization and fragmentation of PM01218 was studied using electrospray tandem mass spectrometry. The collision-induced dissociation spectra of parent ion at *m/z* 278.4 produced an intense fragment ion at *m/z* 184.2 under optimum collision energy at 55 V. The parent mass spectrum and product ion mass spectrum of PM01218 are shown in Fig. 2A and B. The parent mass spectrum and product ion mass spectrum of internal standard are shown in Fig. 3A and B.

The multiple reaction monitoring, based on *m/z* 278.4 → 184.2 transition, was specific for PM01218; based on *m/z* 292.4 → 198.3 transition, was specific for the internal standard. A typical chromatogram from blank plasma is shown in Fig. 4A, showing no interfering endogenous materials with the analysis of PM01218 and its internal standard. Fig. 4B shows chromatogram for blank plasma spiked with 0.05 ng/mL PM01218. Fig. 4C shows chromatogram for blank plasma spiked with 100 ng/mL PM01218 and its internal standard. The retention time of PM01218 was 1.9 min.

The carryover problem was solved successfully by using a CTC PAL autosampler equipped with a dual washing system. With dual extensive washes (15 washes per injection) of the syringe, valve, and injection loop, as described in Section 2, the carryover was reduced to less than 1% of LLOQ, allowing us to reach the LLOQ of 0.05 ng/ml. Clearly, the current assay based on the MRM MS/MS detection provided a highly specific and sensitive measurement of PM01218 in plasma.

3.2. Method validation

The assay was linear over the concentration range of 0.05–100 ng/ml (Fig. 5). The mean correlation coefficient for the calibration curves (weighted by $1/x$, the reciprocal of the concentration ratio) was 0.9992. Typically, the calibration curve was defined by a slope of 5.06 and an intercept of 0.0020. The coefficient of variation (CV) of the slopes was 5.8%. The mean inter-day accuracy for all calibration standards ranged from 94

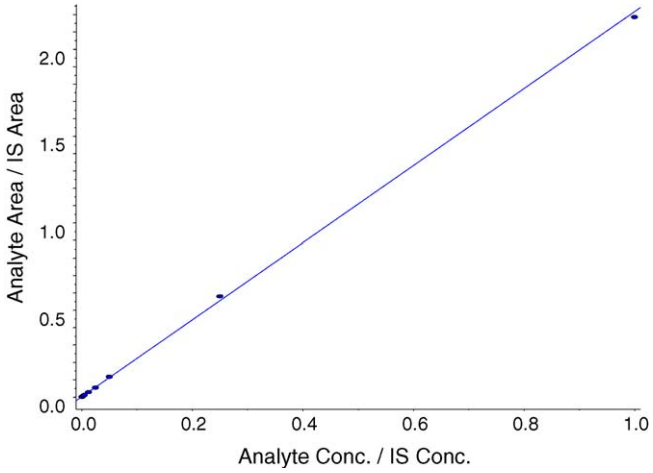

Fig. 5. A typical calibration standard curve for PM01218 spiked into blank (drug-free) mouse plasma. The mouse plasma samples were spiked with 0.05–100 ng/mL of PM01218. The ratio of peak area for PM01218 to its internal standard was used to construct the calibration standard curve.

to 110% (≤10% bias) for PM01218 prepared in mouse plasma and 93 to 105% (≤7% bias) for calibration standards prepared in rat plasma, and the mean inter-day precision for all calibration standards showed %R.S.D. values of 9% or less (Table 1).

The method precision was assessed by the coefficient variation (%R.S.D.). Table 2 shows the summary of the validation of the QC samples. The %R.S.D. values of the intra-day assays range from 1.4 to 12.8% and those for the inter-day assays range from 4.4 to 8.5%. Table 2 also shows that the mean intra- and inter-day assay accuracies, determined at each QC level throughout the validated runs, remained below 15 and 8%, respectively.

Table 1
Summary of the back-calculated PM01218 calibration standards ($n = 9$) in mouse or rat plasma

Nominal concentration (ng/mL)	Concentration found (ng/mL)	R.S.D. (%)	Accuracy (%)
Mouse			
0.050	0.055	9.0	110
0.125	0.131	7.8	105
0.25	0.252	5.5	101
0.50	0.47	2.2	94
1.25	1.19	3.8	95
2.50	2.53	5.2	101
5.0	4.8	4.2	96
25.0	24.4	4.9	97
100.0	100.8	1.3	101
Rat			
0.050	0.050	6.0	99
0.125	0.120	4.5	96
0.25	0.232	5.3	93
0.50	0.49	3.5	98
1.25	1.29	2.9	103
2.50	2.56	6.1	102
5.0	5.2	4.7	105
25.0	26.2	5.4	105
100.0	98.5	1.6	99

R.S.D., relative standard deviation.

Table 2
Summary of the PM01218 quality control samples ($n = 3$ for intra-day and $n = 9$ for inter-day assay) validation in mouse and rat plasma

Nominal concentration (ng/mL)	Concentration found (ng/mL)	R.S.D. (%)	Accuracy (%)
Mouse			
Intra-day assay			
0.050	0.047–0.0584	4.0–9.6	106–110
0.125	0.117–0.140	4.6–9.0	102–105
0.25	0.227–0.267	4.5–6.1	95–102
2.50	2.25–2.69	4.3–6.5	95–103
25.0	24.6–28.0	3.5–6.5	104–106
100.0	91.2–106.0	2.1–8.8	96–100
Inter-day assay			
0.050	0.054	6.3	108
0.125	0.130	5.9	104
0.25	0.249	5.6	100
2.50	2.48	5.7	99
25.0	26.2	4.4	105
100.0	98.4	5.3	98
Rat			
Intra-day assay			
0.050	0.045–0.054	7.6–8.6	98–101
0.125	0.108–0.136	4.4–6.9	91–104
0.25	0.223–0.295	1.4–12.8	95–103
2.50	2.55–2.97	2.7–5.4	105–115
25.0	25.3–29.6	2.1–7.3	105–113
100.0	95.9–115	2.0–6.9	103–109
Inter-day assay			
0.050	0.050	7.2	100
0.125	0.119	8.5	95
0.25	0.249	8.0	100
2.50	2.74	5.5	109
25.0	27.2	5.3	109
100.0	104.9	5.2	105

R.S.D., relative standard deviation.

3.3. Pharmacokinetic studies

Using our validated assay, the plasma concentration versus time profile of PM01218 was determined in SD rats dosed intravenously (Fig. 6). The mean plasma concentration–time curves showed that PM01218 has similar pharmacokinetic pro-

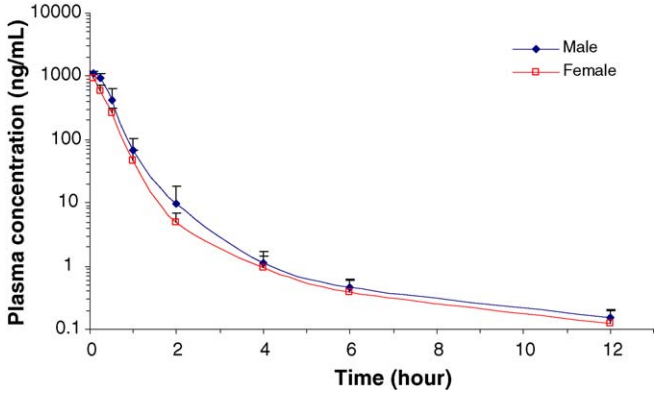

Fig. 6. Mean concentrations of PM01218 in plasma after i.v. administration of 1.0 mg/kg to SD rats ($n = 4$).

Table 3
Estimated pharmacokinetic parameters (mean ± S.D.) after intravenous administration of PM01218 (1.0 mg/kg) to SD rats (four rats per sampling time points)

Gender	C_{max} (μg/mL)	$t_{1/2}$ (h)	$AUC_{0\rightarrow t}$ (μg h/mL)	V_d (L/kg)	CL_p (mL/min/kg)
Male	1.11 ± 0.09	3.5 ± 0.8	0.61 ± 0.14	8.59 ± 2.29	28.43 ± 7.61
Female	0.95 ± 0.15	4.6 ± 2.9	0.44 ± 0.60	16.32 ± 12.80	38.62 ± 5.56

C_{max}, peak plasma concentration; $t_{1/2}$, terminal elimination half live; AUC, area under the plasma concentration–time curve; V_d, apparent volume of distribution; CL_p, plasma clearance.

filing in both female and male mice. The elimination kinetics of PM01218 was biexponential and PK data analysis showed that PM01218 has moderate clearance and larger volume of distribution. The corresponding pharmacokinetic parameters (calculated using the WinNonlin™ Professional Version 4.01) are summarized in Table 3.

4. Summary

The pharmacokinetic analysis of PM01218 relies on a highly sensitive assay, capable of determining PM01218 in plasma at low picogram-per-milliliter concentrations. In preclinical species, the limited volumes of plasma and interferences from the biological matrix, all add to the complexity of the trace analysis of PM01218. The specificity and sensitivity of HPLC–MS/MS assay reported here match these requirements. The assay also required lower volumes of plasma, which allowed for the pharmacokinetic analysis to be conducted in individual animals. The assay has used to measure PM01218 plasma concentrations in support of preclinical studies.

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