LC–MS-MS for the Determination of Ponicidin in Dog Plasma

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Received 15 November 2012; revised 19 December 2012

A highly specific and sensitive liquid chromatography–tandem mass spectrometry method has been developed and validated for the determination of ponicidin in dog plasma. The plasma samples were prepared using liquid–liquid extraction with ethyl acetate as the extraction solvent. Chromatographic separation was accomplished on a Waters X Terra MS C18 column. The extracted ponicidin and the internal standard, oridonin, were detected by tandem mass spectrometry in positive electrospray ionization mode with multiple reaction monitoring. The optimized mass transition ion pairs (m/z) for quantitation were 363.08–345.08 for ponicidin and m/z 365.10–347.06 for the internal standard. The lower limit of quantification was 5 ng/mL. The linear range of the method was from 5 to 5,000 ng/mL. The intra-day and inter-day precision measurements were lower than 5.3 and 6.0% in terms of relative standard deviation and the accuracy was within ± 8.4% in terms of relative error. Additionally, no significant matrix ionization suppression or enhancement was observed. The validated method was successfully applied in a pharmacokinetic study of ponicidin in dogs. The primary pharmacokinetic parameters in dogs were: terminal elimination half-life, 8.14 ± 1.35 h; mean residence time, 12.30 ± 2.08 h; area under the plasma concentration–time curve from time zero to the last measurable concentration, 14.34 ± 1.37 μg/h/mL; area under the plasma concentration–time curve from time zero to infinity, 15.75 ± 1.44 μg/h/mL; apparent volume of distribution, 4.79 ± 1.68 L/kg; total body clearance, 0.41 ± 0.08 L/kg/h.

Introduction

Rabdosia rubescens (Chinese name “Donglingcao”) is an herbal medicine that is traditionally used in China for the treatment of tonsillitis and a variety of cancers, including esophageal carcinoma, gastric cardia carcinoma, primary hepatic carcinoma and breast cancer (1–2). Ponicidin, a diterpenoid extracted from Rabdosia rubescens, is one of the major anti-tumor components of this herb (3–4). Recent laboratory data has suggested that ponicidin has obvious inhibitory effects on human breast cancer cells (5), lung cancer cells (6), hepatocellular carcinoma cells (7), myeloid leukemia cells (8) and monocytic leukemia cells (9) in vitro. Studies have also demonstrated that ponicidin may inhibit the growth and metastasis of prostate cancer due to its significant anti angiogenic activity (10).

As a promising anti-tumor agent, a better understanding of the pharmacokinetics of ponicidin is very important for explaining its mechanism of action and therapeautic effect. According to the literature, only one laboratory until now has used a high-performance liquid chromatography–ultraviolet (HPLC–UV) method for the determination of ponicidin in rats (11). The sensitivity (100 ng/mL) of the HPLC–UV method was found to be inadequate for the pharmacokinetic (PK) profiling of ponicidin in dogs by its administration via conventional routes. Thus, a novel analysis method for ponicidin in dog plasma was established by using liquid chromatography–tandem mass spectrometry (LC–MS-MS).

During recent years, LC–MS-MS method has proven to be a powerful technique for the rapid, quantitative determination of drugs in biological fluids. In this study, a sensitive LC–MS-MS method in positive electrospray ionization (ESI+) mode with multiple reaction monitoring (MRM) was developed and validated to quantify ponicidin in dog plasma, and has been successfully applied to a PK study of ponicidin in dogs.

Experimental

Materials and reagents

Ponicidin (99.8% purity; Figure 1A) and oridonin [internal standard (IS), 99.8% purity; Figure 1B] were supplied by the new drug research and development centre of Zhengzhou University. HPLC-grade methanol and acetoneitrile were purchased from Fisher Scientific (Fair Lawn, NJ). HPLC-grade formic acid was purchased from Tedia (Fairfield, OH). Analytical grade ethyl acetate was purchased from Tianjin Kernel Chemical Reagents Development Centre (Tianjin, China). Ultra-pure water from a Milli-Q Plus water purification system (Millipore, Bedford, MA) was used throughout the study. Male and female beagle dogs (10 ± 0.5 kg) were purchased from Henan Provincial Laboratory Animal Center (Zhengzhou, China). The animals were acclimated for one week and feed standard beagle dog chow and sterilized tap water. Before use, all beagle dogs were maintained under standard laboratory conditions on a 12 h light/dark cycle. All experimental procedures were conducted in accordance with the guidelines of the Animal Care Committee of Henan Provincial Laboratory Animal Center.

Preparation of standard and quality control samples

Ponicidin and IS stock solutions were prepared at 100 μg/mL in 100% methanol. The stock solution of ponicidin was serially diluted with 50% methanol to obtain working solutions at concentrations over 0.05–25 μg/mL. The stock solution of IS was diluted with 50% methanol to obtain a working solution at 1,250 ng/mL. All solutions were stored at 4°C and brought to room temperature before use. The analytical standard and quality control (QC) samples were prepared by spiking 500 μL of blank dog plasma with 10 μL of ponicidin standard working solutions for validation and during each experimental run for the PK study. Calibration samples were made at concentrations of 5, 10, 25, 75, 250, 750, 2,500 and 5,000 ng/mL for ponicidin.
QC samples were at concentrations of 10 (low), 250 (medium) and 4,000 ng/mL (high).

Sample preparation
To 500 μL of dog plasma in a tapered tube, 10 μL of the IS working solution (250 ng/mL of oridonin) was added. After vortex mixing for 30 s, the sample was extracted with 3 mL of ethyl acetate by vortex mixing for 3 min. After centrifugation at 4,000 rpm (1,450 × g) for 10 min, the upper organic layer was separated and evaporated to dryness at 40 °C in a vacuum concentration system (Concentration Plus, Eppendorf AG, Hamburg, Germany). The residue was reconstituted in 500 μL of 50% methanol, vortex mixed for 1 min and centrifuged at 13,000 rpm (15,300 × g) for 10 min. Finally, an aliquot of 5 μL of the supernatant was injected into the LC–MS-MS system for analysis. For samples containing poncidin at a concentration higher than the upper limit of the range in the standard curve, an aliquot of the sample was first diluted with blank dog plasma; then, 500 μL of the diluted sample was treated as described previously.

LC–MS-MS
A Micromass Quattro micro triple quadrupole mass spectrometer (Waters, Manchester, UK) equipped with an ESI interface was used for analysis and detection. The ESI source was set in positive ionization mode. Quantification was performed using MRM mode. Nitrogen was used as desolvation gas (350 L/h). For collision-induced dissociation (CID), argon was used as the collision gas and the collision energy was optimized as 15 eV for poncidin and 13 eV for IS. The capillary voltage was 3.0 kV and cone voltage was 15 V. The entrance and exit energies of the collision cell were set at 1 and 2 V, respectively. The source and desolvation temperatures were kept at 120 and 300 °C, respectively. On the basis of full-scan mass spectra of poncidin and IS, the most abundant ions were selected and the mass spectrometer was set to monitor the transitions of the precursors to the product ions as follows: m/z 363.08–345.08 for poncidin and m/z 365.10–347.06 for oridonin (IS) with a dwell time of 200 ms. Data acquisition, peak integration and calibration were performed with MassLynx 4.0 software (Micromass).

A Waters Alliance 2695 separation module (Avondale, CA) was used for solvent and sample delivery. Chromatographic separation was achieved by using a Waters X Terra MS C18 column (5 μm, 150 × 2.1 mm i.d.; Waters, Milford, MA) with an X Terra MS C18 precolumn (5 μm, 20 mm × 2.1 mm i.d.; Waters). The mobile phase consisted of acetonitrile–water–formic acid (30:70:0.001, v/v/v), pumped at a flow rate of 0.2 mL/min. Total run time was 5 min for each injection.

Method validation
The method was validated for selectivity, linearity, accuracy, precision, recovery and stability. The selectivity of the method was evaluated by analyzing six blank dog plasma samples and six spiked plasma samples at the lower limit of quantification (LLOQ) from six different sources. Peak areas of endogenous compounds co-eluting with the analyte should be less than 20% of the peak area of the LLOQ standard, and the deviation from the nominal concentrations for the LLOQ in these six plasma batches should be within ±20%. Absolute and relative matrix effects were evaluated. To evaluate the absolute matrix effect (ME), six different sources of blank plasma were extracted and then spiked with the analyte at three QC concentrations. The corresponding peak areas of the analyte spiked post-extraction (B) were compared to those of the analyte dissolved in matrix component-free reconstitution solvent (A) at equivalent concentration. The ratio (B/A × 100%) was defined as the ME. The assessment of the relative ME, expressed as relative standard deviation (RSD) (%), was made by a direct comparison of the analyte peak area values between different sources of plasma. The inter-subject variability of ME at each concentration level should be less than 15% (12). The same evaluation was performed for IS (250 ng/mL in plasma). Linearity was assessed by preparing and analyzing poncidin standard samples over 5–5,000 ng/mL in blank dog plasma. Calibration curves were analyzed by weighted least-square linear regression (1/x²) of the peak area of poncidin over that of the IS. The acceptance criterion for each back-calculated standard concentration was 15% deviation from the nominal value. The LLOQ was defined as the lowest concentration on the calibration curve that could be measured with acceptable precision and accuracy, and with at least five times the response compared with the blank response. It was determined in six replicates over three validation days. The precision should be equal to or less than 20% and relative error within ±20% for both intra-day and inter-day. Accuracy and precision were assessed by determining QC samples at three concentration levels (10, 250 and 4,000 ng/mL) in six replicates on three consecutive validation days. The precision was determined as the RSD and the accuracy was expressed as relative error (RE): (observed concentration nominal concentration)/(nominal concentration) × 100%. The intra-day and inter-day precision measurements were required to be below 15% and the accuracy was required to be within ±15%. The extraction recovery for poncidin was estimated at three concentration levels (10, 250 and 4,000 ng/mL) by comparing two groups of control samples: analyte spiked after the extraction of blank plasma
analyte spiked to plasma and prepared normally (pre-extraction) (C). Extraction recovery was calculated as the response ratio \( C/B \times 100\% \). The reproducibility of the extraction procedure was determined as RSD (\%). The extraction recovery of the IS was determined in a similar way. The stability of ponicidin in dog plasma was assessed by analyzing replicates \( (n = 3) \) of plasma samples at concentrations of 10, 250 and 4,000 ng/mL, which were exposed to different conditions (time and temperature). The short-term stability was determined after the exposure of the spiked samples to room temperature (25°C) for 2 h, or after the exposure of the ready-to-inject samples (after extraction, in reconstitution solvent) to the autosampler rack (25°C) for 24 h. The long-term stability was evaluated after storage of the standard spiked plasma samples at \(-20^\circ\text{C}\) for 1 month. The freeze/thaw cycle stability was assessed after three complete freeze/thaw cycles (\(-20\) to 25°C) on consecutive days. The analyte was considered to be stable in dog plasma when 85–115% of the initial concentrations were found.

Application to pharmacokinetic study

Six beagle dogs (three males and three females) were given a single dose of ponicidin (4 mg/kg) via the hindlimb vein. Blood samples (2 mL) were collected in heparinized tubes from the forelimb vein before dosing and at 0.083, 0.25, 0.5, 0.75, 1, 1.5, 2, 4, 6, 9, 12, 24 and 36 h after drug administration; samples were centrifuged at 4,000 rpm (1,450 \( \times \) g) for 10 min to separate the plasma fractions. The collected plasma samples were stored at \(-20^\circ\text{C}\) until analysis.

Results

Figure 2 shows the chromatograms obtained for the analysis of blank plasma (Figure 2A), LLOQ sample (Figure 2B) and a sample of authentic dog plasma taken at 24 h after intravenous administration of 4 mg/kg of ponicidin (Figure 2C). The retention time \( (t_R) \) of ponicidin was 3.27 min and \( t_R \) of the IS was 4.02 min. The mass spectrometric condition was performed in positive ion mode detection. Ponicidin was resolved on a Waters XTerra MS C18 column with a mobile phase consisting of a mixture of acetonitrile–water–formic acid (30:70:0.001, v/v/v).

The analytical method to determine the sample concentration of ponicidin in beagle dog plasma showed a non-significant matrix effect in six beagle dogs.

Tables I–IV show the confidence limits of the analytical methods for the determination of plasma concentrations of ponicidin. The plasma concentration versus time curves are shown in Figure 3. The plasma concentration declined with terminal elimination half-life \( (t_{1/2}) \) of 8.14 ± 1.35 h and mean residence time
**Table I**
Matrix Effect Data in Six Different Lots of Dog Plasma (n = 6)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (ng/mL)</th>
<th>ME (Mean ± SD) (%)</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ponicidin</td>
<td>10</td>
<td>102.4 ± 2.9</td>
<td>4.8</td>
</tr>
<tr>
<td></td>
<td>425</td>
<td>108.5 ± 3.5</td>
<td>5.3</td>
</tr>
<tr>
<td></td>
<td>4,000</td>
<td>99.8 ± 2.1</td>
<td>4.1</td>
</tr>
<tr>
<td>Oridonin (IS)</td>
<td>250</td>
<td>104.7 ± 3.2</td>
<td>3.5</td>
</tr>
</tbody>
</table>

[Note: Samples were analyzed over three consecutive days, six replicates for each day.]

**Table II**
Precision and Accuracy for the Analysis of Ponicidin in Dog Plasma*

<table>
<thead>
<tr>
<th>Added (ng/mL)</th>
<th>Found (ng/mL)</th>
<th>Intra-day RSD (%)</th>
<th>Inter-day RSD (%)</th>
<th>Accuracy, RE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 (LLOQ)</td>
<td>5.4</td>
<td>4.1</td>
<td>4.6</td>
<td>6.1</td>
</tr>
<tr>
<td>10 (low)</td>
<td>10.2</td>
<td>5.3</td>
<td>6.0</td>
<td>8.4</td>
</tr>
<tr>
<td>250 (medium)</td>
<td>3.9</td>
<td>4.4</td>
<td>7.2</td>
<td></td>
</tr>
<tr>
<td>4,000 (high)</td>
<td>4217</td>
<td>3.5</td>
<td>5.4</td>
<td></td>
</tr>
</tbody>
</table>

*Note: Samples were analyzed over three consecutive days, six replicates for each day.

**Table III**
Recovery of Ponicidin and IS in Dog Plasma (n = 6)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (ng/mL)</th>
<th>Recovery (Mean ± SD) (%)</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ponicidin</td>
<td>10</td>
<td>78.7 ± 4.1</td>
<td>5.2</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>83.2 ± 3.4</td>
<td>5.3</td>
</tr>
<tr>
<td></td>
<td>4,000</td>
<td>75.4 ± 2.9</td>
<td>3.8</td>
</tr>
<tr>
<td>Oridonin (IS)</td>
<td>250</td>
<td>76.2 ± 3.5</td>
<td>4.6</td>
</tr>
</tbody>
</table>

**Table IV**
Stability of Ponicidin during Sample Storage, Preparation and Analysis (n = 3)

<table>
<thead>
<tr>
<th>Concentration (ng/mL)</th>
<th>Recovered (%)</th>
<th>25°C, 2 h</th>
<th>−20°C, 1 month</th>
<th>3 freeze-thaw cycles</th>
<th>Autosampler, 25°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>89.2 ± 2.5</td>
<td>92.7 ± 3.2</td>
<td>92.9 ± 2.3</td>
<td>98.5 ± 2.9</td>
<td></td>
</tr>
<tr>
<td>250</td>
<td>93.5 ± 3.9</td>
<td>94.1 ± 4.2</td>
<td>95.3 ± 3.0</td>
<td>101.3 ± 5.5</td>
<td></td>
</tr>
<tr>
<td>4,000</td>
<td>94.4 ± 3.1</td>
<td>95.2 ± 3.7</td>
<td>93.8 ± 4.5</td>
<td>100.9 ± 3.4</td>
<td></td>
</tr>
</tbody>
</table>

**(MRT) of 12.30 ± 2.08 h; the area under the plasma concentration–time curve from time zero to the last measurable concentration (AUC(0–t)) and area under the plasma concentration–time curve from time zero to infinity (AUC(0–∞)) values were 14.34 ± 1.37 and 15.75 ± 1.44 μg/h/mL; the apparent volume of distribution (Vd) was 4.79 ± 1.68 L/kg; total body clearance (CL) was estimated to be 0.41 ± 0.08 L/kg/h.**

**Discussion**

**Mass spectrometric conditions**

Ponicidin and oridonin (IS) exhibited favorable sensitivity in positive ion mode detection. ESI was found to be more sensitive than atmospheric pressure chemical ionization (APCI). Under ESI+ conditions, ponicidin and oridonin (IS) formed predominantly protonated molecules [M + H]+ at m/z 365.08 and 365.10, respectively, in Q1 full scan mass spectra. The corresponding production mass spectra are depicted in Figure 2, where the [M + H]+ of each compound was selected as the precursor ion. Ponicidin showed a major fragment ion at m/z 345.08 and oridonin (IS) showed a major fragment ion at m/z 347.00. The major fragment ion of each compound was derived from the loss of a water molecule. The collision energy in the production MS-MS mode was investigated from 5 to 50 eV to optimize the sensitivity; optimal values were found to be 15 eV for ponicidin and 13 eV for oridonin.

**Chromatographic conditions**

Various combinations of acetonitrile, methanol and water with changed contents of each component were investigated and compared to identify the optimal mobile phase that produced the best sensitivity, efficiency and peak shape. Acetonitrile led to a higher mass spectrometric response and a lower background noise than methanol, and was chosen as the organic phase. Various proportions of acidic modifier (including formic acid and acetic acid) were added to the mobile phase, and it was found that 0.001% formic acid in the mobile phase could improve the MS response of ponicidin and IS two-fold. Therefore, a mobile phase consisting of acetonitrile–water–formic acid (30:70:0.001, v/v/v) was used in the experiment.

**Sample preparation**

Sample preparation is a critical step for accurate and reliable LC–MS-MS assays. Currently, the most widely employed biological sample preparation methodologies are liquid–liquid extraction (LLE) and protein precipitation (PPT). In the early stage of method development, a PPT method was employed to separate ponicidin from plasma samples; however, strong ion suppression occurred from the endogenous substances in plasma and the recovery was very low. Following this, an LLE procedure was adopted. To obtain optimum recovery, five organic extraction solvents were evaluated, including ethyl ether, ethyl acetate, acetone, chloroform and dichloromethane. Ethyl acetate was found to provide high recovery for ponicidin and IS without any significant interference. Therefore, ethyl acetate was chosen as the suitable extraction solvent.

**Method validation**

**Selectivity**

The LC–MS-MS method had high selectivity because only ions derived from the analyte and IS were monitored. Figure 3 shows the typical MRM chromatograms of blank dog plasma (A), blank dog plasma spiked with ponicidin at LLOQ (5 ng/mL; 250 ng/mL for IS) (B) and authentic dog plasma sample taken at 24 h after intravenous administration of 4 mg/kg of ponicidin (C). The figure shows no significant endogenous interferences at the retention times of the analyte and IS. Typical retention times for ponicidin and IS were 3.27 and 4.02 min, respectively.

**Matrix effect**

Because ME can affect the reproducibility of the assay (13), the co-elution effect and potential ion suppression or enhancement were evaluated. An ME value of 100% indicated that no absolute ME was observed. A value of >100% indicated ionization
Figure 3. Typical MRM chromatograms: blank dog plasma (A); blank dog plasma spiked with ponicidin at LLOQ (5 ng/mL) and IS (250 ng/mL) (B); authentic dog plasma sample taken at 24 h after intravenous administration of 4 mg/kg of ponicidin (C). Ponicidin $t_{R}$: 3.27 min; IS $t_{R}$: 4.02 min.
enhancement and a value of <100% indicated ionization suppression. ME data at different QC concentrations in six different sources of dog plasma are presented in Table I. The absolute ME values were 99.8–106.5%, indicating no significant ion suppression or enhancement effects. The variability was acceptable, with RSD values <5.3% at different concentrations. These data confirm that the relative ME for the analyte was not significant. Thus, no matrix ion suppression or enhancement effects were observed and the analytical method was considered to be reliable. In addition, the cross-talk between MS-MS channels of ponidin and IS was assessed by separately injecting a plasma extract containing only ponidin or IS at LLOQ concentration and monitoring the response in the other channel. No cross-talk was observed between channels.

**Linearity and sensitivity**
Acceptable linearity of spiked ponidin concentrations was obtained from 5 to 5,000 ng/mL. The regression coefficients ($r$) were greater than 0.99. A typical regression equation of the calibration curve on a validation run was as follows: $y = 3.9 \times 10^{-7}x + 5 \times 10^{-4}$, $r = 0.9957$, where $y$ represented the peak area ratio of ponidin to IS and $x$ represented the plasma concentration of ponidin. For each point on the calibration curve, the concentration that was back-calculated from the equation of the regression analysis was within acceptable limit for accuracy. Using 500 µL of dog plasma, the LLOQ of ponidin was 5 ng/mL [signal-to-noise ($S/N$) ratio >10], with intra-day and inter-day precision at 4.1 and 4.6%, respectively, and relative error 6.1%. Under the achieved LLOQ of 5 ng/mL, ponidin could be determined in plasma samples up to 36 h after a single intravenous administration dose of 4 mg/kg, which was sensitive enough to investigate the pharmacokinetic behavior of ponidin.

**Precision and accuracy**
The intra-day and inter-day precision and accuracy for ponidin are summarized in Table II. Intra-day and inter-day precisions were measured to be below 5.3 and 6.0%, respectively, with RE from 5.4 to 8.4%. These results demonstrated that the values were within the acceptable range and that the method was accurate and precise.

**Recovery**
The recovery of ponidin and IS from dog plasma are summarized in Table III. The recovery of ponidin ranged over 75.4–83.2%, and was similar at the three QC concentrations without significant concentration dependence. The recovery of IS was 76.2 ± 3.5%.

**Stability**
The results of stability experiments (Table IV) showed that ponidin was stable during sample storage (in plasma at 25°C for 2 h, in plasma at −20°C for 1 month), processing procedures (three freeze-thaw cycles) and post-treatment (in constitution solvent at 25°C for 24 h). The measured concentrations were all within the range of 85–115%, compared with freshly prepared QC samples. In addition, the stock solution of ponidin in methanol was shown to be stable for 1 month at 4°C (RE from 1.2 to 1.9%).

**Dilution effect**
The dilution effect of blank dog plasma was evaluated with spiked samples (7.5 and 25 µg/mL) diluted with blank dog plasma to 750 and 2,500 ng/mL ($n = 6$) and prepared following the same sample preparation procedure. The accuracy and precision were all within the acceptable range and no unacceptable dilution effect was found.

**Application to pharmacokinetic study**
The validated method was applied to determine the plasma concentrations of ponidin and to characterize the pharmacokinetics of ponidin after a single intravenous administration dose of 4 mg/kg ponidin to six dogs. The profile of mean ponidin plasma concentration versus time is presented in Figure 4; the primary pharmacokinetic parameters of ponidin were estimated using the 3P97 computer program written by the Chinese Society of Mathematical Pharmacology. The pharmacokinetic parameters of ponidin in rats have been reported (11). The primary pharmacokinetics of ponidin with I.V. administration were different in dogs and rats; in particular, the $t_{1/2}$ in dogs (8.14 h) was longer than in rats (5.22 h). One reason for this is probably the different species.

**Conclusion**
A rapid, specific and sensitive LC–MS–MS method was developed and fully validated for the determination of ponidin in dog plasma. The chromatographic and mass spectrometric conditions were optimized to achieve an LLOQ of 5 ng/mL. The method allowed the accurate and reproducible determination of ponidin plasma samples and has been successfully applied to characterize the pharmacokinetics of ponidin in dogs.

**References**


