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ORIGINAL ARTICLE

Quantitative analysis of a novel antimicrobial peptide in rat plasma by ultra performance liquid chromatography–tandem mass spectrometry

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Abstract We described the first results of a quantitative ultra performance liquid chromatography–tandem mass spectrometry method for a novel antimicrobial peptide (phylloseptin, PSN-1). Chromatographic separation was accomplished on a Waters bridged ethyl hybrid (BEH) C_{18} (50 mm × 2.1 mm, 1.7 μm) column with acetonitrile–water (25:75, v/v) as isocratic mobile phase. Mass spectrometry detection was performed in the positive electrospray ionization mode and by monitoring of the transitions at *m*/*z* 679.6/120, 509.6/120 (PSN-1) and *m*/z 340.7/165 (Thymopentin, IS). Protein precipitation was investigated and the recovery was satisfactory (above 82%). The method was shown to be reproducible and reliable with intra-day precision below 5.3%, inter-day precision below 14.2%, and linear range from 0.02 to 2 μg/mL with $r > 0.994$. The method was successfully applied to a pharmacokinetic study of PSN-1 in rats after intravenous administration.

1. Introduction

Host defense peptides are known to protect amphibians against a variety of pathogens [1]. The skins of anurans (frogs and toads), an important source of biologically active peptides, are regarded as both potential key to resolve the crisis of multiple drug-resistant strains and potential development into therapeutically valuable pharmaceutical agents [2–4]. Phylloseptins, a new family of antimicrobial peptides, is firstly isolated from the skin secretion of the South American amphibian *Phyllomedusa hypochondrialis*, a kind of frog native to Amazonian, Brazil, which showed a strong antimicrobial effect against Gram-positive and Gram-negative bacteria [5,6]. The phylloseptin (PSN-1) with a molecular mass of 2036.44 Da (Phe-Leu-Ser-Leu-Ile-Pro-His-Ile-Val-Gly-Val-Ala-Ser-Ile-Ala-Lys-His-Phe-amide) is a novel antimicrobial peptide from the skin secretion of the waxy monkey frog

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Phyllomedusa sauvagei [7]. Until now, the pharmacokinetic of phylloseptins *in vivo* has not been reported and the study is necessary for therapeutically valuable pharmaceutical peptides in the future.

In most cases, the development of immunoassays for a peptide is a challenge because its immunogenicity is low and it is small [8]. Recent advances in liquid chromatography–mass spectrometry (LC–MS) techniques made LC–MS enormously useful in polypeptide and protein analysis (e.g., determination of molecular mass, kinetic studies, sequence analysis). However, reports on the application of LC–MS for quantitative analysis of polypeptides/proteins in complex matrix are scarce [9–19]. Isotope dilution (ID) was used in some methods [9,13,14,17–19]. Indeed, because of the relative difficulty and hence high cost for synthesis of stable-isotopically labeled analogs of polypeptides, a diastereomer [15] or a homolog [16] as internal standard was used in the measurement procedure. In our study, an oligopeptide, thymopentin, was used as internal standard because it was easy to obtain. The established method was more applicable than the other methods.

Mass spectrometric analysis of PSN-1 acquired in the positive ion mode detected a sequence of ions with m/z 509.6 ($[M+4H]^{4+}$), m/z 679.6 ($[M+3H]^{3+}$), and m/z 1018.9 ($[M+2H]^{2+}$), which corresponded to the quadruply, triply, and doubly charged species, respectively. Singly charged molecular ion m/z 2037 ($[M+H]^{+}$) almost could not be detected. When choosing one of these ions for quantitative analysis by single ion monitoring (as the method reported [16]) or selective reaction monitoring, the data generated showed unacceptable linearity in the concentration range 0.02–2 μg/mL. The assay's precision was little improved when applying selected ion monitoring (SIM), which combines the ion currents of all three charge states to generate a total area under the curve (as the method [15]), but the linearity was still good enough. Both situations were attributed to variability in the three ions' distribution of intensities when the concentration of PSN-1 changed. However, after adding enough amount acid in the PSN-1 sample solution to overcome the variability, relative standard deviations (RSDs) and linearity were acceptable for pharmacokinetic study. For MS/MS analysis, the transitions at m/z 679.6/120, 509.6/120 were monitored and total area under the curve of combined daughter ion currents was used for calculation. To further optimize accuracy, inclusion of an internal standard was thought to be advisable.

We reported on first results of a quantitative method for plasma PSN-1 that used thymopentin as internal standard, protein precipitation for sample preparation, and UPLC-tandem mass spectrometry (MS/MS) for fast detection. The measurement procedure was optimized and its analytical performance characteristics were thoroughly evaluated. Based on the positive outcome of the latter investigations, the method was successfully applied to a pharmacokinetic study of PSN-1 in rats after intravenous administration.

2. Materials and methods

2.1. Materials

The novel phylloseptin (PSN-1, 98% pure) with a computed molecular mass of 2036.44 Da was synthesized by the Protein Synthesizer (PS3 Protein Technologies, Inc.). Thymopentin (internal standard, IS, Arg-Lys-Asp-Val-Tyr, 99.2% pure) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Acetonitrile and formic acid (Tedia, Fairfield, USA) were of HPLC grade. Stock solutions were prepared by dissolving appropriate amounts of these compounds in water. Double distilled water was used throughout the study.

2.2. Instrumentation

Liquid chromatography was performed on a Waters Acquity^{TM} Ultra Performance Liquid Chromatography (UPLC) system (Waters, Milford, MA). A 6 μL aliquot of the sample was injected into a Waters bridged ethyl hybrid (BEH) C_{18} (50 mm × 2.1 mm, 1.7 μm) column held at 30 °C. Mass spectrometric detection was carried out on a Micromass Quattro micro^{TM} API mass spectrometer (Waters Corp., Milford, MA, USA) with an electrospray ionization (ESI) interface and triple quadruple mass analyzer.

2.3. Sample pretreatment

Blood samples were collected from 6 rats after intravenous administration. All the samples were then transferred into heparinized tubes and immediately centrifuged at 3500 rpm (890*g*) for 10 min. The plasma was stored at –20 °C until utilized for the UPLC/MS/MS analysis.

To a 200 μL aliquot of plasma samples, 20 μL internal standard solutions (2 μg/mL) was added, and then 500 μL of acetonitrile (contain 0.2% formic acid, for increasing the solution of PSN-1) was added for protein precipitation. The mixture was vortexed for 1 min. After centrifugation at 11,200*g* for 10 min at 4 °C, the supernatant was transferred and evaporated to dryness at 30 °C under a gentle stream of nitrogen. The dried residue was then reconstituted in 200 μL of 10% formic acid water (v/v). The content was transferred to 2 mL glass vials and an aliquot of 6 μL was injected for UPLC/MS/MS analysis.

2.4. UPLC/MS/MS conditions

UPLC/MS/MS measurements were performed in the positive electrospray tandem MS mode, i.e., monitoring the transitions at m/z 679.6/120, 509.6/120 (total area of these two transitions was used for calculation), and 340.7/165 (Thymopentin, IS). The MS/MS settings were: collision energy 37 eV, cone 33 V, for m/z 679.6/120; collision energy 27 eV, cone 27 V, for m/z 509.6/120; collision energy 19 eV, cone energy 25 V, for m/z 340.7/165. Capillary voltage was set at 3 kV. Nitrogen was used as desolvation and cone gas with the flow rate of 600 and 50 L/h, respectively.

Isocratic elution was employed with the mobile phase composed of water and acetonitrile (75:25, *v*/*v*). The flow rate was set at 0.2 mL/min. Under these chromatographic conditions, the retention time for PSN-1 and thymopentin were 1.15 and 0.71 min, respectively. Analysis time was 1.8 min for one injection.

2.5. Analytical performance

Spiked plasma standards at seven concentrations over the range from 0.02 to 2 μg/mL were prepared and analyzed in

three consecutive analytical runs, with two groups in each run. The calibration curves were constructed by weighted ($1/x^2$) least-squares linear regression analysis of observed PSN-1-to-IS peak-area ratios against concentration. For the determination of LLOQ by analysis of six replicate plasma samples, the relative standard deviation (RSD) was less than 20% and signal-to-noise (S/N) was above 10. Quality control (QC) samples at three concentrations (0.05, 0.2, and 1.6 μg/mL) were analyzed to assess the precision and accuracy of the method. Six replicates were analyzed in each of three consecutive analytical runs. Accuracy was expressed as relative error (RE) and precision as relative standard deviation (RSD). Recovery of the extraction procedure was also evaluated at three concentration levels, by comparing the mean peak areas ($n=6$ for each concentration) obtained from plasma samples spiked before extraction with those from plasma samples spiked after extraction. To evaluate sample stability both after freeze–thaw cycles and at room temperature, six replicate QC samples at 0.05, 0.2, and 1.6 μg/mL were subjected to three freeze–thaw cycles (−20 to 25 °C) or were stored at room temperature for 4 h before sample processing. Six replicate QC samples at 0.05, 0.2, and 1.6 μg/mL were also processed and stored in an autosampler for 8 h. Long-term cold storage stability was also evaluated at three concentrations. Samples were assumed to be stable if their bias fell within ±15% of the theoretical value.

2.6. *Application to pharmacokinetic study*

Six male Wister rats (220–250 g) were purchased from the Experimental Animal Center of Shenyang Pharmaceutical University. Before drug administration, the rats were fasted overnight. The rats were given PSN-1 intravenously (3.0 mg/kg). Blood samples (approx. 0.5 mL) were collected from the ocular vein before (0 h) and 0.017, 0.05, 0.083, 0.17, 0.25, 0.5, 0.75, 1, 1.5, 2, 4, 6, and 8 h after dosing. For three rats, the sampling time was 0, 0.05, 0.17, 0.5, 1, 2, and 6 h. For the other three rats, the sampling time was 0.017, 0.083, 0.25, 0.75, 1.5, 4, and 8 h. All the samples were then transferred to heparinized tubes and immediately centrifuged at 3500 rpm (890g) for 10 min. The plasma obtained was stored at –20 °C until analysis.

3. Results and discussion

3.1. *Sample pretreatment and UPLC/MS/MS conditions*

Acetonitrile and methanol were investigated for protein precipitation. A higher recovery achieved by using acetonitrile. The solubility of peptide could be changed with the fluctuation of pH. PSN-1 was easily dissolved by adding 0.2% formic acid in water and 0.2% formic acid in acetonitrile was responsible for the increase of solubility.

In ESI negative ion mode, any charged molecular ions of PSN-1 almost could not be used for quantitation in this study, so ESI positive ion mode was employed. In current study, without adding acid in the sample, stably charged ions using 0.2% formic acid in mobile phase were investigated. It was found stably quadruply or triply charged ions were not produced and the linearity did not meet the criterion of pharmacokinetic study. While in the method reported [20], 0.2% formic acid in mobile phase is enough to produce stably doubly charged ions. The reason may be that the molecular weight of PSN-1 is much higher than that of Ximelagatran in the reported method [20]. PSN-1 was more complicated and 0.2% formic acid in mobile phase could not produce stably quadruply or triply charged ions.

Without adding acid in the sample for injecting into the UPLC/MS/MS system, full scan ESI mass spectra (m/z 100–2048) were generated in the positive ion mode and showed the quadruply, triply, and doubly charged molecular ions of PSN-1 with m/z 509.6 ($[M+4H]^{4+}$), m/z 679.6 ($[M+3H]^{3+}$), and m/z 1018.9 ($[M+2H]^{2+}$). Singly charged molecular ion m/z 2037.6 ($[M+H]^{+}$) almost could not be found (Fig. 1A).

In ESI-MS analysis, lowering the pH of the sample by addition of formic or acetic acid (ca. 1–10%) favors the ionization process by enhancing the degree of protonation of peptides in solution [21]. After adding enough amount acid in the PSN-1 sample solution to overcome the variability, RSD and linearity were acceptable.

After adding 10% formic acid in the reconstitution solution, m/z 509.6 ($[M+4H]^{4+}$), m/z 679.6 ($[M+3H]^{3+}$) could be found in the full scan ESI mass spectra, and the intensity of quadruply charged ion was twice as much as that of triply charged ion. The quintuply and doubly charged molecular ions (m/z 408.2, m/z 1018.9) almost could not be detected. Internal standard thymopentin with m/z 227.2 ($[M+3H]^{3+}$), m/z 340.7 ($[M+2H]^{2+}$), and m/z 680.9($[M+H]^{+}$) (Fig. 1B) was

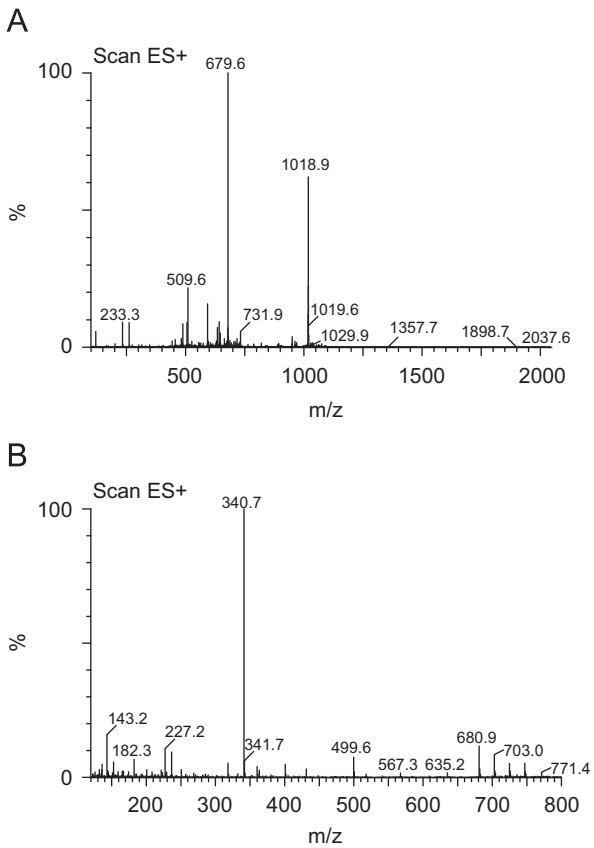

Figure 1 Full scan ESI^{+}mass spectra of PSN-1 generated in the positive ion mode (A, without adding acid in the sample) and full scan ESI mass spectra of thymopentin (B, adding 10% formic acid in the sample).

shown in the mass spectra, only m/z 340.7 was chosen as parent ion.

Mass spectrometric analysis of PSN-1 acquired in the positive ion mode, and the transitions monitored were m/z 509.6/120 ($[M+4H]^{4+}$) and m/z 679.6/120 ($[M+3H]^{3+}$). When choosing one of them for quantitative analysis by selective reaction monitoring(SRM), the data generated showed RSDs were not good enough. However, the assay's precision was significantly improved when the daughter ion currents of two charged states were combined to generate a total area. The typical multiple reaction monitoring (MRM) chromatograms are shown in Fig. 2. By comparing the ratio of peak areas of two ion species in selected reaction monitoring (SRM) mode with that in selected ion monitoring (SIM) mode, the ratios were the same in different mode. When the concentration was 0.05 μg/mL, the ratios of peak area of 4+ to that of 3+ ions were about 1.7 in both modes. When the concentration was 1.6 μg/mL, the ratios were about 1.1 in both modes. Specificity for SRM was better than that for SIM, so SIM mode was replaced by SRM mode.

The investigation of linearity was the key for the determination of PSN-1. When choosing one of charged ions for quantitative analysis by single ion monitoring or selective reaction monitoring, or combining the ion currents of all charge states to generate a total area under the curve in our study, the data generated showed unacceptable linearity over the concentration range 0.02–2 μg/mL. Both situations were attributed to variability in the each ion's distribution of intensities when the concentration of PSN-1 changed and pH played an important role in the distribution of intensities. For the example in our study, 0.2 and 2 μg/mL PSN-1 were prepared in 0.2% formic acid, respectively, and m/z 679.6/120 ($[M+3H]^{3+}$) was chosen for quantitative analysis by single ion monitoring, the peak area of 2 μg/mL PSN-1 was only about three times as much as that of 0.2 μg/mL PSN-1 under the same UPLC/MS condition, though the ten times of concentration existed between two samples. The RSD% of peak areas of PSN-1 was not acceptable. The concentration of formic acid in reconstitution solution was investigated. The different concentrations of peptide (PSN-1) in 0%, 0.2%, 2%, 5%, 8%, and 10% (v/v) formic acid water were injected into the UPLC/MS/MS system. When 10% formic acid was applied, the linearity was best among them. Larger amount of formic acid was not investigated and 10% formic acid was not used in the mobile phase, because low pH could do harm to the column and mass system.

The peak of PSN-1 was divided to 2 peaks, which was due to the difference between reconstitution solution and mobile

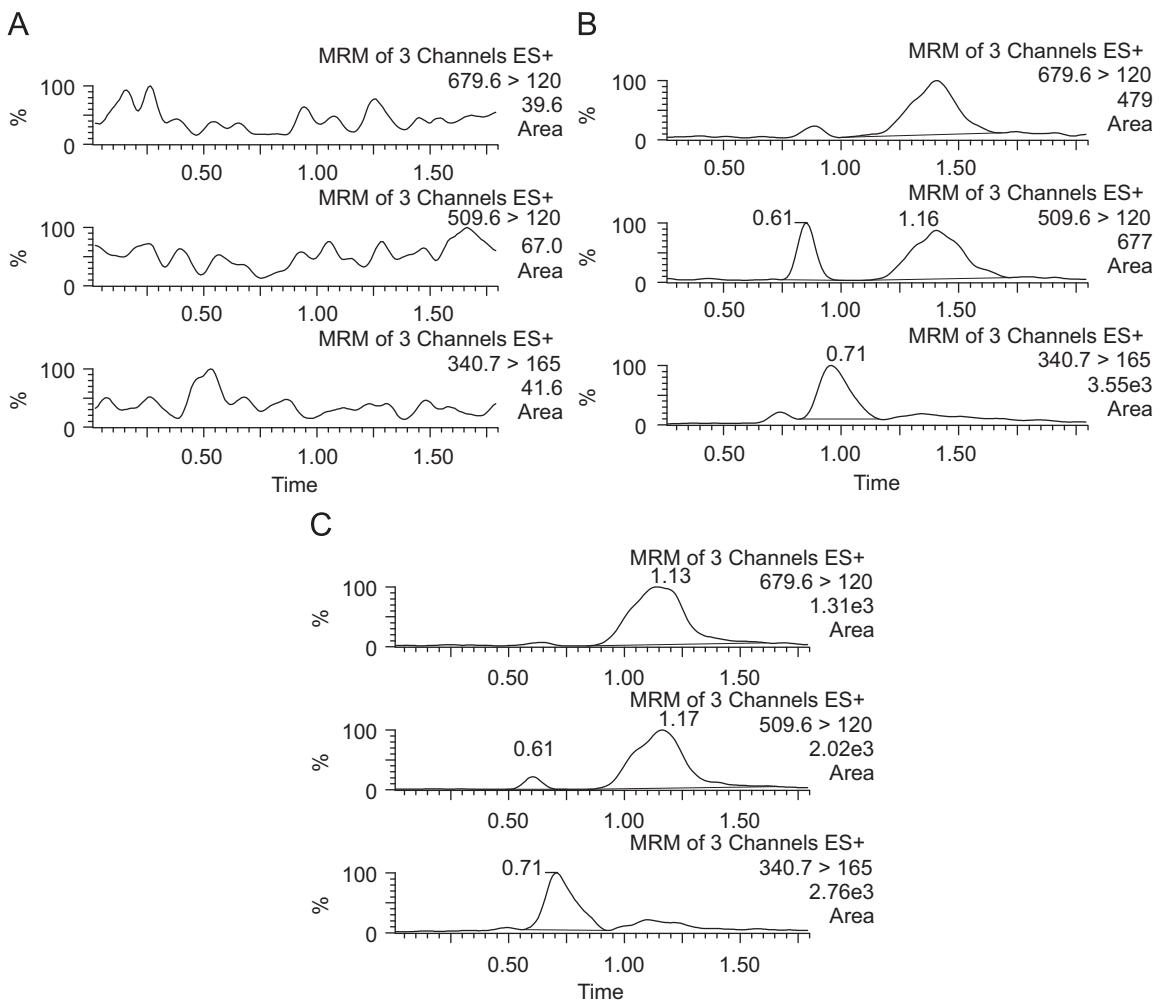

Figure 2 MRM chromatograms of blank plasma (A), blank plasma spiked with PSN-1 (m/z 679.6/120, m/z 509.6/120, 0.02 μg/mL, LLOQ) and thymopentin (m/z 340.7/165, IS) (B), and subject plasma sample obtained from a rat 2 h after intravenous administration (C).

phase [22]. The peak ahead was much smaller than the behind and the retention time was 0.61 and 1.16 min, respectively. The total area of 2 peaks was used for calculation.

3.2. Calibration and assay validation

Spiked plasma standards at six concentrations over the range 0.02–2 μg/mL were prepared and analyzed in three consecutive analytical runs, with two groups in each run. The calibration plots were constructed by weighted ($1/x^2$) least-squares linear regression analysis of observed peptide-to-IS peak-area ratios against concentration. The mean regression equation for the calibration plot was $y=11.99x-0.135$, with a correlation coefficient >0.994. Analysis of six replicate plasma samples showed the LLOQ of the method was 0.02 μg/mL, S/N was above 10 and the RSD was 12.9%. Quality control (QC) samples at three concentrations (0.05, 0.2, and 1.6 μg/mL) were analyzed to assess the precision and accuracy of the method. Six replicates were analyzed in each of three consecutive analytical runs. Accuracy was expressed as RE and precision as RSD. Recovery of the extraction procedure was also evaluated at three concentrations, by comparing the mean peak areas ($n=6$ for each concentration) obtained from plasma samples spiked before extraction with those from plasma samples spiked after extraction.

The result listed in Table 1 was indicative of good analytical characteristics of the assay. To evaluate sample stability both after freeze–thaw cycles and at room temperature, six replicate QC samples at 0.05, 0.2, and 1.6 μg/mL were subjected to three freeze–thaw cycles (−20 to 25 °C) or were stored at room temperature for 4 h before sample processing. Six replicate QC samples at 0.05, 0.2, and 1.6 μg/mL were also processed and stored in an autosampler for 8 h. Long-term cold storage stability was also evaluated at three concentrations. Samples were assumed to be stable if their bias fell within ±15% of the actual value. Results from testing of the freeze–thaw, short-term, post-preparative, and long-term stability of peptide, summarized in Table 2, showed that peptide is sufficiently stable under these conditions.

Suppression of ionization was evaluated by comparing the absolute peak area from a control plasma sample extracted and then spiked with a known amount of analyte, to that of a neat standard prepared in the same reconstitution solvent. The samples at 0.05, 0.2, and 1.6 μg/mL concentrations were analyzed. The ratios of the absolute peak area at the same concentration were within 0.94–1.06. The results showed that the influence of the matrix effect could be ignored. 10% formic acid in reconstitution solvent had a positive effect on the ionization of PSN-1.

3.3. Pharmacokinetic study of PSN-1 in rats

The plasma concentrations of PSN-1 at different time are expressed as mean ± SD, and mean concentration–time curve was plotted (Fig. 3).

All of the pharmacokinetic data listed in Table 3 were obtained on the basis of non-compartmental analysis (DAS 2.1 statistical software; Pharmacology Institute of China). Pharmacokinetic study of PSN-1 in rats after intravenous administration was investigated. A complete plasma concentration–time curve was obtained. The fate of PSN-1 in rats after intravenous administration (3.0 mg/kg) was indicative of fast elimination process with $t_{1/2}$ of 2.08 h.

Table 1 Precision, accuracy, and recovery for the determination of PSN-1 in rat plasma ($n=6$).

Concentration (μg/mL)		RSD (%)		RE (%)	Recovery (%)
Added	Found (mean)	Intra-assay	Inter-assay		
0.05	0.048	5.2	14.2	−3.1	89.5 ± 11.1
0.2	0.19	5.3	12.9	−4.6	86.5 ± 6.1
1.6	1.49	3.6	10.3	−7.0	82.4 ± 5.1

Table 2 Summary of stability of PSN-1 in rats plasma ($n=6$).

Stability	Concentration added								
	0.05 μg/mL			0.2 μg/mL			1.6 μg/mL		
	Found (mean, μg/mL)	Precision (RSD, %)	Accuracy (RE, %)	Found (mean, μg/mL)	Precision (RSD, %)	Accuracy (RE, %)	Found ((mean, μg/mL)	Precision (RSD, %)	Accuracy (RE, %)
Three freeze–thaw cycles	0.050	9.9	−0.8	0.207	8.7	3.4	1.579	7.4	−1.3
Short term (room temperature for 4 h)	0.053	2.3	5.2	0.189	3.7	−5.5	1.515	4.9	−5.3
Long term (−20 °C for 10 days)	0.048	4.4	−4.9	0.180	5.6	−10.1	1.571	2.7	−1.8
Post-preparative (25 °C for 8 h)	0.049	3.7	−1.2	0.183	2.9	−8.6	1.535	2.1	−4.1

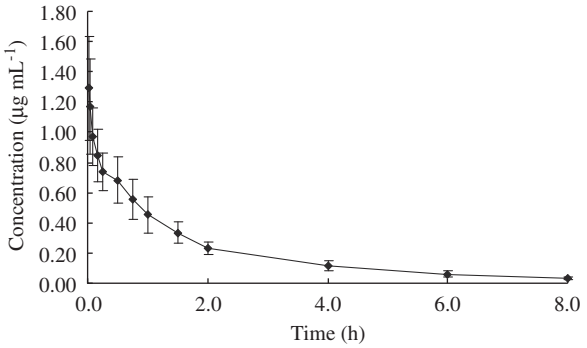

Figure 3 Mean plasma concentration–time profile after intravenous administration of PSN-1 to rats ($n=6$).

Table 3 Pharmacokinetic data in rats after intravenous administration of 3.0 mg/kg PSN-1 ($n=6$).

Parameter	Estimate (mean ± SD)
t_{max} (h)	0.017 ± 0
C_{max} (μg/mL)	1.29 ± 0.34
$t_{1/2}$ (h)	2.08 ± 0.31
Vd (L/kg)	5.23 ± 0.84
Clp [L/(h kg)]	1.782 ± 0.405
AUC_{0-t} (μg h/mL)	1.658 ± 0.356
$AUC_{0-\infty}$ (μg h/mL)	1.759 ± 0.404

4. Conclusions

A new method had been established for analysis of PSN-1 in plasma. The recovery of the method was satisfactory (in the range 82–90%). This method is especially suitable for determination of peptide from biological matrixes. Combined with UPLC/MS/MS detection, it can be highly effective in the extraction and analysis of peptide from rat plasma. Pharmacokinetic study was necessary for more and more therapeutically active peptides in the future. This method has been shown to enable simple, rapid, and reliable assay of peptide in biological samples.

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