Determination of Meropenem in Rabbit Plasma by LC–MS/MS

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SUMMARY. A sensitive and selective liquid chromatography tandem mass spectrometry (LC–MS/MS) method for determination of meropenem in rabbit plasma was developed. After addition of triazolam as internal standard (IS), protein precipitation by acetonitrile was used in sample preparation. Chromatographic separation was achieved on a Restek Allure (TM) PFP Propyl (2.1 mm × 100 mm, 5 μm) column with acetonitrile-0.1 % formic acid as mobile phase with gradient elution. Electrospray ionization (ESI) source was applied and operated in positive ion mode; multiple reaction monitoring (MRM) mode was used to quantification using target fragment ions m/z 384.1 → 339.9 for meropenem and m/z 342.7 → 307.8 for the IS. Calibration plots were linear over the range of 0.1-40 μg/mL for meropenem in plasma. Lower limit of quantification (LLOQ) for meropenem was 0.1 μg/mL. Mean recovery of meropenem from plasma was in the range 85.6 %-96.5 %. CV of intra-day and inter-day precision were both less than 15 %. This method is simple and sensitive enough to be used in pharmacokinetic research for determination of meropenem in rabbit plasma.

INTRODUCTION

Meropenem is an ultra-broad spectrum injectable antibiotic used to treat a wide variety of infections, including meningitis and pneumonia. Unlike imipenem, meropenem is stable against renal dehydropeptidase I (DHP-I) 1 and does not need to be administered with a DHP-I enzyme inhibitor such as cilastatin 2. Meropenem has therefore, advantages in Intensive Care Unit (ICU), notably in critically ill patients with renal failure, a population at risk for accumulating co-administered drugs or metabolites 3.

Several analytical methods have been published for determination of meropenem in different biological samples, such as capillary electrophoresis 4-6, checkerboard and time-kill methodology 7, high-performance liquid chromatography 3,8-14. The method coupled with mass spectrometry (MS) detector has a much higher selectivity than that with ultraviolet detector, and it can separate analytes from co-eluents based on their mass-to-charge ratios. Several liquid chromatography/tandem mass spectrometry methods 15-18 for the determination of meropenem in biological fluids have been reported, however, these bioanalytical methods were not full validated.

Meropenem was a hydrophilic and acid compound. Consequently, buffer salts or ion-pairing agents and a mobile phase with a low organic content are needed to ensure adequate retention on a typical C18 stationary phase. Retention can be adequate under these conditions, but the highly aqueous mobile phase causes poor MS response, due to inefficient desolvation, and the salts cause ion suppression during ESI. In contrast, a high-organic mobile phase can be used with an Allure PFP Propyl column 19,20. This combination provides adequate retention and excellent sensitivity.

In this paper, a simple and sensitive LC-MS/MS method for the determination of meropenem in rabbit plasma using one-step protein precipitation was developed and validat-
ed. The LC-MS/MS method successfully applied to a pharmacokinetic study of meropenem after intravenous administration to rabbits.

MATERIAL AND METHODS

Chemicals and Reagents

Meropenem and triazolam (both purity > 90 %) were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). LC-grade acetonitrile and methanol were from Merck Company (Darmstadt, Germany). Ultra-pure water was prepared by a Millipore Milli-Q purification system (Bedford, MA, USA).

Instrument and Conditions

All analysis was performed with a 1200 Series liquid chromatograph (Agilent Technologies, Waldbronn, Germany) equipped with a quaternary pump, a degasser, an autosampler, a thermostatted column compartment, and a Bruker Esquire HCT ion-trap mass spectrometer (Bruker Technologies, Bremen, Germany) equipped with an electrospray ion source and controlled by ChemStation software (Version B.01.03 [204], Agilent Technologies, Waldbronn, Germany).

Chromatographic separation was achieved on an Restek Allure (TM) PFP Propyl (2.1 mm x100 mm, 5 µm) column at 40 °C, with acetonitrile–0.1 % formic acid as mobile phase. The flow rate was 0.3 mL/min. A gradient elution program was conducted for chromatographic separation with mobile phase A (0.1 % formic acid), and mobile phase B (acetonitrile) as follows: 0–2.0 min (85-50 % B), 2.0–6.0 min (50–50 % B), 6.0–7.0 min (50-85 % B), 7.0–10.0 min (85–85 % B).

Drying gas flow and nebuliser pressure was set at 6 L/min and 20 psi. Dry gas temperature and capillary voltage of the system were adjusted at 350 °C and 3500 V, respectively. LC-MS/MS was performed with MRM mode using target ions at m/z 384.1 → 339.9 for meropenem (Fig. 1a) with fragmentation energy of 0.15 v and m/z 342.7 → 307.8 for triazolam (IS, Fig. 1b) with fragmentation energy of 0.23 v, in positive ion electrospray ionization interface, respectively.

Calibration Standards and Quality Control Samples

Individual stock solutions of meropenem (1.0 mg/mL) and triazolam (IS) (100 µg/mL) were prepared in methanol. Working solutions for calibration and controls were prepared from the stock solution by dilution using methanol. 5 µg/mL working standard solution of IS was prepared by dilution of the IS stock solution with methanol. All of the solutions were stored at 4 °C and were brought to room temperature before use.

Meropenem calibration standards were prepared by spiking blank rabbit plasma with appropriate amounts of the working solutions. Calibration plots were constructed in the range 0.1–40 µg/mL for meropenem in rabbit plasma (concentrations 0.1, 0.2, 0.5, 2, 5, 10, 20 and 40 µg/mL). Quality-control (QC) samples were prepared by the same way as the calibration standards, and three different plasma concentrations were 0.2, 5 and 30 µg/mL. The analytical standards and QC samples were stored at –20 °C.

Sample Preparation

Before analysis, the plasma sample was thawed to room temperature. In a 1.5 mL centrifuge tube, an aliquot of 10 µL of the internal standard working solution (5 µg/mL) was added to 100 µL of collected plasma sample followed by the addition of 200 µL acetonitrile. The tubes were vortex mixed for 0.5 min. After centrifugation at 15000 rpm for 10 min, the supernatant (5 µL) was injected into the LC-MS/MS system for analysis.
Method Validation

The selectivity of the method was evaluated by analyzing blank rabbit plasma, blank plasma spiked meropenem and IS and a rabbit plasma sample.

Calibration curves were constructed by analyzing spiked calibration samples on three separate days. Peak area ratios of meropenem to IS were plotted against analyte concentrations, and standard curves were well fitted to the equations by linear regression with a weighting factor of the reciprocal of the concentration squared ($1/x^2$) in the concentration range of 0.1–40 µg/mL. The LLOQ was defined as the lowest concentration on the calibration curves.

To evaluate the matrix effect, blank rabbit plasma were protein precipitated and then spiked with the analyte at 0.2, 5 and 30 µg/mL. 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 matrix effect (ME). The matrix effect of IS was evaluated at the working concentration (0.5 µg/mL) in the same manner.

Accuracy and precision were assessed by the determination of QC samples at three concentration levels in six replicates (0.2, 5 and 30 µg/mL) in three validation days. The precision was expressed by coefficient of variation (CV) and the accuracy by relative error (RE).

The recoveries of meropenem at three QC levels ($n = 6$) were determined by comparing peak-area of the analytes in QC samples to which the analytes were added post-protein precipitation at equivalent concentrations. The recovery of the IS was determined in a similar way.

The stabilities of meropenem in rabbit plasma were evaluated by analyzing three replicates of plasma samples at the concentrations of 0.2 and 30 µg/mL, which were exposed to different conditions. These results were compared with those obtained for freshly prepared plasma samples. The short-term stability was determined after the exposure of the spiked samples at room temperature for 2 h, and the ready-to-inject samples (after protein precipitation) in the HPLC autosampler at room temperature for 6 and 24 h. The long-term stability was assessed after storage of the standard spiked plasma samples at -80 °C for 30 days. The stability of the IS (0.5 µg/mL) was evaluated in a similar way.

Pharmacokinetic Study

Six Japanese male rabbits (2.1–2.3 kg) raised from Laboratory Animal Center of Wenzhou Medical College (Wenzhou, China) were used for this study. All experimental procedures and protocols were reviewed and approved by the Animal Care and Use Committee of Wenzhou Medical College and were in accordance with the Guide for the Care and Use of Laboratory Animals. Rabbits were intravenously given with meropenem via marginal ear vein at a dose of 20 mg/kg within 0.5 min. Blood samples (0.3 mL) were collected from the marginal ear vein into heparinized 1.5 mL polythene tubes at 0, 5, 10, 15, 30, 45, 60 min after dosing. The samples were immediately centrifuged at 5000 rpm for 5 min. The plasma obtained (100 µL) was stored at -80 °C until analysis. Plasma meropenem concentration versus time data for each rabbit was analyzed by DAS software (Version 2.0, Wenzhou Medical College, China).

RESULTS AND DISCUSSION

Method Development

The feasibility of electrospray in positive and negative ion modes of detection was evaluated during the early stages of assay development. It was found that electrospray ionization with positive ion detection resulted in the better signal-to-noise ratio.

Liquid chromatographic condition optimization is also very important during method development. In order to avoid the interference from matrix and to enhance the selectivity and sensitivity, several types of analytical columns including Zorbax SB C18, Zorbax Extend C18 and Allure PFP propyl column were tested. By comparison, the Allure PFP propyl column was found more suitable for meropenem analysis because of its high retention excellent selectivity, and better compatibility with high-organic mobile phases for better ESI-MS sensitivity.\(^{19,20}\)

The mobile phase played a critical role in achieving good chromatographic behavior (including peak symmetry and short analysis time) and appropriate ionization. Various combinations of acetonitrile, methanol, water and 0.1 % formic acid in water with changed content of each component were investigated and compared to identify the optimal mobile phase. Acetonitrile was chosen as the organic solvent because of its suitable sharper peak shape, lower pressure and more stable compared to methanol. Formic acid added into the mobile phase could improve the peak shape, therefore acetonitrile-0.1 % formic acid was chosen as mobile phase. Gradient elution provided better peak symmetry, proper retention time, and avoided the matrix effects for the analyte com-
pared to isocratic elution. A flow rate of 0.3 mL/min produced good peak shapes and permitted a run time of 10 min.

Plasma protein precipitation was used for pretreatment of rabbit plasma samples as it is rapid, widely used and has a high recovery. The supernatant was directly injected into the LC-MS/MS system for analysis. Methanol, acetonitrile and 10 % trichloroacetic acid in water (w/v) were investigated as precipitation reagents, and acetonitrile proved to be the best reagent in terms of the peak shape obtained by LC-MS/MS.

Selectivity and Matrix Effect

Figure 2 shows the typical chromatograms of a blank plasma sample, a blank plasma sample spiked with meropenem and IS, and a plasma sample. No interfering endogenous substances were observed at the retention times of the analyte and IS.

The ME for meropenem at concentrations of 0.2, 5 and 30 µg/mL were measured to be 87.3, 94.7 and 97.8 % (n = 6), respectively. The ME for IS (0.5 µg/mL) was 92.1 % (n = 6). As a result, ME from plasma was negligible in this method.

Calibration Curve and Sensitivity

The linear regressions of the peak area ratios versus concentrations were fitted over the concentration range 0.1–40 µg/mL for meropenem in rabbit plasma. Typical equation of the calibration curve was: \( y = 0.448x - 0.0712 \), \( r = 0.9988 \), where \( y \) represents the ratios of meropenem peak area to that of IS and \( x \) represents the plasma concentration.

The LLOQ for the determination of meropenem in plasma was 0.1 µg/mL. The precision and accuracy at LLOQ were 14.1 % and 86.6 %, respectively. The LOD, defined as a signal–noise ratio of 3, was 0.04 µg/mL for meropenem in plasma.

Precision, Accuracy and Recovery

The precision of the method was determined by calculating CV for QCs at three concentration levels over three validation days. Intra-day precision was 14 % or less and the inter-day precision was 13 % or less at each QC level. The accuracy of the method ranged from 88.5 to 106.3 % at each QC level. Mean recoveries of meropenem were better than 85.6 %. The recovery of the IS (0.5 µg/mL) was 90.3 %.

**Figure 2.** Representative LC-MS/MS chromatograms of meropenem (1) and triazolam (IS, 2), (a) blank plasma; (b) blank plasma spiked with meropenem (0.5 µg/mL) and IS (0.5 µg/mL); (c) a rabbit plasma sample 30 min after intravenous administration of single dosage 20 mg/kg meropenem.
Assay performance data are presented in Table 1. The above results demonstrate that the values are within the acceptable range and the method is accurate and precise.

**Stability**

The stability results showed that meropenem spiked into rabbit plasma was stable for 2 h at room temperature, for 30 days at -80 °C. Stability of meropenem extracts in the sample solvent on autosampler was accepted over 6 h period, but not stable over 24 h period. The results of stability experiments are listed in Table 2.

**Application**

The method was applied to a pharmacokinetic study in rabbits. The mean plasma concentration–time curve after administration of a single 20 mg/kg intravenous dose of meropenem was shown in Figure 3. The main pharmacokinetic parameters from two-compartment model analysis were summarized in Table 3.

**CONCLUSION**

A sensitive, simple and specific LC–MS/MS method for the determination of meropenem in rabbit plasma was developed and validated over the concentration range of 0.1–40 µg/mL. The simple and rapid protein precipitation by acetonitrile was used for pretreatment of plasma samples. The LC-MS/MS method was successfully applied to a pharmacokinetic study of meropenem after intravenous administration of single dosage 20 mg/kg to rabbits.

<table>
<thead>
<tr>
<th>Concentration (µg/mL)</th>
<th>CV (%)</th>
<th>RE (%)</th>
<th>Mean Recovery (%)</th>
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<tr>
<td></td>
<td>Intra-day</td>
<td>Inter-day</td>
<td>Intra-day</td>
</tr>
<tr>
<td>0.2</td>
<td>13.1</td>
<td>12.9</td>
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<td>5</td>
<td>8.5</td>
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<td>30</td>
<td>6.3</td>
<td>7.3</td>
<td>-7.5</td>
</tr>
</tbody>
</table>

*Table 1. Precision and accuracy for meropenem of quality control sample in rabbit plasma (n = 6).*

<table>
<thead>
<tr>
<th>Condition</th>
<th>CV (%)</th>
<th>Concentration (µg/mL)</th>
<th>RE (%)</th>
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<tr>
<td></td>
<td>Added</td>
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<tr>
<td>Ambient, 2h</td>
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<td>0.19</td>
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<td></td>
<td>30</td>
<td>28.65</td>
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<tr>
<td></td>
<td>(IS) 0.5</td>
<td>0.53</td>
<td>6.3</td>
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<tr>
<td>-80 °C, 30 days</td>
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<td>0.19</td>
<td>10.3</td>
</tr>
<tr>
<td></td>
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<td>31.34</td>
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<tr>
<td></td>
<td>(IS) 0.5</td>
<td>0.46</td>
<td>10.4</td>
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<tr>
<td>Autosampler ambient 6 h</td>
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<td>0.18</td>
<td>5.9</td>
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<tr>
<td></td>
<td>30</td>
<td>30.12</td>
<td>7.6</td>
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<tr>
<td></td>
<td>(IS) 0.5</td>
<td>0.47</td>
<td>5.7</td>
</tr>
<tr>
<td>Autosampler ambient 24 h</td>
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</tr>
<tr>
<td></td>
<td>30</td>
<td>25.63</td>
<td>7.9</td>
</tr>
<tr>
<td></td>
<td>(IS) 0.5</td>
<td>0.47</td>
<td>5.8</td>
</tr>
</tbody>
</table>

*Table 2. Summary of stability of meropenem and IS under various storage conditions (n = 3).*

![Figure 3](image-url)
<table>
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<tr>
<th>Pharmacokinetic parameters</th>
<th>Mean (± SD)</th>
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<tr>
<td>$t_{1/2}$ (min)</td>
<td>8.0 ± 0.6</td>
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<tr>
<td>MRT(0-t) (min)</td>
<td>10.45 ± 0.25</td>
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<tr>
<td>MRT(0-∞) (min)</td>
<td>10.76 ± 0.30</td>
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<td>CL/(L/min)</td>
<td>0.04 ± 0.01</td>
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<tr>
<td>AUC(0-t) (min µg/mL)</td>
<td>507.12 ± 112.22</td>
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<tr>
<td>AUC(0-∞) (min µg/mL)</td>
<td>509.79 ± 113.57</td>
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</tbody>
</table>

Table 3. The main pharmacokinetic parameters after intravenous administration of single dosage 20 mg/kg meropenem in 6 rabbits.

REFERENCES