Determination of Voriconazole in Human Plasma by HPLC–ESI-MS and Application to Pharmacokinetic Study

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A fast, sensitive, high-performance liquid chromatography–tandem mass spectrometry method was developed for the determination of voriconazole in human plasma. Carbamazepine was used as the internal standard and the sample pretreatment involved one-step protein precipitation. Chromatographic separation was conducted on an Ultimate C18 column with a mobile phase consisting of acetonitrile–water (containing 0.1% formic acid; 40:60, v/v) at a flow rate of 0.3 mL/min. The detection of voriconazole was performed on a triple-quadrupole tandem mass spectrometer by multiple reaction monitoring with an electrospray ionization source in the positive mode. The standard curve was linear (r² ≥ 0.99) within the concentration range of 2.49–293 ng/mL. The intra-day and inter-day precision values were below 5.3%, and the accuracy was within −4.3–5.7%. The method was applicable to the clinical study of the pharmacokinetics of voriconazole in healthy volunteers following oral administration.

Introduction

Voriconazole [(2R, 3S)-2-(2,4-difluorophenyl)-3-(5-fluoro-1-pyrimidinyl)-1-(1H-1, 2, 4-triazol-1-yl)-2-butan-2-ol] is a novel broad-spectrum antifungal agent with cidal activity against Aspergillus species. The chemical structure of voriconazole is derived from that of fluconazole by the replacement of one triazole moiety with a fluoropyrimidine group and subsequent alpha methylation (Supplementary Figure 1). This modification results in an enhanced spectrum of antifungal activity and increased in vitro potency. Voriconazole binds to the cytochrome P-450 enzyme lanosterol 14-alpha-demethylase, which prevents the conversion of lanosterol to ergosterol (1). This alters the function and permeability of the cell membrane, resulting in cell dysfunction and growth arrest. However, voriconazole has several disadvantages. First, the drug is apparently more poorly tolerated than other antifungal agents. Transient visual abnormalities have been observed in up to 30% of patients receiving voriconazole treatment (2). In humans, approximately 80% of voriconazole is hepatically eliminated, primarily via cytochrome P450 (CYP) 2C9 and CYP2C19, and to a lesser extent via CYP3A4 (3). Patients at risk for acquiring fungal infections, such as those under bone marrow or solid organ transplantation, typically require multiple medications and are prone to drug-drug interactions (4). Second, a non-linear pharmacokinetic behavior has also been observed in healthy volunteers (5), and patients taking voriconazole (2). Therefore, monitoring voriconazole in plasma is very important.

Several high-performance liquid chromatography (HPLC) assays using ultraviolet (UV) detection have been reported for the determination of voriconazole in biological samples (6). However, the sensitivity of HPLC–UV methods is inadequate for pharmacokinetic studies. Liquid chromatography coupled with mass spectrometry (LC–MS) is a powerful tool for the analysis of drug samples in biological media because of its superior sensitivity and selectivity. The first use of LC–MS for voriconazole quantification in biological liquids, such as aqueous humor, was reported by Zhou et al. (7). Three LC–tandem MS (MS-MS) methods (8–10) for voriconazole determination in human serum and two LC–MS-MS methods (11, 12) for voriconazole determination in human plasma have subsequently been proposed. However, the method described by Verdier et al. (11) required time-consuming and expensive sample extraction. The methods described by Chahbouni et al. (8), Egle et al. (9), Verdier et al. (11) and Cheng et al. (12) required long analysis times (longer than 6 min) and the methods described by Chahbouni et al. (8), Egle et al. (9), Verdier et al. (11) resulted in high lower limits of quantification (LLOQs) (higher than 20 ng/mL).

This paper describes a fast, selective and highly sensitive approach to the determination of voriconazole at 2.49 ng/mL in plasma with good accuracy using HPLC–MS-MS. The analysis time was only 3 min and the sample preparation comprised only one simple step of protein precipitation. The method was fully validated and applied to the pharmacokinetic study of voriconazole in healthy volunteers.

Experimental

Reagents and chemicals

Reference standards of voriconazole (99.9% purity, batch number 100862-200701) and carbamazepine [internal standard (IS), 99.9% purity; batch number 0142-9502; Figure 1] were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Acetonitrile, methanol and formic acid (HPLC grade) were purchased from Dikma (Richmond Hill, NY). All other chemicals were analytical grade. Water was purified by redistillation and filtered through a 0.22 μm membrane filter before use.
**Apparatus and operation conditions**

**Liquid chromatography**
Chromatography was performed on an Ultimate C18 column (50 × 2.1 mm, 3.5 μm), with a cooling autosampler and column oven that allowed the temperature of the column to be maintained at 40°C. An isocratic mobile phase was composed of 40% acetonitrile and 60% water (containing 0.1% formic acid, v/v) at a flow rate of 0.30 mL/min. The injected sample volume was 5 μL.

**Mass spectrometry**
Voriconazole and carbamazepine were detected using a Sciex API 4000 Qtrap MS system equipped with a Turbo Ionspray interface. The mass spectral operation settings in the positive ion mode (ESI+) were as follows: ion source voltage, 5,500 V; ion source temperature, 550°C; collision gas (N₂), medium curtain gas, 10 psi; nebulizer gas, 55 psi; auxiliary gas, 55 psi. Quantification was performed using multiple reaction monitoring (MRM) of the transitions of m/z 350.2 → 127.2 for voriconazole and m/z 237.2 → 194.3 for carbamazepine, respectively. Data acquisition and analysis were performed using the software Analyst 1.5.

**Preparation of standards and quality control samples**
Standard stock solutions of voriconazole and carbamazepine were separately prepared in methanol at concentrations of 113 and 116 μg/mL, respectively. The IS solution was diluted with methanol to 1.12 × 10⁶ ng/mL. The voriconazole solution was then serially diluted with methanol to provide working standard solutions with desired concentrations. All stock solutions were stored at 4°C.

Calibration standards were prepared by spiking 0.2 mL of blank human plasma with 20 μL of the appropriate working standard solutions of voriconazole. The effective concentrations in the standard plasma samples were 2.49, 9.97, 59.8, 199, 499, 1.47 × 10³ and 2.93 × 10³ ng/mL. One calibration curve was constructed on each analysis day using freshly prepared calibration standards. The quality control (QC) samples were prepared using blank plasma with low, middle and high concentrations of 6.23, 499 and 2.53 × 10³ ng/mL, respectively, and then stored at 4°C after preparation. The standards and QCs were extracted on each analysis day, by the same procedure for plasma samples, as described in the following.

**Plasma sample preparation**
A 0.2 mL aliquot of plasma sample, 20 μL of IS (1.12 × 10⁶ ng/mL) and 600 μL of methanol were mixed in a 1.5 mL centrifuge tube. The mixture was thoroughly vortexed for 1 min and then centrifuged at 13,000 rpm for 5 min. The supernatant was directly injected into the HPLC–MS-MS system.

**Method validation**
The method was validated for specificity, linearity, accuracy, precision, extraction recovery, matrix effect, and stability according to the guidelines of the Food and Drug Administration.
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Friedman and associates (12) for validation of bioanalytical methods. Validation runs were conducted on three consecutive days. Each validation run consisted of one set of calibration standards and six replicates of QC plasma samples at three concentrations. The peak area ratios of QC plasma samples at three concentrations were interpolated from the calibration curves on the same day to give the concentrations of voriconazole. The results for the QC plasma samples obtained in three runs were used to evaluate the precision and accuracy of the method.

The specificity of this method was investigated by preparing and analyzing six individual human blank plasma samples with corresponding plasma samples spiked with voriconazole and carbamazepine, and plasma samples after oral administration of voriconazole tablets.

The linearity was determined using seven standard plasma samples, with concentrations ranging from 2.49–2.93 × 10⁻¹⁰ ng/mL, by plotting the peak area ratio of voriconazole to IS against the nominal concentration of voriconazole in plasma. The calibration curves were constructed by weighted (1/x²) least-squares linear regression. The LLOQ was defined as the lowest concentration on the calibration curve at which acceptable accuracy [relative error (RE)] within ± 20% and precision [relative standard deviation (RSD)] below 20% were obtained.

The precision and accuracy were determined by six replicate analyses at each QC level (low, medium and high) on three different days. To determine the intra-day accuracy and precision, a replicate analysis of QC plasma samples of voriconazole was performed on the same day. The run consisted of a calibration curve and six replicates of each QC sample at low, medium and high concentrations. The inter-day accuracy and precision were determined by analyzing three batches on different days. The precision was defined as the RSD and the accuracy was expressed as the RE.

The recovery was calculated by comparing the peak areas of voriconazole added to the blank plasma and extracted using the protein precipitation procedure with those obtained from the compound spiked into the post-extraction supernatant at three QC concentration levels. This procedure was repeated for the three QC concentrations of 6.23, 499 and 2.53 × 10⁻⁸ ng/mL.

The matrix effect was measured by comparing the peak responses of the sample spiked post-extraction (A) with that of the standard solution containing equivalent amounts of the two compounds (B). The matrix effect was calculated by A/B × 100%. The extraction recovery and matrix effect of the IS were also evaluated by the same procedure.

The long and short-term stability, freezing and thawing stability of voriconazole in plasma and stability in post-preparation samples were investigated as described in the following. To determine the long-term stability, five aliquots of QC samples at low, medium, and high concentrations were stored at −20°C for 20 days. The samples were then processed and analyzed. Five aliquots of QC samples at the three concentration levels were also stored at room temperature for 4 h to determine the short-term stability of voriconazole in human plasma. The effect of freeze and thaw cycles on the stability of plasma samples containing voriconazole was determined by subjecting five aliquots of QC samples at the three concentration levels to three freeze (−20°C) and thaw (room temperature) cycles.

After the completion of three freeze–thaw cycles, the samples were analyzed. Post-preparative stability was studied by analyzing the extracted QC samples kept in the autosampler at 4°C for 8 h. All measured concentrations were compared with the nominal values to evaluate the stability.

Application to pharmacokinetic study

The HPLC–MS–MS method was used to determine the plasma concentrations of voriconazole from a clinical trial comprising 20 healthy male volunteers who received voriconazole tablets (200 mg). The pharmacokinetic study was approved by the local Ethics Committee and all volunteers gave their signed informed consent to participate in the study according to the principles of the Declaration of Helsinki. Blood samples were collected before and 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 6.0, 8.0, 12 and 24 h post-dosing. Samples were promptly centrifuged, and plasma was separated and stored at −20°C until analysis.

The maximum plasma concentrations (Cmax) and their times (Tmax) were directly obtained from the measured data. The elimination rate constant (kₑ) was calculated by linear regression of the terminal points of the semi-log plot of plasma concentration against time. The elimination half-life (t₁/₂) was calculated using the formula t₁/₂ = 0.693 / kₑ. The area under the plasma concentration-time curve (AUC₀–t) to the last measurable plasma concentration (Cₜ) was calculated by the linear trapezoidal rule. The area under the plasma concentration-time curve to time infinity (AUC₀–∞) was calculated as AUC₀–∞ = AUC₀–t + Cₜ / kₑ.

Results and Discussion

Sample preparation

Liquid–liquid extraction (LLE) and solid-phase extraction are often used to prepare biological samples due to their ability to improve the sensitivity and robustness of assays. However, these methods are both time-consuming and expensive, and may result in environmental pollution. In this study, a simple protein precipitation method was used to reduce the sample preparation time. No further concentration procedure was needed. To obtain high levels of extraction efficiency, two different protein precipitation agents (acetoneitrile and methanol) were investigated. Higher extraction efficiency was obtained using methanol as the precipitation solvent rather than using acetoneitrile. High extraction efficiency was also achieved when methanol was applied to the IS. Methanol was chosen as the precipitation solvent, and the mean recoveries of voriconazole were all above 85%, with RSDs above 7.0%. The protein precipitation sample preparation procedure was much simpler and less expensive than an LLE method previously used in the literature (11). Thus, this method met the requirements of high sample throughput in bioanalysis.

Chromatography and mass spectrometry

Analyte ionization is affected by the composition of the mobile phase. Ammonium acetate and formic acid were used to augment the ionic strength. Formic acid was better than
ammonium acetate at improving the response of voriconazole. The effect of formic acid (0.05, 0.1 and 0.2% in the aqueous phase) on the response to voriconazole was investigated, and 0.1% formic acid was found to be the best concentration. Acetonitrile was better than methanol at improving the shapes of the voriconazole peaks. A mixture of water with 0.1% formic acid–acetonitrile was finally adopted as the mobile phase.

The LLOQ of voriconazole in this method was 2.49 ng/mL with an injection volume of 5 μL. This is much lower than that reported in literature using HPLC–MS-MS, which is higher than 20 ng/mL (8–11).

The total run time was 3 min per sample (Fig. 1), which was lower than previously reported analysis times (longer than 6 min) (8, 9, 11, 12). This short analysis time indicated that this method better met the requirement of high sample throughput in bioanalysis.

The mass spectrometer was tuned in both positive and negative ionization modes with ESI for voriconazole analysis. The signal intensity in the positive mode was approximately 1.5e6, which was much greater than that in the negative mode. In the precursor ion full-scan spectra, the most abundant ions were the protonated molecules [M + H]⁺ m/z 350.2 and 237.2 for voriconazole and the IS, respectively. The production scan spectra showed a high abundance of fragmentations at m/z 127.2 and 194.3 for voriconazole and the IS, respectively (Figure 1). MRM using the precursor to production transitions of m/z 127.2 → 127.2 and m/z 237.2 → 194.3 was performed to quantify voriconazole and the IS, respectively.

**Method Validation**

**Selectivity**

Selectivity was assessed by comparing the chromatograms of six different batches of blank human plasma with the corresponding spiked plasma. As shown in Figure 2A, no interference from any endogenous substance was observed at the retention times of voriconazole and the IS.

**Linearity and LLOQ**

The standard calibration curve for voriconazole was linear within the concentration range of 2.49–293 × 10⁻⁶ ng/mL, using weighted least-squares linear regression analysis with a weight factor of 1/x². The typical regression equation for the calibration curves of voriconazole was y = 0.955x – 1.25 × 10⁻⁶, r = 0.9978.

The LLOQ for voriconazole was 2.49 ng/mL, and the precision (RSD < 20%) and accuracy (RE within ± 20%) data are presented in Table I. A corresponding chromatogram is shown in Figure 2B.

**Precision and accuracy**

The intra-day and inter-day precision and accuracy data for the HPLC–MS-MS method are listed in Table I. The intra-day and inter-day precision value for low, medium and high QC levels of voriconazole were below 5.3%, and the accuracy was within −4.3–5.7%. The precision and accuracy of the present method conformed to the criteria for the analysis of biological samples according to the guidance of the FDA (13), which requires the precision (RSD) determined at each concentration level not to exceed 15%, and the accuracy (RE) to be within ±15% of the actual value.

**Extraction recovery and matrix effect**

The extraction recoveries of voriconazole from human plasma were 95.3 ± 7.0%, 92.8 ± 2.2% and 96.5 ± 5.1% at the concentrations of 6.23, 499 and 2.53 × 10⁻⁵ ng/mL, respectively. The extraction recovery of the IS was 93.2 ± 3.5%.

In terms of the matrix effect, all previously defined ratios were between 85 and 115%. Thus, no matrix effect on voriconazole exists in this method.

**Stability**

The stock solutions of voriconazole and the IS were found to be stable at room temperature for 4 h and at 4°C for 30 days. The accuracy values of voriconazole from the stock solutions of voriconazole and the IS were 103.8 ± 3.3% and 105.4 ± 1.4%, respectively. The results of all stability tests (Table II) demonstrated the stability of voriconazole across all determination steps. Therefore, the proposed method is applicable in routine analysis.

**Pharmacokinetic application**

The present method was successfully applied to the pharmacokinetic study of voriconazole after oral administration in healthy volunteers. After the administration of voriconazole (200 mg), Cₘₐₓ and Tₘₐₓ were 1.21 × 10⁻⁵ ± 343 ng/mL and 1.4 ± 0.9 h, respectively. The plasma concentration declined

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**Figure 2.** Mean plasma concentration–time curve of voriconazole in 20 volunteers after a single oral dose of 200 mg.

**Table I**

Precision and Accuracy for the Determination of Voriconazole in Human Plasma*

<table>
<thead>
<tr>
<th>Concentrations (ng/mL)</th>
<th>RSD (%)</th>
<th>Relative error (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Added</td>
<td>Found ± SD</td>
<td>Within batch</td>
</tr>
<tr>
<td>2.493</td>
<td>2.124 ± 0.3</td>
<td>4.1</td>
</tr>
<tr>
<td>6.232</td>
<td>5.968 ± 0.4</td>
<td>4.2</td>
</tr>
<tr>
<td>498.6</td>
<td>480.3 ± 61.6</td>
<td>4.2</td>
</tr>
<tr>
<td>2.527</td>
<td>2.672 ± 240.2</td>
<td>2.0</td>
</tr>
</tbody>
</table>

*Note: intra-day, n = 6; inter-day, n = 6 series per day, three days.
with \( t_{1/2} = 6.4 \pm 3.4 \) h. The mean (±SD) AUC\(_{0-\infty}\) for voriconazole in plasma was \( 7.02 \times 10^3 \pm 3.48 \times 10^3 \) ng/h/mL (Fig. 2). The results were similar to reported pharmacokinetic parameters (6), indicating the applicability of this method to the pharmacokinetic study of voriconazole.

**Conclusion**

A sensitive, selective and rapid HPLC–MS-MS method was described for the determination of voriconazole in human plasma. Compared with published methods, the method had superior sensitivity, with an LLOQ of 2.49 ng/mL for voriconazole, satisfactory selectivity, a shorter run time of 3 min and a simpler protein precipitation procedure for sample preparation. Based on FDA criteria (13), the proposed method can be used to monitor and determine the pharmacokinetic parameters of voriconazole.

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**References**


