A fast and reliable liquid chromatography coupled with atmospheric pressure chemical ionization tandem mass spectrometry (LC-APCI-MS/MS) method was developed and validated for the quantification of voriconazole in human plasma. The proposed method was validated in a linear range of 50–10,000 ng/ml, and the total run time was 1.5 min. This method was successfully used to support routine therapeutic drug monitoring of voriconazole.

Key words: voriconazole; liquid chromatography coupled with atmospheric pressure chemical ionization tandem mass spectrometry (LC-APCI-MS/MS); human plasma

Voriconazole, (2R,3S)-2-(2,4-difluorophenyl)-3-(5-fluoro-4-pyrimidinyl)-1-(1H-1,2,4-triazol-1-yl)-2-butanol, is a triazole antifungal with enhanced activity against a broad spectrum of fungal pathogens, including Aspergillus and Candida species.1 It is characterized by non-linear pharmacokinetic behavior2–4 and high inter-individual variability of its pharmacokinetic parameters, and it is involved in many pharmacokinetic interactions involving immunosuppressive agents, antiretroviral protease inhibitors, and anti-tuberculosis drugs.5,6 Due to the high inter-individual variability expected, therapeutic drug monitoring is suggested.

The quantification of voriconazole in biological fluids by HPLC10–12 and by LC-MS13–20 methods has been reported. Because voriconazole determination to monitor therapeutic regimens is not yet established, often only a few samples have to be measured in a single run. Thus, taking into account the inclusion of calibrators and quality-control samples, the determination of only a few samples can be rather time-consuming, considering typical chromatographic run times of up to 12 min for HPLC methods. On the other hand, clinicians often require results on the same day, which makes the turnaround time for drug analysis critical, especially if other drugs have to be measured with the same equipment. Recently, several different methods have been described using LC-MS or LC-MS/MS techniques for the determination of voriconazole in the aqueous humor,13 serum,14,15 and plasma,16,17 or simultaneously quantitate concentrations of different antifungal drugs including voriconazole in human plasma18,19 or serum.20 However, the analysis times were found be 4 to 13 min for these methods. This is still not fast enough for high throughput sample analysis.

ESI and APCI are the most commonly used soft-ionization sources in mass spectrometry, and ESI has been found to be more susceptible to the matrix effect than APCI.21–23) Endogenous matrix components can lead to many analytical problems, such as retention time shifts, elevated baseline, and divergent curves. For these reasons, sample extraction, chromatographic separation, and the selection of an internal standard should be more carefully evaluated in ESI than in APCI. On this basis, here we describe the development and validation of a fast, reliable, sensitive LC-APCI-MS/MS method for the quantification of voriconazole in human plasma. The run time was only 1.5 min. It was successfully applied to the routine quantitative determination of voriconazole in plasma samples from patients.

Acetonitrile, tert-butyl methyl ether (Fisher Chemical, Boston, MA) and ammonium formate (Dikma Technologies Inc., Boston, MA) were HPLC grade. Voriconazole (99.8% purity), and internal standard (IS) fentanyl (methanolic solution) were prepared by the China National Institute for the Control of Biological Products (Beijing, China). Standard stock solution was prepared in methanol, and consisted of 2.0 mg/ml of voriconazole. The concentration of the IS fentanyl (methanolic solution) was 100 ng/ml. All standard solutions were stored at 4 °C.

Prior to chromatographic analysis, 80 μl of calibrator, quality control (QC), or patient samples was spiked with 10 μl of IS, and 50 μl of a 20 mM phosphate buffer (pH 8.5), and was vortex-mixed for 1 min. Then 900 μl of tert-butyl methyl ether was added to the samples. The mixture was vortex-mixed for 2 min, and then centrifuged at 15,000 rpm for 5 min. The organic phase was transferred to another vial and evaporated to dryness at 35 °C under a gentle flow of nitrogen. The residue was reconstituted in 100 μl mobile phase, and a 1 μl aliquot was injected into the LC-MS/MS system.

LC separation was performed on an Agilent 1200 system (Agilent Technologies, Palo Alto, CA). The analytical column was a reversed-phase XDB C18 (50 × 4.6 mm I.D., 1.8 μm, Agilent). The column was maintained at 30 °C. The mobile phase was composed of

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Abbreviations: IS, internal standard; LC-APCI-MS/MS, liquid chromatography coupled with atmospheric pressure chemical ionization tandem mass spectrometry; MRM, multiple reaction monitoring; QC, quality control; LLOQ, lower limit of quantitation; ICU, intensive care unit
acetonitrile-10 mM ammonium formate (55:45). The flow rate was set at 1.0 ml/min. Mass spectrometry detection was performed on an Agilent 6410 triple quadrupole mass spectrometer (Agilent Technologies) equipped with an APCI source that was run in positive ion mode. The ion source conditions were set as follows: gas temperature, 350 °C; nebulizer gas, 60 psi; gas flow, 61/min; capillary voltage, 4,500 V; Corona current, 4 μA; fragmentor 100 V for voriconazole and 135 V for IS; collision energy, 15 V for voriconazole and 25 V for IS; and dwell time 100 ms. Voriconazole and IS were monitored in MRM mode using the following transitions: 350 → 281 (voriconazole), and 337 → 188 (IS).

The specificity of the method was determined by screening blank plasma of six different donors. Figure 1 illustrates the influence of the biological matrix with typical chromatograms of blank plasma (Fig. 1(a)) and pooled human plasma spiked with 50 ng/ml of voriconazole (Fig. 1(b)), as well as a plasma sample obtained from a patient undergoing voriconazole treatment at a concentration of 3,845 ng/ml (Fig. 1(c)). No peaks were seen to interfere with the peaks of either the drugs or the internal standard. The retention times were 0.90 min for voriconazole, and 0.58 min for IS, and the total run time was within 1.5 min per sample.

This method was evaluated for linearity, accuracy, and precision (expressed as the percent coefficient of variation %CV). We constructed calibration curves for voriconazole at seven concentrations, ranging from 50 to 10,000 ng/ml (50, 100, 250, 500, 1,000, 2,500, 5,000, and 10,000 ng/ml). The calibration curves showed an excellent linear relationship between the peak height ratio for drug and IS. The correlation coefficient (r) was greater than 0.996 (n = 5). Intra- and interday precision and accuracy were assessed by analyzing three quality control samples (nominal values of 150, 1,500, and 8,000 ng/ml) at each concentration on the same day and mean values of three QCs for 5 d. The intra- and interday accuracy and precision data of voriconazole are summarized in Table 1. The lower limit of quantitation (LLOQ) was determined as the lowest concentration of the standard (50 ng/ml).

The stability tests were designed to cover anticipated conditions that the clinical samples may experience. The stability of voriconazole during sample processing (in plasma at room temperature in freeze-thaw cycles), chromatographic analysis (extracts), and sample storage at −20 °C were investigated. Three freeze/thaw cycles and ambient temperature storage of the QC samples for up to 12 h appeared to have little effect on quantitation. The QC samples stored in a freezer at −20 °C remained stable for at least 82 d. Extracted QC samples were allowed to stand in the autosampler tray for 24 h prior to injection.

Recovery experiments was performed by comparing the analytical results for the extracted samples (n = 5) with unextracted standards that represented 100% recovery. The mean extraction recoveries of the method for voriconazole using five replicates of QC at three concentration levels (150, 1,500, and 8,000 ng/ml) were found to be 86.2%, 95.2%, and 96.3%, respectively. The recovery for IS was 96.2%. The absolute magnitude of matrix suppression was determined by comparing the

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**Fig. 1.** Representative Chromatograms of Voriconazole and IS in Human Plasma.
(a) Blank human plasma; (b) pooled human plasma spiked with 50 ng/ml of voriconazole; (c) plasma sample obtained from a patient with a voriconazole concentration of 3,845 ng/ml.

**Table 1.** Intra- and Inter-Day Accuracy and Precision Data for the Measurement of Voriconazole in Human Plasma

<table>
<thead>
<tr>
<th>Amount of analyte added (ng/ml)</th>
<th>Intraday run (n = 5)</th>
<th>Interday run (n = 5 runs)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (ng/ml) ± SD</td>
<td>CV (%)</td>
</tr>
<tr>
<td>150.0</td>
<td>145.6 ± 2.3</td>
<td>1.56</td>
</tr>
<tr>
<td>1,500</td>
<td>1,566 ± 35</td>
<td>2.24</td>
</tr>
<tr>
<td>8,000</td>
<td>8,134 ± 329</td>
<td>4.04</td>
</tr>
</tbody>
</table>
Determination of Voriconazole in Plasma by LC-APCI-MS/MS

A regression analysis of LC-APCI-MS/MS against LC-UV is shown in Fig. 2. The two methods found similar concentrations. The concentrations measured by the LC-APCI-MS/MS method correlated significantly ($r^2 = 0.874$). The regression coefficient, 0.874, was low because of the limited patient samples available. More patient samples are needed for analysis and comparison in this assay to improve the coefficient.

As a practical application, six patients’ samples were also analyzed by the published LC-UV method. A regression analysis of LC-APCI-MS/MS against LC-UV is shown in Fig. 2. The two methods found similar concentrations. The concentrations measured by the two methods correlated significantly ($p < 0.05$) for voriconazole. The regression coefficient, 0.874, was low because of the limited patient samples available. More patient samples are needed for analysis and comparison in this assay to improve the coefficient.

A therapeutic drug monitoring laboratory is often faced with the problem that different analytical methods have to be applied to only a few patient samples, and the results have to be forwarded to clinicians on the same day. Our LC-APCI-MS/MS enables rapid, sensitive, and reliable quantitation of voriconazole in human plasma. Compared with the published methods, a shorter run-time with a total analysis time of 1.5 min enabled high sample throughput. It provides a practical tool for in vivo detection of voriconazole.

References