Gradient Elution LC-ESI-MS Determination of Cilostazol in Rat Plasma and its Application

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SUMMARY. A sensitive and simple liquid chromatography/electrospray mass spectrometry (LC-ESI-MS) method for determination of cilostazol in rat plasma using one-step protein precipitation was developed. After addition of midazolam as internal standard (IS), protein precipitation by acetonitrile was used as sample preparation. Chromatographic separation was achieved on an SB-C18 (2.1 × 50 mm, 5.0 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; selected ion monitoring (SIM) mode was used to quantification using target fragment ions \( m/z \) 370.0 for cilostazol and \( m/z \) 325.9 for the IS. Calibration plots were linear over the range of 10-2000 ng/mL for cilostazol in rat plasma. Lower limit of quantification (LLOQ) for cilostazol was 10 ng/mL. Mean recovery of cilostazol from plasma was in the range 90.14-95.10 %. RSD 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 cilostazol in rat plasma.

INTRODUCTION

Cilostazol, 6-[4-(1-cyclohexyl-1H-tetrazol-5-yl)butoxy]-3,4-dihydro-2(1H)-quinolinone, is a potent, selective phosphodiesterase III inhibitor which has the ability to increase availability of cyclic adenosine monophosphate (cAMP). cAMP mediates many agonist-induced platelet inhibitory, vasodilatory and vascular antiproliferative responses. Cilostazol inhibits platelet aggregation and stimulate vasodilation through the accumulation of cAMP. Its main effects are dilation of the arteries supplying blood to the legs and decreasing platelet coagulation. So it is a medication approved for the treatment of patients with intermittent claudication due to peripheral vascular disease 1-6.

Several methods have been published for the determination of cilostazol in different biological matrices, such as high performance liquid chromatography (HPLC) 7-11 and high performance liquid chromatography-mass spectrometry (LC-MS) 12-13.

Liquid chromatography in combination with mass spectrometry (LC-MS) is a well established analytical tool in many fields of application. However, the high selectivity of LC-MS-MS does not guarantee the effective elimination of interferences from endogenous impurities. An operational strategy is to modify the chromatographic conditions to shift the retention time of the target analytes far away from the area affected by signal suppression or enhancement 15. In this paper, an easy and effective way to do this adjustment is to modify gradient conditions. A simple and sensitive LC-ESI-MS method for the determination of cilostazol in rat plasma using one-step protein precipitation with gradient elution was developed and validated. The developed method was successfully applied to pharmacokinetic studies of cilostazol in rats following oral administration.

MATERIAL AND METHODS

Chemicals and Reagents

Cilostazol (purity > 98.0 %) was purchased from Suizhou Tianfeng Chemical Technology Co., Ltd (Wuhan, China). Midazolam (purity > 98.0 %) was purchased from Institute of Forensic Science under the Ministry of Justice (Shanghai, China). LC-grade acetonitrile and methanol were from Merck Company (Darmstadt, Germany). While LC-grade formic acid was Tedia Company (Cincinnati, USA). Ultra-pure water was prepared by a Millipore Milli-Q purification system (Bedford, MA, USA).

KEY WORDS: Cilostazol, Gradient elution, LC-ESI-MS, Rat plasma.

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**Instrumentation 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 Agilent Zorbax SB-C18 (2.1 × 150 mm, 5.0 µm) column at 30 °C, with acetonitrile-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>Methanol %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>400</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>3.0</td>
<td>400</td>
<td>20</td>
<td>80</td>
</tr>
<tr>
<td>7.0</td>
<td>400</td>
<td>20</td>
<td>80</td>
</tr>
<tr>
<td>8.0</td>
<td>400</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>11.0</td>
<td>400</td>
<td>90</td>
<td>10</td>
</tr>
</tbody>
</table>

Table 1. HPLC gradient for detection of cilostazol in rat plasma.

Drying gas flow and nebuliser pressure was set at 7 L/min and 30 psi. Dry gas temperature and capillary voltage of the system were adjusted at 350 °C and 3,500 V, respectively. LC-MS was performed with SIM mode using target ions at m/z 370.0 for cilostazol and m/z 325.9 for Midazolam (IS) in positive ion electrospray ionization interface.

**Calibration Standards and Quality Control Samples**

Individual stock solutions of cilostazol (1.0 mg/mL) and midazolam (internal standard, IS) (100 µg/mL) were prepared in methanol. Working solutions for calibration and controls were prepared from the stock solution by dilution using methanol. 5.0 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.

Cilostazol calibration standards were prepared by spiking blank rat plasma with appropriate amounts of the working solutions. Calibration plots were constructed in the range 10-2000 ng/mL for cilostazol in rat plasma (concentrations 10, 20, 50, 100, 200, 500, 1000, and 2000 ng/mL). Quality-control (QC) samples were prepared by the same way as the calibration standards, three different plasma concentrations (20, 200, and 2000 ng/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 (5.0 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 14900 g for 10 min, the supernatant (10 µL) was injected into the LC-ESI-MS system for analysis.

**Method Validation**

The selectivity of the method was evaluated by analyzing blank rat plasma, blank plasma spiked with cilostazol and IS, and a rat plasma sample.

Calibration curves were constructed by analyzing spiked calibration samples on three separate days. Peak area ratios of cilostazol to IS were plotted against analyte concentrations, and standard curves were well fitted to the equa-
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 cilostazol in rat plasma were evaluated by analyzing three replicates of plasma samples at the concentrations of 20, 200, and 2000 ng/mL, which were exposed to different conditions. 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 24 h. The freeze/thaw stability was evaluated after three complete freeze/thaw cycles (-20 to 25 °C) on consecutive days. The long-term stability was assessed after storage of the standard spiked plasma samples at -20 °C for 30 days.

Pharmacokinetic study

Male Sprague-Dawley rats (200-220 g) obtained from Laboratory Animal Center of Wenzhou Medical College (Wenzhou, China) were used to study the pharmacokinetics of cilostazol. All six rats were housed at Wenzhou Medical College Laboratory Animal Research Center. Animals were housed under controlled conditions (25 ± 1 °C, RH 55 ± 10 %) with a natural light-dark cycle. The animals were allowed to adapt to the housing environment for at least 1 week before the study. Diet was prohibited for 12 h before the experiment but water was freely available. Blood samples (0.3 mL) were collected from the tail vein into heparinized 1.5 mL polythene tubes at 0.0833, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, and 12 h after oral administration of cilostazol (15 mg/kg). The samples were immediately centrifuged at 2,500 g for 5 min. The plasma obtained (100 µL) was stored at -20 °C until analysis. Plasma cilostazol concentration versus time data for each rat was analyzed by DAS software (Version 2.0, Wenzhou Medical College, China).

RESULTS AND DISCUSSION

Method Development

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 formic acid in water with changed content of each component were investigated and compared to identify the optimal mobile phase. In our study, acetonitrile was chosen as the organic solvent because it offers sharper peak shape than methanol. Often the best sensitivity in ESI is achieved when the analyte is ionized already in a liquid phase by using acidic mobile phase for basic analytes 16. Formic acid added into the mobile phase could improve the sensitivity, 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 and IS compared to isocratic elution 17-20. A flow rate of 0.4 mL/min produced good peak shapes and permitted a run time of 10 min (Fig. 1).

An efficient clean-up for bio-samples to remove protein and potential interferences prior to LC-MS analysis was an important point in the studies. The simple and effective protein precipitation was employed in our work. Acetonitrile was chosen as the protein precipitation solvent because it exhibited better effect than methanol and trichloroacetic acid (10 %), which could provide acceptable recoveries.

Selectivity and Matrix Effect

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

The ME for cilostazol at concentrations of 20, 200, and 2000 ng/mL were measured to be 90.14, 93.82 and 95.10 % (n = 6), respectively. The ME for IS (500 ng/mL) was 93.37 % (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 10-2000 ng/mL for cilostazol in rat plasma. Typical equation of the calibration curve was: 

\[
y = 0.0016x + 0.0228, \quad r = 0.9982,\]

where y represents the ratios of cilostazol peak area to that of IS and x represents the plasma concentration. For cilostazol, the present LC-ESI-MS method gave an LLOQ of 10 ng/mL with an accuracy of 16.75 % in terms of RE and a precision of 16.57 % in terms of RSD.

**Precision and Accuracy**

The precision of the method was determined by calculating RSD for QCs at three concentration levels over three validation days. Intra-day precision was 10 % or less and the inter-day precision was 15 % or less at each QC level (20, 200, and 2000 ng/mL). The accuracy of the method ranged from 90.36 to 108.03 % at each QC level.

Assay performance data are presented in Table 2. The above results demonstrate that the values are within the acceptable range and the method is accurate and precise.

**Recovery**

Mean recoveries of cilostazol were 84.72, 89.11, and 93.01 % (n = 6) at concentrations of 20, 200, and 2000 ng/mL, respectively. The recovery of the IS was 98.62 % (n = 6).

**Stability**

The auto-sampler, freeze-thaw and long-term (30 days) stability results indicated that cilostazol was stable under the storage conditions described above since the accuracy were within 85-115 % of their nominal values.

<table>
<thead>
<tr>
<th>Concentration (ng/mL)</th>
<th>RSD( %)</th>
<th>Accuracy( %)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intra-day</td>
<td>Inter-day</td>
</tr>
<tr>
<td>20</td>
<td>9.15 %</td>
<td>14.30 %</td>
</tr>
<tr>
<td>200</td>
<td>9.44 %</td>
<td>11.18 %</td>
</tr>
<tr>
<td>1000</td>
<td>5.17 %</td>
<td>7.40 %</td>
</tr>
</tbody>
</table>

Table 2. Precision and accuracy for cilostazol of quality control sample in rat plasma (n = 6).
Application of the Method

The method was applied to a pharmacokinetic study in rats. The plasma samples with analyte concentration above upper limit of quantitation were diluted with blank rat plasma. The main pharmacokinetic parameters from one compartment model analysis were summarized in Table 3. The mean plasma concentration-time curves after oral administration of cilostazol (15 mg/kg) in 6 rats are shown in Figure 3.

CONCLUSION

A sensitive, simple and specific LC-ESI-MS method with gradient elution for the determination of cilostazol in rat plasma was developed and validated over the concentration range of 10-2000 ng/mL. This chromatographic condition of gradient elution is successfully shift the retention time of the target analytes far away from matrix effect. The method was validated to meet the requirements for pharmacokinetic determination of the cilostazol in rat plasma.

REFERENCES


### Table 3. The main pharmacokinetic parameters after oral administration of single dosage 15 mg/kg cilostazol to rats (n = 6).

<table>
<thead>
<tr>
<th>Pharmacokinetic parameters</th>
<th>Mean (± SD)</th>
</tr>
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<tbody>
<tr>
<td>t1/2(h)</td>
<td>3.63 ± 2.63</td>
</tr>
<tr>
<td>Cl (L/h)</td>
<td>1.53 ± 0.52</td>
</tr>
<tr>
<td>Cmax (ng/mL)</td>
<td>3362.31 ± 1509.91</td>
</tr>
<tr>
<td>AUC0(t) (h ng/mL)</td>
<td>94±2239.89</td>
</tr>
<tr>
<td>AUC0-∞ (h ng/mL)</td>
<td>10697.59 ± 4040.14</td>
</tr>
</tbody>
</table>

![Figure 3](image-url) Mean (± SD) plasma concentration time profile with one compartmental model after oral administration of cilostazol (15 mg/kg) in 6 rats.