Determination of Voriconazole in Rat Plasma by UPLC-MS/MS and its Pharmacokinetic Study

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SUMMARY. In this study, a simple, rapid and sensitive ultra performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) method is described for determination of voriconazole in rat plasma samples using pirfenidone as the internal standard (IS) from pharmacokinetic assays. Sample preparation was accomplished through a simple protein precipitation with acetonitrile, and chromatographic separation was performed on an Acquity BEH C18 column (2.1 x 50 mm, 1.7 μm) with gradient profile at a flow of 0.4 mL/min. The linearity of this method was found to be within the concentration range of 10-5000 ng/mL for voriconazole in rat plasma. Only 3.0 min was needed for an analytical run. The method was applied to a pharmacokinetic study of voriconazole in rats.

RESUMEN. En este estudio se describe un método de espectrometría de masas en tándem de cromatografía líquida de ultra desempeño (UPLC-MS/MS) simple, rápido y sensible para la determinación de voriconazol en muestras de plasma de rata usando pirfenidona como patrón interno (IS) a partir de ensayos farmacocinéticos. La preparación de la muestra se llevó a cabo a través de una sencilla precipitación de proteínas con acetonitrilo, y la separación cromatográfica se realizó en una columna Acquity BEH C18 (2.1 x 50 mm, 1.7 μm) con perfil de gradiente a un caudal de 0.4 mL/min. La linealidad del método está dentro del rango de 10 a 5.000 ng/mL para voriconazol en plasma de rata. Se necesitaron sólo 3.0 min para una serie de análisis. El método se aplicó a un estudio de farmacocinética de voriconazol en ratas.

INTRODUCTION

Voriconazole (Fig. 1) is a second-generation, extended-spectrum triazole antifungal that works by inhibiting cytochrome P450 14-alpha-demethylase, an enzyme responsible for sterol biosynthesis in fungi. Principally, clinicians administer voriconazole for the treatment of invasive aspergillosis, candidiasis, scedosporiosis, and fusariosis 1. However, voriconazole exhibits large intra- and interindividual variability because of non-linear pharmacokinetics, drug-drug interactions, fasting state during dosing, age, and cytochrome P450 polymorphisms 2-5. These challenges are frequent, especially in institutions with large immunosuppressed or ethnically diverse patient populations 7-8. As a result, therapeutic drug monitoring of voriconazole has become an integral part of clinical management.

Several high-performance liquid chromatography (HPLC) assays using ultraviolet (UV) detection have been reported for the determination of voriconazole in biological samples 9-15. However, the sensitivity of HPLC-UV methods is inadequate for pharmacokinetic studies. Liquid chromatography tandem with mass spectrometry (LC-MS/MS) is a powerful tool for the analysis of drug samples in biological media because of its superior sensitivity and selectivity. Several methods for voriconazole determination in plasma have also subsequently been proposed 16-22. In pharmacokinetic studies, the proposed methods should be simple and able to process hundreds of samples in a limited time. While some of these methods are successful in the determination of voriconazole, these methods have several limitations. It is well known that these limitations...
methods required long analysis times, high lower limit of quantification (LLOQ), time-consuming and expensive sample extraction.

The purpose of this study is to develop a simple ultra performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) method for the determination of voriconazole in rat plasma with a simplified sample pretreatment procedure and a short running time. This method has been successfully applied to pharmacokinetic studies in rats.

MATERIALS AND METHODS

Chemicals and reagents

Voriconazole (purity > 98.0 %) and pirfenidone (purity > 98.0 %) were purchased from Sigma (St. Louis, MO, USA). LC-grade acetonitrile and methanol were from Amethyst Chemicals. Formic acid was an analytical grade and purchased from the Beijing Chemical Reagents Company (Beijing, China). Ultra-pure water (resistance > 18 mΩ) was prepared by a Millipore Milli-Q purification system (Bedford, MA, USA).

Instrumentation and conditions

Liquid chromatography was performed on an Acquity ultra performance liquid chromatography (UPLC) unit (Waters Corp., Milford, MA, USA) with an Acquity BEH C18 column (2.1 × 50 mm, 1.7 µm particle size) and inline 0.2 µm stainless steel frit filter (Waters Corp., Milford, MA, USA). The mobile phase consisted of acetonitrile and water containing 0.1% formic acid. A gradient elution program was conducted for chromatographic separation with mobile phase A (0.1% formic acid), and mobile phase B (acetonitrile) as follows: 0-1.0 min (20-95% B), 1.0-2.0 min (95-95% B), 2.0-2.1 min (95-20% B), and 2.1-3.0 min (20-20% B). The flow rate was 0.4 mL/min. The overall run time was 3.0 min.

A XEVO TQD triple quadruple mass spectrometer equipped with an electro-spray ionization (ESI) source was used for mass spectrometric detection. The detection was operated in the multiple reaction monitoring (MRM) mode under unit mass resolution in mass analyzers. The MRM transitions were m/z 351.0→281.1 and m/z 186.2→92.1 for voriconazole and IS, respectively. After optimization, the source parameters were set as follows: curtain gas, 30 psig; nebulizer gas, 50 psig; turbo gas, 60 psig; ion spray voltage, 4.0 kV; and temperature, 600 °C. The Masslynx 4.1 software (Waters Corp., Milford, MA, USA) was used for data acquisition and instrument control.

Preparation of standard and quality control (QC) samples

The stock solution of voriconazole that was used to make the calibration standards and quality control (QC) samples was prepared by dissolving 10 mg in 10 mL methanol to obtain a concentration of 1.00 mg/mL. The working solutions for calibration and quality controls were made from the stock solution by diluting with methanol. Calibration curve standards were prepared by spiking blank rat plasma with appropriate amounts of the working solutions at final drug concentrations of 10, 20, 50, 100, 200, 500, 1000, 2000, and 5000 ng/mL for voriconazole. The preparation of QC samples were the same, with the three levels of plasma concentrations (20, 400, and 4000 ng/mL). IS stock solution was made at an initial concentration of 1 mg/mL. The IS working solution (200 ng/mL) was made from the stock solution using acetonitrile for dilution. All of the solutions were stored in a refrigerator at 4 °C.

Sample preparation

Before analysis, the plasma sample was thawed to room temperature. In a 1.5 mL centrifuge tube, an aliquot of 200 µL of the IS working solution (200 ng/mL in acetonitrile) was added to 100 µL of collected plasma sample. The tubes were vortex mixed for 1.0 min and spun in a centrifuge at 13,000 g for 10 min. The supernatant (2 µL) was injected into the UPLC-MS/MS system for analysis.

Method validation

Specificity was determined by analysis of blank rat plasma samples from six different rats,
every blank sample was handled by the procedure described before and confirmed that endogenous substances did not have the possible interference with the analyte and the IS.

Plasma samples were quantified using the calibration curve. Calibration curves were constructed validated by analyzing spiked calibration samples on three days in a row. Peak area ratio of voriconazole to IS was plotted against analyte concentrations, and standard curves were fitted by weighted \( \frac{1}{\chi^2} \) least squares linear regression in the concentration of 10-5000 ng/mL for voriconazole. A correlation of more than 0.99 was desirable for all the calibration curves. The sensitivity of the method was determined by quantifying the LLOQ. The LLOQ was defined as the lowest acceptable point in the calibration curve which were determined at an acceptable precision and accuracy.

To determine the matrix effect, six different blank plasma samples were utilized to prepare QC samples and used for assessing the lot-to-lot matrix effect. Matrix effect was evaluated at three QC levels by comparing the peak areas of analytes obtained from plasma samples spiked with analytes after extraction to those of the pure standard solutions at the same concentrations. The matrix effect of IS (200 ng/mL) was evaluated at the working concentration in the same manner.

The extraction recoveries of voriconazole at three QC levels \((n = 6)\) were determined by comparing peak area of the analytes in samples that were spiked with the analytes prior to extraction with those of samples to which the corresponding solution was added after extraction. The extraction recovery of the IS at the working concentration was determined in a similar way as a reference.

The intra-day precision and accuracy of voriconazole were evaluated by analyzing QC samples \((20, 400, \text{and } 4000 \text{ ng/mL})\) with six replicates for each concentration. The inter-day precision and accuracy were evaluated by analyzing QC samples with six replicates for each concentration over 6 days. The precision was expressed by coefficient of variation \((\text{RSD})\) and the accuracy by relative error \((\text{RE})\).

The stabilities of voriconazole in rat plasma were tested by analyzing five replicates of plasma samples at three concentration levels \((20, 400, \text{and } 4000 \text{ ng/mL})\) in different conditions. The short-term stability was determined after the exposure of the spiked samples at room temperature for 3 h, and the ready-to-inject samples (after extraction) in the autosampler at room temperature for 24 h. The freeze-thaw stability was evaluated after three complete freeze-thaw cycles \((-20 \text{ to } 25 \, ^\circ\text{C})\) on consecutive days. The long-term stability was assessed after storage of the standard spiked plasma samples at \(-20 \, ^\circ\text{C}\) for 35 days.

**Pharmacokinetic study**

Male Sprague-Dawley rats \((180-220 \, \text{g})\) were obtained from Laboratory Animal Center of Wenzhou Medical University (Zhejiang, China) used to study the pharmacokinetics of voriconazole. All experimental procedures and protocols were reviewed and approved by the Animal Care and Use Committee of Wenzhou Medical University and were in accordance with the Guide for the Care and Use of Laboratory Animals. Diet was prohibited for 12 h before the experiment but water was freely available. Blood samples \((0.3 \, \text{mL})\) were collected from the tail vein into heparinized 1.5 mL polythene tubes at 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 12, and 24 h after oral administration of voriconazole \((40 \, \text{mg/kg})\). The samples were immediately centrifuged at 4000 g for 8 min. The plasma obtained \((100 \, \mu\text{L})\) was stored at \(-20 \, ^\circ\text{C}\) until analysis. Plasma voriconazole concentration versus time data for each rat was analyzed by DAS (Drug and statistics) software (Version 2.0, Shanghai University of Traditional Chinese Medicine, China).

**RESULTS AND DISCUSSION**

**Method development and optimization**

The choice of mobile phase should be concerned based on the consideration of ionization efficiency before the analyte enters the MS/MS system in order to obtain nice resolution and high sensitivity. As for the choice of strong elution mobile phase, methanol and acetonitrile were considered as two candidates. Results showed that the response of the analyte with acetonitrile as the mobile phase was higher than that with methanol under ESI positive mode. To obtain the maximum sensitivity, we investigated the effects of pH with various mobile phases on the ionization efficiency. The analyte and IS were found to have the highest response and the best peak shapes in the mobile phase containing 0.1% formic acid. The LC mobile phase was optimized with varying percentages of organic solvent and different modifiers in water to obtain high sensitivity. Analyte and IS were separated on an Acquity UPLC BEH C18 column.
with a gradient mobile phase consisting of acetonitrile and 0.1% formic acid. The whole separation of the analyte and IS was completed within only 3.0 min per sample.

**Specificity and matrix effect**

Fig. 2 shows the typical chromatograms of a blank plasma sample, a blank plasma sample spiked with voriconazole and IS, and a plasma sample from a rat after oral administration. No endogenous interferences were observed in the blank plasma samples for the analytes.

The matrix effect for voriconazole at concentrations of 20, 400 and 4000 ng/mL were measured to be 108.4, 98.5 and 102.4% (n = 6), respectively. The ME for IS (200 ng/mL) was 105.5% (n = 6). As a result, matrix effect from plasma was negligible in this method.

**Linearity of calibration curve and sensitivity**

The linear regressions of the peak area ratios versus concentrations were fitted over the concentration range of 10-5000 ng/mL for voriconazole in rat plasma. The typical equations of the calibration curve was as follow: 

\[ y = 0.1176x + 0.0065, \quad r = 0.9921, \]

where \( y \) represents the ratio of peak area to that of IS, and \( x \) represents the plasma concentration. The LLOQ was estimated in the process of calibration curve construction and defined as the concentration giving a signal-to-noise ratio of 10, was 10 ng/mL for voriconazole.

**Precision, accuracy and recovery**

The precision of the method was evaluated by calculating RSD for QCs at three concentration levels (20, 800, and 8000 ng/mL) over three validation days. The intra-day precision for voriconazole was 6.9% or less, and the inter-day precision was 6.5% or less at each QC level. The accuracy of the method ranged from -5.1 to 8.4% for voriconazole at three QC levels. Mean recoveries of voriconazole were better than 78.6%. The recovery of the IS (200 ng/mL) was 85.9%. Assay performance data are presented in Table 1. The above results demonstrated that the values were within the acceptable range and the method was accurate and precise.

**Stability**

All the stability studies of voriconazole in plasma were conducted at three concentration levels (20, 400, and 4000 ng/mL) with five determinations for each under different storage conditions. The RSD of the mean test responses was within 15% in all stability tests of voriconazole in plasma. No effect on the quantitation was observed for plasma samples kept at room temperature for 3 h and 24 h in an autosampler. There was also no significant degradation when samples of voriconazole in plasma were taken through three freeze (-20 °C)-thaw (25 °C) cycles. And it was also stable at -20 °C for 35 days.

**Application of the method**

The method described above was successful...
ly applied to determine the concentration of voriconazole in rat plasma. After oral administration of 40 mg/kg voriconazole, the main pharmacokinetic parameters of voriconazole for six rats were estimated. The mean plasma concentration-time curve of voriconazole was displayed in Fig. 3, and the main pharmacokinetic parameters of voriconazole were calculated and are summarized in Table 2.

CONCLUSIONS

An UPLC-MS/MS method for the determination of voriconazole in rat plasma was developed and validated. The method offered sample preparation with a simple protein precipitation with acetonitrile and shorter run time of 3.0 min. The method meets the requirement of high sample throughput in bioanalysis and has been successfully applied to the pharmacokinetic study of voriconazole in rats.

REFERENCES


Figure 3. Mean plasma concentration time profile after oral administration of 40 mg/kg voriconazole in six rats.

Table 2. The main pharmacokinetic parameters after oral administration of 40 mg/kg voriconazole in six rats.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Voriconazole</th>
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<tbody>
<tr>
<td>( t_{1/2} ) (h)</td>
<td>3.48 ± 2.46</td>
</tr>
<tr>
<td>( C_{\text{max}} ) (ng/mL)</td>
<td>3504.77 ± 461.10</td>
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<tr>
<td>( T_{\text{max}} ) (h)</td>
<td>3.50 ± 1.80</td>
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<tr>
<td>( AUC_{0-t} ) (ng/mL.h)</td>
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<td>( AUC_{0-\infty} ) (ng/mL.h)</td>
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