Drug Monitoring and Toxicology: Quantification of Antifungal Drug Voriconazole in Human Plasma and Serum by High-Performance Liquid Chromatography with Fluorescence Detection

By Dr. Peter H. Tang
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I. Introduction

Voriconazole (2R,3S-2-(2,4-difluorophenyl)-3-(5-fluoropyrimidin-4-yl)-1-(1H-1,2,4-triazol-1-yl)butan-2-ol) (Figure 1) is a triazole antifungal drug that is generally used to treat invasive fungal infections including invasive aspergillosis, invasive candidiasis, fusariosis, scedosporiosis, and severe fungal corneal infections. Voriconazole exhibits its antifungal activity by inhibiting fungal cytochrome P450-dependent 14α-sterol demethylase (1), an enzyme responsible for ergosterol biosynthesis, which leads to disruptions of the structure and function of the fungal cell membrane.

Voriconazole is metabolized primarily by hepatic cytochrome P450 isoenzyme 2C19 (2), where CYP2C19 contributes largely to pharmacokinetic variability. This drug appears to display non-linear pharmacokinetics, most likely due to saturation of metabolism (3). Many patient factors such as body weight, age, sex, food, drug interactions, and hepatic disease state affect voriconazole plasma or serum concentrations, which leads to significant variability (4-11). In virtue of wide variability of voriconazole plasma or serum concentrations within and between patients, the use of therapeutic drug monitoring is strongly recommended. Determination of voriconazole concentration in plasma or serum is a most useful way for adjusting antifungal drug dosage, individualizing and improving the treatment regimen, and resulting in better efficacy and minimal side effects.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) has been the most powerful technique used for the determination of voriconazole concentration. LC-MS/MS or LC-MS methods require small sample sizes (12-17) and are superior in sensitivity and specificity. However, the purchase, maintenance, and running costs of LC-MS/MS and LC-MS are high. In contrast, high-performance liquid chromatography (HPLC) methods with fluorescence (FL) or ultraviolet (UV) detection (18-26) allow cost-effective operations and appropriate sensitivities for clinically relevant drug concentrations (1-5.5 µg/mL). A validated HPLC/UV method (26) has been previously developed at the Cincinnati Children’s Hospital Medical Center (Cincinnati, OH) and used routinely for measuring voriconazole concentration in human plasma and
serum. The validated HPLC/UV method has provided satisfactory service for therapeutic drug monitoring of voriconazole in patients. To meet increasing demands of drug monitoring and challenges from combination of antifungal therapy, an accurate and sensitive fluorescence detection for the determination of voriconazole concentration in human plasma or serum has come to the forefront. The development of an alternative assay is particularly critical to therapeutic drug monitoring of voriconazole, which accurately monitors plasma or serum drug concentrations in real time and allows more flexibility in managing test schedules and better turnaround time. In this paper, a new HPLC/FL method for the determination of voriconazole concentration in human plasma and serum is described.

II. EXPERIMENTAL

Chemicals and reagents: Voriconazole (analytical standard grade), ketoconazole (pharmaceutical secondary standard grade), trichloroacetic acid (TCA), trifluoroacetic acid (TFA), zinc sulfate heptahydrate and ammonium acetate were obtained from Sigma (St. Louis, MO). HPLC-grade acetone, acetonitrile, ethanol, methanol and n-propanol were purchased from Fisher Scientific (Hampton, NH). Lyphochek® Therapeutic Drug Monitoring Control was from Bio-Rad (Hercules, CA).

Patient plasma or serum samples used for this study were de-identified and approved by the IRB. Normal pooled human plasma or serum (analyte-free) were used as blank. Ammonium acetate solution (0.1 M) was prepared by dissolving 7.7 g ammonium acetate in a 1000 mL deionized water. The mobile phase consisted of 0.1 M ammonium acetate solution, acetonitrile and TFA (409:590:1, v/v/v).

Instrumentation: Automated Hitachi Chromaster™ system (Tarrytown, NY) was equipped with Model 5110 quaternary pump, Model 5210 autosampler, Model 5310 column oven and Model 5440 FL detector. The EZChrom Elite® software was used for monitoring output signal and processing result. The analytical column was a 250-mm x 4.6-mm ODS HYPERSIL column (Thermo Scientific, Sunnyvale, CA) with 5-µm spherical particles connected to a Security Guard (Phenomenex, Torrance, CA) equipped with C18 cartridge (4-mm x 3-mm). MAGNA nylon filter (0.2-µm, 47-mm diameter) was from GE Water & Process Technologies (Minnetonka, MN) for the filtration of mobile phase. Details of HPLC settings are described in Table 1.

Preparation of calibrator and quality control samples in plasma: Stock solutions of voriconazole and ketoconazole were prepared separately by dissolving pure chemical in acetonitrile to have a concentration of 100 µg/mL. Two series of calibrators for voriconazole in the range of 0.1–10 µg/mL were prepared separately by diluting the stock solution of voriconazole with normal pooled human plasma and serum, respectively. Quality controls (QCs) in normal pooled human plasma or serum were similarly prepared containing 0.3, 1, 4, and 8 µg/mL. All solutions were stored at refrigerator for 24 hrs, an aliquot of 100 µL calibrator or QC sample was then dispensed in a 1.5-mL polypropylene screw-top tube. Internal standard ketoconazole solution (1 µg/mL) was prepared in acetonitrile from the ketoconazole stock solution. All solutions and samples were stored at −20 °C until required.

External standard calibration for voriconazole and ketoconazole: In these experiments, two series of calibrators for both voriconazole and ketoconazole were prepared separately by diluting their stock solutions with acetonitrile to produce final concentrations in the range of 0.1-10 µg/mL. External standard calibration curves for voriconazole and ketoconazole were individually generated by HPLC analysis of their fluorescent responses, respectively. Experiments were performed in triplicate. Linearity was assessed by a least squares linear regression of the analyte peak height versus the analyte concentration.

### Table 1: HPLC Settings

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Analytical column</td>
<td>ODS HYPERSIL, 5-µ m, 250 x 4.6 mm</td>
</tr>
<tr>
<td>Mobile phase</td>
<td>Ammonium acetate buffer: acetonitrile: TFA (409/590/1, v/v/v)</td>
</tr>
<tr>
<td>Mode</td>
<td>Isocratic elution</td>
</tr>
<tr>
<td>Column oven</td>
<td>45° C</td>
</tr>
<tr>
<td>Autosampler</td>
<td>5° C</td>
</tr>
<tr>
<td>Flow rate</td>
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</tr>
<tr>
<td>Injection volume</td>
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</tr>
<tr>
<td>Detector settings</td>
<td>254 nm (excitation) and 372 nm (emission)</td>
</tr>
<tr>
<td>Chromatographic time</td>
<td>&lt;7 min</td>
</tr>
</tbody>
</table>
Protein precipitation and extraction procedures: To evaluate the protein precipitation and extraction efficiency of selected reagents, normal pooled human plasma or serum fortified with voriconazole (0.3, 1, 4, and 8 µg/mL) or ketoconazole (1 µg/mL) were used. Ketoconazole solution was prepared by diluting its stock solution (100 µg/mL) with normal pooled human plasma to give a final concentration of 1 µg/mL and refrigerated for 24 hrs prior to analysis. The following reagents were selected and prepared as protein precipitants: TCA (10%, w/v), zinc sulfate heptahydrate (10%, w/v), acetone, acetonitrile, ethanol, methanol and n-propanol. Each precipitant was added to the fortified plasma or serum containing either voriconazole or ketoconazole in volume ratio of 3:1 in triplicate. Mixtures were vortexed for 1 min and centrifuged for 10 min at 10,000 rpm. The supernatant was transferred to an autosampler vial, capped, and 10 µL was injected directly onto the HPLC system. Concentrations of voriconazole and ketoconazole in supernatant were determined by using the external standard calibration curves for both analytes and compared to that of unextracted analytes where extraction efficiency = ([extracted analyte]/[unextracted analyte]) x 100.

Internal standard calibration for voriconazole: Frozen calibrators of voriconazole (0.1–10 µg/mL) prepared in human plasma or serum in 1.5-mL polypropylene screw-top tubes were thawed at room temperature. The internal standard ketoconazole in acetonitrile, 100 µL of 1 µg/mL, was then added to each tube followed by 200 µL of acetonitrile. Mixtures were processed in the same manner as the fortified plasma and serum described in the aforementioned section for extraction and protein precipitation. Internal standard calibration curve for voriconazole was generated by a least squares linear regression of the voriconazole-internal standard peak height ratio versus the voriconazole concentration.

Sample preparation: The internal standard ketoconazole in acetonitrile, 100 µL of 1 µg/mL, was added to 100 µL each of patient plasma or serum, normal pooled human plasma or serum, or QC, and followed by adding 200 µL of acetonitrile. Mixtures were processed in the same manner as the fortified plasma described in the aforementioned section for extraction and protein precipitation procedures. The isolated supernatant (10 µL) was injected directly onto the HPLC system for measuring voriconazole and ketoconazole. After analyzing the voriconazole-ketoconazole peak height ratio, voriconazole concentration was determined from the internal standard calibration curve for voriconazole.

Selectivity: To explore possible interference, ten serum samples from patients treated with other drugs and the Lyphochek® Therapeutic Drug Monitoring Control were examined. Human blood and lyophilized products contains endogenous components, metabolites, decomposition products, concomitant medication or exogenous xenobiotics. These subjects contained multiple substances such as acetaminophen, amikacin, amiodarone, amitriptyline, caffeine, carbamazepine, chloramphenicol, cortisol, cyclosporine, desipramine, digoxin, disopyramide, estriol, ethosuximide, felbamate, flecainide, gabapentin, gentamicin, haloperidol, imipramine, lacosamide, lamotrigine, levetiracetam, lidocaine, lithium, methotrexate, methsuximide, micafungin, milrinone, mycophenolic acid, mycophenolic acid glucuronide, N-acetylprocainamide, netilmicin, nortriptyline, oxcarbazepine, phenobarbital, phenytoin, posaconazole, primidone, procainamide, propranolol, quinidine, rufinamide, salicylate, T3, T4, theophylline, tobramycin, tricyclic antidepressants, thyroid-stimulating hormone, valproic acid, vancomycin and zonisamide. These subjects were treated as patient samples and processed in the same manner as described in the sample preparation section.

Accuracy and precision: Accuracy and precision of within-run or between-run were evaluated by six times processing and analyses of the LLOQ and four QCs (0.3, 1, 4 and 8 µg/mL). Accuracy was expressed as percentage of the deviation of mean from the true value, determined with the formula [(mean measured concentration - true concentration)/true concentration] x 100, whereas precision was expressed as coefficient of variation (CV) calculated as follows: [CV% = (standard deviation/mean of measured values) x 100]. Criteria for accuracy is within ±15% deviation from the actual value except at the lower limit of quantitation (LLOQ), where it should not deviate by more than 20% (27). Criteria for precision is within 15% of the CV% except for the LLOQ, where it should not exceed 20% of the CV% (27).

Reproducibility and stability: To evaluate between-run reproducibility, the LLOQ and four QCs were analyzed on different days. Stabilities of voriconazole and ketoconazole in QCs were evaluated under a variety of storage and handling conditions: freeze-thaw cycles at room temperature; bench-top stability experiments were conducted at room temperature under normal laboratory light for up to 24 hrs; long-term stability (stored samples at −20 °C for 5 days and then thawed for 30 min at room temperature); the stability of stock solutions of voriconazole and ketoconazole were examined; and finally the stability of processed samples sitting on the bench-top for up to 24 hrs were examined.

Cross-validation: Cross-validation was performed on 30 patient samples which were separated into paired sets and stored at −20 °C until required. One set of samples was submitted to the HPLC/UV analysis. The second set of patient samples were analyzed using the current method. To confirm the reliability of this method, paired results from the two different methods were compared.
III. Results and Discussion

Chromatography: Typical chromatograms obtained from a normal pooled human plasma, the 1.25 µg/mL calibrator, and a representative plasma of patient treated with voriconazole are shown in Figure 2. Peaks of voriconazole and internal standard ketoconazole were eluted at ~4.0 and ~5.7 min, respectively. A chromatography was completed in <7 min. The current method allows good resolutions of voriconazole and ketoconazole without ambiguity in identification in the chromatogram.

Figure 2: Chromatograms obtained from a normal pooled human plasma (A), the 1.25 µg/mL calibrator (B), and a representative plasma of patient treated with voriconazole (C) are shown. The retention times of voriconazole and ketoconazole were ~4.0 and ~5.7 min, respectively.

Analytical method validation: Method validation was carried out according to the FDA Guidance for Industry Bioanalytical Method Validation (27). The fundamental parameters for the current bioanalytical method validation included selectivity, accuracy, precision, sensitivity, reproducibility, and stability. Measurements for voriconazole and ketoconazole in plasma and serum were validated. In addition, the stability of voriconazole in fortified samples was determined.

For selectivity, human plasma and serum obtained from ten patients were evaluated. In addition, lyophilized products such as the Lyphochek® Therapeutic Drug Monitoring Controls were also examined. Samples free of voriconazole and ketoconazole did not show any interference with the voriconazole and ketoconazole signals. None of other drugs tested showed chromatographic interference with voriconazole or ketoconazole. Selectivity was also ensured at the LLOQ. The ability of the current method to differentiate and quantify both voriconazole and ketoconazole in the presence of other components in human plasma and serum was proven.

Quantitative recovery of voriconazole in human plasma and serum has recently been achieved by using methanol as an extracting solvent in a single dilution step (26). Most recently, a single dilution step procedure for simultaneous protein precipitation and analytes extraction has been proved as an excellent sample preparation for acquiring fast sample clean-up and disruption of protein-drug binding (28). It is evident that significant loss of analyte due to adsorption at the precipitate exists during protein precipitation. Therefore, addition of internal standard during sample preparation was necessitated to compensate the loss of analyte. After investigating several compounds, ketoconazole was found to best fit as an internal standard in this study. According to Polson et al. (29), the most efficient protein precipitants for protein removal were zinc sulfate, acetonitrile and TCA. These three precipitants were found to remove plasma protein effectively at 2:1 and greater volumes of precipitant to plasma. Therefore, the precipitant to plasma volume ratio of 3:1 was chosen in the current study. Seven protein precipitants were evaluated for their extraction efficiencies of voriconazole (4 µg/mL) and ketoconazole (1 µg/mL) in human plasma. The concentrations of extracted voriconazole and ketoconazole were determined by using the external standard calibration curves. The extraction efficiency...
results for these protein precipitants are shown in Table 2. Each extraction efficiency is an average of three replicates.

**Table 2: Comparison of extraction efficiency for various protein precipitants**

<table>
<thead>
<tr>
<th>Precipitants*</th>
<th>% Analyte extraction efficiency** (% RSD)</th>
<th>Voriconazole</th>
<th>Ketoconazole</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetonitrile</td>
<td>96.2 (1.5)</td>
<td>93.7 (0.8)</td>
<td>95.0</td>
<td></td>
</tr>
<tr>
<td>Methanol</td>
<td>95.6 (1.1)</td>
<td>90.5 (0.7)</td>
<td>93.1</td>
<td></td>
</tr>
<tr>
<td>Ethanol</td>
<td>93.9 (0.8)</td>
<td>90.9 (0.7)</td>
<td>92.4</td>
<td></td>
</tr>
<tr>
<td>Acetone</td>
<td>89.1 (1.9)</td>
<td>90.3 (1.1)</td>
<td>89.7</td>
<td></td>
</tr>
<tr>
<td>n-Propanol</td>
<td>89.6 (1.2)</td>
<td>87.7 (1.5)</td>
<td>88.6</td>
<td></td>
</tr>
<tr>
<td>Zinc sulfate (10%, w/v)</td>
<td>22.5 (2.8)</td>
<td>26.4 (2.0)</td>
<td>24.5</td>
<td></td>
</tr>
<tr>
<td>TCA (10%, w/v)</td>
<td>43.7 (2.0)</td>
<td>3.0 (1.5)</td>
<td>23.3</td>
<td></td>
</tr>
</tbody>
</table>

*Each precipitant was carried out three times (n = 3).

**% Analyte extraction efficiency = ([extracted analyte] / [unextracted analyte]) x 100.**

With the exception of zinc sulfate and TCA, all protein precipitants were on average at least 88% effective in extraction. Both zinc sulfate and TCA had decreased concentrations of voriconazole and ketoconazole in the supernatant due to the loss of analytes by adsorption at the precipitate. Because voriconazole and ketoconazole are more soluble in organic solvents, both analytes were effectively extracted by the organic solvents. Acetonitrile, aside from being excellent in precipitating protein, was chosen based on its optimal extraction efficiency toward voriconazole and ketoconazole. It is believed that recovery pertains to the extraction efficiency of an analytical method. Recovery of voriconazole from human plasma and serum were carried out in triplicate by comparing the analytical results for extracted drug at four concentrations (0.3, 1, 4 and 8 µg/mL) with unextracted drug. Recoveries of voriconazole from plasma and serum were similar at four concentrations, and the mean recovery of voriconazole was in the range 94.7-98.1%.

The internal standard calibration curve of voriconazole prepared in plasma was linear ($r^2 > 0.99$) over the concentration range of 0.1 to 10 µg/mL. The slopes of internal standard calibration curves in the six different preparations for voriconazole were practically the same. The mean linear regression equation of internal standard calibration curve was $y = 8.615 x - 0.0302$, where $y$ represents the concentration of voriconazole and $x$ represents the ratio of voriconazole peak height to that of the internal standard. The LLOQ was 0.1 µg/mL, whereas the limit of detection (LOD) was 0.04 µg/mL. The internal standard calibration curve prepared in serum was $y = 8.628 x - 0.0413$, while the internal standard calibration curve prepared in acetonitrile was $y = 8.128 x - 0.013$. All calibration curves were linear ($r^2 > 0.99$). However, matrix effects were detected when compared the slopes of calibration curves prepared in plasma or serum with that prepared in acetonitrile ($p<0.05$). Since the plasma and serum calibration curves were parallel, no matrix effects was detected between plasma and serum. As such, the linear regression of the plasma curve was used to derive the voriconazole concentrations in human plasma and serum.
The accuracy and imprecision were evaluated at the LLOQ and four QC concentrations (Table 3). Bias was calculated as the found minus the nominal concentration, expressed as a percentage of the nominal concentration. Imprecision was calculated as within- and between- runs coefficient of variation (CV). The bias for LLOQ and QCs were <4%. Imprecision was <15% at the LLOQ and QC concentrations, as indicated by both within- and between- runs. For QCs, within-run precision was between 1.3% and 7.5% and between-run precision was between 4.5 and 9.2%. Overall accuracy was between 96.1% and 102.1%. The minimal deviation of the mean from the true value indicates the excellent accuracy of the method. Table 4 provides the method reproducibility performed by five technicians for patient samples. Overall CVs were less than 9%.

The stability of QC samples at -20°C was evaluated at 2-week intervals for 3 months. The stability of stock standard solutions at -20°C for 6 months was evaluated. The effects of freezing and thawing on voriconazole were studied using QC samples, which were subjected three free-thaw cycles before analysis (freeze samples at -20°C and then thaw for 30 min at room temperature). The stability of the processed samples sitting at 5°C (the temperature of the autosampler) for 24 hrs were evaluated. Voriconazole and internal standard ketoconazole were stable in all storage and handling conditions.

Cross-validation: A cross-validation was also carried out, using a total of 30 de-identified patient samples with voriconazole concentrations ranging from 0.1 to 7.2 µg/mL. Results from the current method were compared with data generated using a validated HPLC-UV method (26) served as the reference. These data are shown in Figure 3. The least squares linear regression equation for correlation where y is the current method and x is the reference method described here was $y = 1.024x - 0.018$ where $r^2 = 0.981$ with a standard error value of 0.027.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Replicate</th>
<th>Found (µg/mL)</th>
<th>Precision (% CV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient #1</td>
<td>5</td>
<td>1.23±0.101</td>
<td>8.2</td>
</tr>
<tr>
<td>Patient #2</td>
<td>5</td>
<td>6.86±0.349</td>
<td>5.1</td>
</tr>
<tr>
<td>Patient #3</td>
<td>5</td>
<td>3.05±0.146</td>
<td>4.8</td>
</tr>
<tr>
<td>Patient #4</td>
<td>5</td>
<td>0.994±0.085</td>
<td>8.6</td>
</tr>
<tr>
<td>Patient #5</td>
<td>5</td>
<td>4.41±0.192</td>
<td>4.4</td>
</tr>
<tr>
<td>Patient #6</td>
<td>5</td>
<td>2.58±0.154</td>
<td>6.0</td>
</tr>
</tbody>
</table>
Analytical method comparison: Up to now, three HPLC/FL methods (18-20) for measuring voriconazole in biological fluids have been previously described. Michael et al. (18) reported the first HPLC-FL method for the determination of voriconazole in 0.3 mL of human plasma or saliva by using an internal standard UK-115 794 which was not readily available. Human sample was extracted twice with n-hexane-ethyl acetate, two extracts were then combined and evaporated to dryness. The residue was reconstituted and 20 µL of reconstitutant was injected onto the HPLC system with a run time of 12 min. Heng et al. (19) described a procedure to manipulate 4 mL of human bronchoalveolar lavage fluid by using a freeze dryer. After 48 hrs of drying, the lyophilized powder was reconstituted with water and extracted with acetonitrile. Following centrifugation, an aliquot (70 µL) of the extract was injected onto the HPLC system. Ogata et al. (20) used ethyl acetate to extract voriconazole and naproxen (as an internal standard) from 0.2 mL of human plasma. After protein precipitation, tedious procedures such as evaporation of ethyl acetate supernatant to dryness and reconstitution of residue with mobile phase were used for sample preparation prior to HPLC analysis. Finally, a lengthy chromatography of longer than 22 min per injection was required for the analysis. In comparison with the reported HPLC/FL methods, the current method employs a single dilution step with acetonitrile for protein precipitation and extraction. Once again, a single dilution step procedure proved to be the most rapid and simplest procedure for sample preparation. The current method only needed a small injection volume (10 µL) and small sample volume (0.1 mL) to achieve the LLOQ of 0.1 µg/mL (1 ng on column). Injection of a small portion of extract (10 µL) has allowed the HPLC system to load ~1500 injections without replacing pre-filter columns. It is cost-effective and time-saver, because the replacements of pre-filter columns are frequently required after loading up to ~700 injections with large portions of extract (20 µL or greater) per injection. Finally, the current method completes a chromatographic run in <7 min per injection with the optimized conditions.

IV. Conclusion

A validated HPLC/FL method for the determination of voriconazole concentration in human plasma or serum has been described. Ketoconazole is readily available and has been successively used as an internal standard. The current method uses acetonitrile as protein precipitant and extraction solvent in a single dilution step procedure which provides rapid sample clean-up and excellent extraction efficiency. By avoiding complex liquid-liquid extraction, tedious solid-phase extraction, evaporation of extract or supernatant, and residual reconstitution procedures, the current method substantially decreases set-up time. This method is simple, rapid, sensitive, accurate and practical for use in the analytical and clinical laboratories for therapeutic drug monitoring of voriconazole in human plasma and serum.

Acknowledgement

The author wishes to thank the Department of Pathology and Laboratory Medicine, Cincinnati Children’s Hospital Medical Center for the internal funding, the Special Chemistry Laboratory and Clinical Chemistry Laboratory for the work performed in this study.
References Références Referencias

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