Determination of Pantoprazole in Rat Plasma by LC–MS/MS and its Application to Pharmacokinetics

Xiangjun QIU 1, Ren-ai XU 2, Yuancai ZHENG 2, Haichao ZHAN 3, Zhe WANG 3 & Lufeng HU 2 *

1 Department of Pharmacology, Medical College of Henan University of Science and Technology, Luoyang, 471003, China
2 The First Affiliated Hospital of Wenzhou Medical College, Wenzhou, 325000, China
3 Pharmacy School of Wenzhou Medical College, Wenzhou, 325035, China

SUMMARY. A highly sensitive liquid chromatographic mass spectrometric (LC-MS/MS) method for determination of pantoprazole in rat plasma using omeprazole as the internal standard (IS) was developed. Plasma samples were precipitated by acetonitrile and separated on a Zorbax SB-C18 column with gradient profile at a flow of 0.4 mL/min. Detection was carried out by SIM mode on an ion-trap LC-MS/MS system with an electrospray ionization interface. The lower limit of quantification (LLOQ) was 5 ng/mL. Calibration curve was linear over the range from 5 to 5000 ng/mL. The intra- and inter-run relative standard deviations of the assay were less than 7 %. The mean absolute recoveries determined at the concentrations of 25, 400, and 4000 ng/mL were 87.40 ± 4.40 %, 87.77 ± 3.30 %, and 92.78 ± 5.02 %, respectively. The method was applied to the pharmacokinetic of 15 mg/kg of pantoprazole in six rats.

INTRODUCTION
Pantoprazole, 5-((difluoromethoxy)-2-((3,4-dimethoxy-2-pyridyl)methylsulfinyl)[1H]benzimidazole, is a selective and irreversible third proton pump inhibitor (PPI) widely used in the treatment of duodenal and gastric ulcers by decreasing the amount of acid produced in the stomach 1,2. It is highly useful for the relief of symptoms and healing of gastro esophageal reflux disease, peptic ulcer, Helicobacter pylori infection, and other gastric-related disorders 3.

Pantoprazole is metabolized in the liver by Cytochrome P450 system. Metabolism mainly consists of ethylation by CYP2C19 and oxidation by CYP3A4 4. CYP2C19 plays an important role in the metabolism of pantoprazole and the enzyme type of CYP2C19 is related to the plasma concentration 5,6. The plasma concentration of pantoprazole in extensive metabolizers (EM) was lower than that of poor metabolizers (PM) in healthy person 7. A recently study showed that the stable isotope [13C]-pantoprazole can serve as a safe, rapid, and noninvasive phenotype marker of CYP2C19 activity in vivo 8. Therefore, it is necessary to identify the individual differences of the curative effect of pantoprazole in order to promote individualized medical care 3.

So far, a number of analytical methods for the quantitative determination of pantoprazole in pharmaceutical formulations and biological fluids have been reported, including cyclic voltammetry (CV) 9,10 and differential pulse voltammetry (DPV) 11, spectrophotometry 12,13, high-performance liquid chromatography 3,14-19 and LC-MS/MS 20. Those electrochemical methods and HPLC methods with UV detection suffered from long run times, and the sample prepared procession was complex, such as the clear supernatant need to be filtered through 0.45 µm millipore filter in electrochemical methods 11. And in some cases they were not sensitive enough for the determination of low level of pantoprazole concentration levels.

KEY WORDS: LC-MS/MS, Pantoprazole, Pharmacokinetics, Rat plasma.

* Author to whom correspondence should be addressed. E-mail: hulufeng79@sina.com
Compared to the methods mentioned above, the LC–MS methods is time saving and greater sensitivity. The aim of the present study was to develop a new, simple, rapid and sensitive liquid chromatography electrospray ionization mass spectrometry method for its determination of pantoprazole. This robust method allowed pantoprazole in plasma level to be determined with acceptable accuracy and precision to support a variety of pharmacokinetic study of pantoprazole.

**MATERIALS AND METHODS**

**Chemicals and Reagents**

Pantoprazole (> 94.6 %) and omeprazole (purity 98.0 %) used as the internal standard (IS) were bought from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, P.R. China). LC-grade acetonitrile and methanol were purchased from Merck Company (Darmstadt, Germany), and other reagents and solvents were of analytical grade. Ultra-pure water (resistance > 18 mΩ) prepared by a Millipore Milli-Q purification system (Bedford, USA) was used to make mobile phase and all other solutions.

**Animals**

Six adult male Sprague-Dawley rats (200-250 g) raised from Wenzhou Medical College Laboratory Animal Center (Wenzhou, China), were used to study the pharmacokinetics of pantoprazole. All these rats were housed at Wenzhou Medical College Laboratory Animal Research Center. The experimental procedures and protocols were approved by the Animal Care and Use Committee of Wenzhou Medical College.

**Instrumentation and Analytical Conditions**

The analytical instruments include an Agilent 1200 Series HPLC (high - performance liquid chromatography) equipped with a quaternary pump, a degasser, an autosampler, a thermostated column compartment, and a Bruker Esquire HCT mass spectrometer (Bruker Technologies, Bremen, Germany) equipped with an electrospray ion source and controlled by ChemStation software.

Chromatographic separation was achieved on an Agilent Zorbax SB-C18 (2.1 mm x 50 mm, 3.5 μm) column at 25 °C, with methanol-0.1 % formic acid as mobile phase. The flow rate was 0.4 mL/min. The HPLC gradient profile can be seen in Table 1.

<table>
<thead>
<tr>
<th>Load time (min)</th>
<th>Pump flow (µL/min)</th>
<th>Formic acid %</th>
<th>Acetonitrile %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>400</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>1.5</td>
<td>400</td>
<td>15</td>
<td>85</td>
</tr>
<tr>
<td>5.5</td>
<td>400</td>
<td>15</td>
<td>85</td>
</tr>
<tr>
<td>6.0</td>
<td>400</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>10.0</td>
<td>400</td>
<td>90</td>
<td>10</td>
</tr>
</tbody>
</table>

**Table 1.** HPLC gradient for detection of pantoprazole in rat plasma.

Drying gas flow and nebuliser pressure was set at 6 L/min and 15 psi. Dry gas temperature and capillary voltage of the system were adjusted at 350 °C and 3,500 V, respectively. LC-MS/MS was performed with MRM mode using target ions at m/z 383.9 → 199.7 for pantoprazole and m/z 346.0 → 197.7 for omeprazole (IS), respectively, in positive ion electrospray ionization interface. Figure 1 showed the product ion mass spectra of the analytes.

![Figure 1](image-url)

**Figure 1.** Full-scan product ion spectra of pantoprazole (A) and omeprazole(IS, B).
sion energy was determined by observing the response obtained vs selectivity response for the fragment ion for each compound. The best collision energies set were 35.0 eV for pantoprazole and 25.0 eV for IS obtaining fragments m/z 199.7 and m/z 197.7 from the respective protonated compound.

**Preparation of Calibration Standards and Quality-Control (QC) Samples**

A standard stock solution of pantoprazole was prepared by dissolving 10 mg pantoprazole sodium in 10 mL methanol (1 mg/mL). The stock solution was appropriately diluted with methanol to obtain working solutions for calibration at 50, 100, 250, 500, 1000, 2000, 5000, 10000, 20000, and 50000 ng/mL of pantoprazole.

The IS stock solution of omeprazole was prepared by dissolving about 10.0 mg omeprazole sodium in 10 mL methanol to give a nominal concentration of 0.5 mg/mL. The IS working solution was prepared by diluting the stock solution of IS with methanol. All of the solutions were stored at 4 °C before use.

Pantoprazole calibration standards were prepared by spiking blank rat plasma with different amounts of the working solutions. Eight calibration standards ranging from 5 to 5,000 ng/mL were prepared by adding 10 µL of a known working solution of pantoprazole to blank rat plasma (concentrations 5, 10, 25, 50, 100, 200, 500, 1000, 2000, and 5000 ng/mL). Three different Quality-control (QC) samples (concentrations 25, 400, and 4000 ng/mL) were prepared by the same way. They were used to check that the system performs correctly in control.

**Sample preparation**

The frozen plasma sample was thawed at room temperature, then 10 µL of the internal standard working (1 µg/mL) was added to 100 µL rat plasma sample. After that, 200 µL of acetonitrile was added for precipitation of protein, then vortex mixed for 2 min and centrifuged at 13,000 rpm for 10 min, finally, the supernatant (5 µL) was injected for LC–MS/MS analysis.

**Method validation**

**Linearity**

Plasma samples were quantified using the calibration curve. Linearity was evaluated using freshly prepared spiked plasma samples in the concentration range of 5-5,000 ng/mL. The calibration curves were constructed by plotting the ratios of the peak area of pantoprazole to that of IS vs. concentrations of pantoprazole. Five such linearity curves were analyzed. A correlation of more than 0.99 was desirable for all the calibration curves.

**Assay Specificity**

The selectivity of the method was evaluated by analyzing five control drug-free rat plasma and five samples of spiked plasma at LLOQ level from different sources. The SIM chromatograms of blank plasma samples were compared with those at LLOQ. Peak area of endogenous compounds coeluting with the analyte needs to be less than 20 % of the peak area of the LLOQ standard. To determine the extent to which endogenous plasma components may contribute to the interference at retention time of analyte and IS.

**Matrix Effect**

To evaluate the matrix effect, five different lots of blank plasma were protein precipitated and then spiked with the analyte. The corresponding peak areas were then compared to those of neat standard solutions at equivalent concentrations, and this peak area ratio is defined as the absolute matrix effect (ME). The assessment of the relative ME was made by a direct comparison of the analyte peak-area values between different lots of plasma. The variability in the values, expressed as RSD(%), is a measurement of the relative ME for the analyte. The matrix effect of IS was evaluated at the working concentration (400 ng/mL) in the same manner.

**Precision, Accuracy, and Recovery**

The intra-day precision and accuracy of pantoprazole were evaluated by analyzing QC samples (25, 400, and 4000 ng/mL) with five replicates for each concentration. The inter-day precision and accuracy were evaluated by analyzing QC samples with five replicates for each concentration over 5 days.

The recoveries of pantoprazole at three QC levels (n = 5) were determined by comparing peak-area of extracted QC samples with those of reference QC solutions which the pantoprazole was added post-protein precipitation at equivalent concentrations. The recovery of the IS was determined in a similar way.

**Stability**

Sample stability was tested by analyzing QC samples (25, 400, and 4000 ng/mL) after short-term (6 h) storage at room temperature, 12 h storage in an autosampler, after three freeze (-20
°C)–thaw (room temperature) cycles, and after long-term (15 days) storage at -20 °C then immediate extraction. The stability experiments were aimed at testing all possible conditions that the samples might experience prior the analysis.

Pharmacokinetic Study

After overnight fast (12 h), six rats were given intragastrically with pantoprazole at a dose of 15 mg/Kg. Water was freely accessible, but no food was allowed until the rat had been given the drug for 12 h. Blood samples 0.5 mL were directly collected into the heparinized tube from the tail veins at 0 (prior to dosing), 0.083, 0.167, 0.333, 0.667, 1, 1.5, 2, 3, 4, 5, 6, 8, 10, and 12 h after administration. The plasma was separated after centrifugation at 3,000 rpm for 10 min, and stored at -20 °C until analysis. Plasma pantoprazole concentration versus time data for each rat was analyzed by DAS software (Version 2.0, Medical College of Wenzhou, China).

RESULTS AND DISCUSSION

Linearity

The standard calibration curves showed good linearity within the range of 5 (LLOQ) to 5000 ng/mL using least squares regression analysis. The linearity for pantoprazole was investigated by linear regression of peak area ratios against concentrations. The regression equation for the calibration plot was $y = (0.00370 \pm 0.00036) x - (1.98233 \pm 0.10107)$, $R^2=0.99867 \pm 0.00115$ ($y$ is the peak ratio of pantoprazole to IS, and $x$ is the concentration of pantoprazole in plasma). The LLOQ for the determination of pantoprazole in plasma was 5 ng/mL.

Assay Specificity

The analyte and IS were well separated under the described chromatographic conditions at retention time (RT) of 3.5 and 3.4 min, respectively. The total run time was 4.5 min. Therefore, under the conditions described in the experimental part, the assay was highly specific.
and no endogenous plasma materials interfered with the peak of pantoprazole or IS. (Fig. 2).

**Matrix Effect**

To avoid interference from exogenous compounds co-eluted with the target compound, MS/MS detection, offering unique selectivity against matrix background and requires very limited sample preparation was performed. Ionization of analytes was carried out using the electrospray ionization (ESI) technique with positive polarity and multiple reaction monitoring (MRM) mode. In order to further reduce the matrix effect, we took only 100 µL of the plasma sample was precipitated with acetonitrile, injecting 5 µL. The ME for pantoprazole at concentrations of 25, 400, and 4000 ng/mL and IS (400 ng/mL) were showed in Table 2. As a result, ME from plasma was negligible in this method.

<table>
<thead>
<tr>
<th>Concentration (ng/mL)</th>
<th>Matrix Effect (Mean ± SD)</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>111.90 ± 3.83</td>
<td>3.43</td>
</tr>
<tr>
<td>400</td>
<td>103.32 ± 8.80</td>
<td>8.51</td>
</tr>
<tr>
<td>4000</td>
<td>101.60 ± 9.30</td>
<td>9.15</td>
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</table>

Table 2. Matrix effect and Recovery of pantoprazole from rat plasma (n = 5).

**Drug Concentration Matrix Effect RSD**

<table>
<thead>
<tr>
<th>Concentration (ng/mL)</th>
<th>Concentration measured (ng/mL)</th>
<th>Recovery (%) Mean ± SD</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>26.30 ± 0.50</td>
<td>105.19 ± 2.00</td>
<td>1.90</td>
</tr>
<tr>
<td>400</td>
<td>394.69 ± 17.54</td>
<td>98.67 ± 4.38</td>
<td>4.44</td>
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<tr>
<td>4000</td>
<td>4185.60 ± 287.37</td>
<td>104.64 ± 7.18</td>
<td>6.87</td>
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</tbody>
</table>

Table 4. Recovery of pantoprazole and IS from rat plasma after the extraction procedure. (n = 5). Mean: average of five determination, SD: standard deviation. RSD (%) (relative standard deviation) = (SD / Mean) x 100 %.

**Precision, Accuracy, and Recovery**

The precision of the method was determined by calculating RSD for QCs at three concentration levels over three validation days. Intra-day precision was 8 % or less and the inter-day precision was 10 % or less at each QC level (25, 400, and 4000 ng/mL).

Intra-day and inter-day precisions were determined for calibration curves prepared on the same day and different days. As shown in Table 3, this method allowed good precision and accuracy. The recovery of pantoprazole from rat plasma was more than 70 % (Table 4).

**Stability**

All the stability studies of pantoprazole in plasma were conducted at three concentration levels (25, 400, and 4000 ng/mL) with five determinations for each under different storage conditions. The RSD of the mean test responses was within 10 % in all stability tests of pantoprazole in plasma.

No effect on the quantitation was observed for plasma samples kept at room temperature for 6 and 12 h in an autosampler. There was also no significant degradation when samples of pantoprazole in plasma were taken through three freeze (-20 °C)-thaw (room temperature) cycles. And it was also stable at -20 °C for 15 days.

**Application of the Method**

The developed LC-MS/MS method had been successfully applied in a pharmacokinetic study of pantoprazole in rats following oral administration. The representative pantoprazole concentration versus time profiles of six rats receiving a single 15mg/kg oral dose of pantoprazole were presented in Figure 3.
The data of pantoprazole plasma concentrations-time were fitted using DAS ver. 2.0 and the values of AIC (Akaike’s Information Criterion). The main pharmacokinetic data for pantoprazole are listed in Table 5.

**CONCLUSION**

A sensitive LC-MS/MS method has been developed and validated for quantitative determination of pantoprazole in rat plasma. A simplified plasma extraction procedure and lower limit of quantification of 5 ng/mL was achieved in this method. It is successfully employed in a pharmacokinetic study of pantoprazole in rats.

**REFERENCES**


**Table 5.** Pharmacokinetic parameters for pantoprazole after single dose administration in rats (15 mg/kg; n = 6).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean</th>
<th>SD</th>
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<tbody>
<tr>
<td>AUC&lt;sub&gt;0→t&lt;/sub&gt;</td>
<td>8528.315</td>
<td>2506.378</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;0→∞&lt;/sub&gt;</td>
<td>8547.716</td>
<td>2484.836</td>
</tr>
<tr>
<td>MRT</td>
<td>1.54</td>
<td>0.351</td>
</tr>
<tr>
<td>T&lt;sub&gt;1/2&lt;/sub&gt;</td>
<td>1.068</td>
<td>0.266</td>
</tr>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt;</td>
<td>5093.523</td>
<td>514.984</td>
</tr>
<tr>
<td>V&lt;sub&gt;z&lt;/sub&gt;</td>
<td>3082.4</td>
<td>1905.702</td>
</tr>
<tr>
<td>CL</td>
<td>1895.637</td>
<td>657.208</td>
</tr>
</tbody>
</table>

**Figure 3.** Profile of mean plasma concentration-time of pantoprazole and pharmacokinetic profile modeled with two-compartment model after oral administration of pantoprazole (15 mg/kg) in 6 rats.

The data of pantoprazole plasma concentrations-time were fitted using DAS ver. 2.0 and the values of AIC (Akaike’s Information Criterion). The main pharmacokinetic data for pantoprazole are listed in Table 5.

<table>
<thead>
<tr>
<th>AUC&lt;sub&gt;0→t&lt;/sub&gt; (ng/mL.h)</th>
<th>AUC&lt;sub&gt;0→∞&lt;/sub&gt; (ng/mL.h)</th>
<th>MRT (h)</th>
<th>T&lt;sub&gt;1/2&lt;/sub&gt; (h)</th>
<th>C&lt;sub&gt;max&lt;/sub&gt; (ng/mL)</th>
<th>V&lt;sub&gt;z&lt;/sub&gt; (L/kg)</th>
<th>CL (L/h/kg)</th>
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