Development and Validation of a Liquid Chromatography–tandem Mass Spectrometry Method for the Determination of Voriconazole and Posaconazole in Serum Samples from Patients with Invasive Mycoses

Loretta Franceschi*, Sara D’Aronco and Mario Furlanut

Institute of Clinical Pharmacology and Toxicology, DISM, University of Udine, Udine, Italy

Abstract
Posaconazole and voriconazole, two triazole antifungal agents, are used for the prophylaxis and treatment of invasive mycoses in patients with acute myeloid leukaemia and/or immunocompromised, Inter- and intra-patient variability of pharmacokinetics, drug-drug interactions, serum concentration related toxicity and success of therapy has stressed the need of frequently therapeutic drug monitoring of both drugs. Therefore, a rapid, selective and sensitive isotopic reversed-phase HPLC assay coupled with Mass spectrometry detection for quantification of posaconazole and voriconazole in serum samples has been developed.

Analytes were extracted on solid-phase cartridges (SPE) and chromatographic separation was achieved on a C8 column and detected by mass spectrometry in positive ion mode with the select ion monitoring (SIM) mode. The total chromatographic running time was 6 minutes. The method was successfully used for a pharmacokinetic study but, thanks to its rapidity and selectivity, it’s also suitable for routinely therapeutic drug monitoring (TDM).

Keywords: Voriconazole; Posaconazole; hplc; Mass spectrometry detection

Introduction
Incidence of invasive mycoses (IFIs) has increased significantly in the last two decades [1] due to an increased number of immunocompromised patients owing to advances in transplantation, the emergence of AIDS and a rise in the number of invasive surgical procedures. IFIs are associated with significant morbidity and mortality and it’s important to ensure appropriate antifungal therapy in order. Historically, the treatment of invasive fungal infections has been marked by few options for therapy because of the limited number of available agents, antifungal drug resistance and drug toxicity [2]. However, in the last decade, therapeutic options for IFIs have doubled thanks the development and approval of new anti-fungal compounds. Voriconazole and posaconazole are two of these new drugs [3].

Posaconazole (PSC) and voriconazole (VRC), like the other triazole antifungal agents (fluconazole and itraconazole), bind the active site of the P450-dependent enzyme lanosterol 14a-demethylase. This enzyme is involved in the syntesis of ergosterol, which is an essential lipid constituent of the cell membrane of fungi and not present in mammalian [4].

PSC [5,6] and VRC [7] possess potent activity against a broad spectrum of clinically significant fungal pathogens: Candida albicans, C. glabrata and C. krusei, dimorphic fungi, Aspergillus fumigatus, A. terreus, Fusarium spp., Dematiaceous fungi, Zygomicetes [8,9]. PSC is available as a lyophilised powder for oral administration. Tissues are not homogeneus compartements and VRC is not evenly distributed [9]. VRC shows nonlinear pharmacokinetics and dose dependent clearance and t1/2β is about 6 hours [12].

Due to the pharmacokinetic variability (inter- and intra-patient), drug-drug (i.e immunosuppressant drugs, anticoagulant drug, proton pumps inhibitors) and drug-food interactions (i.e. carbonated soft drinks, food with high content of fat) [13], age, genetic polymorphism of the cytochrome P450, changes in volume of distribution, and hepatic dysfunction, antifungal therapeutic drug monitoring (TDM) represents a valid instrument for an appropriate dosing to optimize drug administration for favourable patient outcome and minimizing the risk of treatment-related toxicity and an accurate, sensitive, specific and rapid drug assay represent a valid helpful for clinician.

Several methods for quantification of PSC and VRC in human serum by high-performance liquid chromatography coupled with UV [3,4,14-17], fluorescence [18] or mass spectrometry [19,20] detection have been reported. In some reported methods serum samples are simply deproteinized by means of acetonitrile [16,20]. Deproteinization by precipitation is a simple and rapid procedure but the disadvantage is the eventual loss of analytes by absorption at the precipitate and samples obtained are not enough clean, in fact supernatant still contain large amounts of salts, small molecules, fatty acids and triglycerides and endogenous materials may increase ion suppression during LC/MS detection [21]. Other methods require double liquid-liquid extraction with n-hexane-ethyl acetate [18] or diethyl ether [14].

*Corresponding author: Loretta Franceschi, Institute of Clin Pharm & Toxicol – DISM, University of Udine, Piazzale SM della Misericordia, 1533100 Udine – Italy. Tel: +39 (0)432 559833; Fax: +39 (0)432 559819; E-mail: loretta.franceschi@uniud.it

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Up to now, only three HPLC assay has been published for their simultaneous quantitation [3,14,17]. PSC and VRC are not co-administered for treatment of fungal infections and we decided to use VRC as internal standard (IS) for PSC quantitation and PSC for VRC quantitation. Diazepam [3] and linezolid [17], which were used as IS, are not an optimal choice because it is possible a co-administration with PSC or VRC.

This report describes the optimisation and validation of an HPLC assay coupled with mass spectrometry (MS) detection for quantification of PSC and VRC. Sample handling and chromatographic run times were minimized to provide quantitative results while maintaining high sensitivity, specificity, accuracy and precision for routinely TDM and for pharmacokinetic evaluation.

Experimental

Chemicals and reagents

PSC and VRC were supplied, respectively, by Schering-Plough (Ireland) and Pfizer (United Kingdom). Acetonitrile, ammonium acetate (Merck KgaA - Darmstadt, Germany), methanol (Carlo Erba - Milan, Italy), and formic acid (Sigma-Aldrich - Milan, Italy) were analytical-grade reagents. MilliQ water was filtered and deionised with an Ultra Pure Water System, MilliQ-plus (Millipore, USA). The solid-phase extraction (SPE) cartridges Bondelut C8 (EC) containing 200 mg of stationary phase and with a volume of 3 mL were purchased from StepBio (Bologna, Italy).
Chromatographic analysis

Chromatographic equipment consisted of High Performance Liquid Chromatography (HPLC) LC-200 pump (Perkin Elmer, USA). The analytes were eluted at room temperature from a Ultra C8 column (150 mm x 2.1 mm - 5 µm) (Resteck, USA) with a solution mixture [A: acetonitrile/water/formic acid (95/5/0.1) and ammonium acetate (2 mM); B: water/acetonitrile/formic acid (95/5/0.1) and ammonium acetate (2 mM)] that changed linearly, in 3 minutes, from A/B 90/10 to A/B 100/0%, at a flow rate of 0.2 mL/min. Under these conditions, VRC and PSC retention times were 3.11 and 3.18 min, respectively (Figure 1). Total run time was 7 min for each injection. Column effluent was introduced into the mass spectrometer using a fused silica capillary.

A Q-trap LC/MS/MS Systems (MDS Sciex - Ontario, Canada) was equipped with an electrospray source, operating in positive ion mode (ESI). Data were acquired and processed with Analyst 1.4.1. (Applied Biosystems Package, MDS Sciex - Ontario, Canada). Samples were detected in selected ion monitoring (SIM) (m/z: PSC 701.8 and VRC 350.0). In order to optimise the MS parameters, standard solutions of each analyte were infused into the mass spectrometer using an infusion pump. The optimised mass spectrometer parameters for PSC and VRC detection were reported in Table 1.

Preparation of stock and work solutions

Stock solutions of PSC and VRC were prepared separately in methanol at the concentration of 1 mg/mL. Working solutions, for the preparation of calibration curves and quality control samples, were made by diluting, in methanol, stock solutions. Stock and work solutions were stored at -80°C.
Analyte dependent MS parameter

<table>
<thead>
<tr>
<th>Parameter</th>
<th>PSC</th>
<th>VRC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q1 mass</td>
<td>701.8</td>
<td>350.0</td>
</tr>
<tr>
<td>DP</td>
<td>96</td>
<td>41</td>
</tr>
<tr>
<td>EP</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>CEP</td>
<td>4</td>
<td>26</td>
</tr>
</tbody>
</table>

Other MS parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IS</td>
<td>5.5 kV</td>
</tr>
<tr>
<td>CUR</td>
<td>25 units</td>
</tr>
<tr>
<td>CAD</td>
<td>2 units</td>
</tr>
<tr>
<td>TEM</td>
<td>350 °C</td>
</tr>
<tr>
<td>GS1</td>
<td>35 units</td>
</tr>
<tr>
<td>GS2</td>
<td>45 units</td>
</tr>
</tbody>
</table>

Q1 mass: molecular weight (+ 1 amu); DP: Declustering Potential; EP: Entrance Potential; CEP: Collision Cell Entrance; IS: Ion Spray Voltage; CUR: Curtain gas; CAD: Collision gas; TEM: Temperature; GS1: gas 1; GS2: gas 2

Table 1: Optimised mass spectrometer (MS) parameters for PSC and VRC detection.

Intra-day accuracy

<table>
<thead>
<tr>
<th>Parameter</th>
<th>PSC (ng/mL) (n=3)</th>
<th>VRC (µg/mL) (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>149.33</td>
<td>1.5</td>
</tr>
<tr>
<td>Dev. Std.</td>
<td>1.53</td>
<td>0.6</td>
</tr>
<tr>
<td>CV%</td>
<td>1.02</td>
<td>0.3</td>
</tr>
</tbody>
</table>

Table 2: Intra-day and inter-day accuracy.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>PSC (ng/mL) (n=5)</th>
<th>VRC (µg/mL) (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>149.20</td>
<td>1.49</td>
</tr>
<tr>
<td>Dev. Std.</td>
<td>2.17</td>
<td>0.94</td>
</tr>
<tr>
<td>CV%</td>
<td>1.45</td>
<td>0.80</td>
</tr>
</tbody>
</table>

Table 3: PSC serum concentrations.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>OS admin</td>
<td>116.0 ± 63.1</td>
</tr>
<tr>
<td>IV admin</td>
<td>116.0 ± 63.1</td>
</tr>
<tr>
<td>Range</td>
<td>39.2 – 236</td>
</tr>
</tbody>
</table>

Table 4: VRC serum concentrations.

Calibrators and quality control samples

Calibrators and quality control samples containing PSC and VRC were prepared adding known amounts of analytes to blank serum. They were included in each batch of patient samples. Calibration curves and quality control samples ranged from 100 to 1000 ng/mL and 1 to 10 µg/mL for PSC and VRC respectively.

Sample preparation

PSC: We combined 500 µl of serum sample with 10 µl of IS, VRC (10 ng/µl).

VRC: We combined 250 µl of serum sample with 10 µl of IS, PSC (100 ng/µl).

Analytes extraction

After mixing, the sample was transferred into an extraction cartridge conditioned with 3 mL of methanol and then 3 mL of water. After washing the extraction cartridge with 3 mL of water, the sample was eluted with 7.5 mL of methanol. After evaporation of the organic phase, under a nitrogen stream at 40°C, the residue was dissolved with 100 µl of mobile phase and 20 µl was injected into the HPLC system.

Patients

To evaluate PSC pharmacokinetic variability, after approval by the Ethics Committee of the Policlinico Universitario of the University of Udine and after obtaining informed consent, 10 haematological patients candidates to PSC treatment, were prospectively admitted to the study. All patients received a total daily dose of 600 mg of PSC (200 mg every 8 hours). Blood samples were collected, on day 1, 4 and 8, immediately before PSC administration and 2, 4, 6 and 8 hours after its administration.

To ensure optimisation of VRC treatment on patients hospitalized in intensive care, infectious disease, cardiothoracic surgery and onco-haematologic units, serum concentrations were quantitated on blood samples collected immediately before VRC administration and 30 minutes and 2 hours after IV and OS administration respectively.

Blood samples were centrifuged at 3000 rpm for 10 min and serum samples were transferred to polypropylene tubes and transferred at -80°C until processing.

Method validation

The developed HPLC method was validated according to international guidelines [22-24] for bioanalytical methods to evaluate selectivity, accuracy, precision, recovery, calibration curve, sensitivity, reproducibility, stability and matrix effect [25].

The selectivity of the method was investigated by analysing 6 different batches of drug-free human serum for the exclusion of any endogenous co-eluting interferences at the peak region of each analyte.

Accuracy and precision were evaluated by analysing, as described above, aliquots of blank serum enriched with known amount of analytes.

The extraction recovery was determined comparing the analyte results for extracted samples at 5 concentration with unextracted standard.

The calibration standard were prepared and assayed in triplicate on 5 different days to demonstrate the linearity of the method. Intra- and inter-day precision and accuracy were assessed by extracting and analysing 5 replicates of each of 6 quality control concentration levels. The limit of detection (LOD) was defined at a signal-to-noise (S/N) ratio of 3:1; the limit of quantification (LOQ) was defined the lowest quantifiable concentration of analyte with accuracy within 20% and a precision <20%.
Stability of PSC and VRC in methanolic stock solutions and in human serum were demonstrated under various storage conditions: room temperature, +4°C, -80°C. Analyte stability were also determined after three freeze and thaw cycle.

The presence of matrix effect was determined by applying the procedures recommended by Annesley [25].

Results

Selectivity

The selectivity of the method was evaluated for potential endogenous interferences or other sources at the same transition and retention times of PSC and VRC by analysing blank serum samples from six different batches. Figure 2 show typical SIM chromatograms for extracted blank human serum sample. This process was repeated five times and no interferences were observed in any of these samples.

Linearity and recovery

Linearity and limit of linearity (LOL), defined as the concentration at which the calibration curve departs from linearity, were evaluated analyzing quality control samples with concentration lower and greater than range of calibration curves.

The linearity of our method, checked by analysing QC samples in triplicate, was in the range of 40-2300 ng/mL and 0.05-20 µg/mL for PSC and VRC respectively. Correlation coefficients, respectively for PSC and VRC, were 0.999 and 0.998.

The lower (LOL) and the upper LOL (ULOL) were respectively, 40 and 2300 ng/mL for PSC and 0.05 and 20 µg/mL for VRC. Figure 3 and Figure 4 show concentrations at which the depart from linearity.

Extraction recovery, evaluated comparing instrument response for extracted QC samples and unextracted standards, ranged from 89.05 to 90.12% for both analytes. These values were estimated at 5 different concentrations.

Calibration curve

Calibrators and QC samples containing PSC or VRC were prepared adding known amounts of analytes to blank serum and they were included in each batch of patient samples. Each analytical batch included 5 calibrators ranging from 100 to 1000 ng/mL for PSC (Figure 5A) and from 1 to 10 µg/mL for VRC (Figure 5B) and 3 QC samples (low, medium and high).

Limit of quantification (LOQ) and limit of detection (LOD)

The sensitivity of the method is satisfactory for TDM analysis and pharmacokinetic studies. The limit of quantification (LOQ) was set at the LLOL (PSC 40 ng/mL; VRC 0.05 µg/mL). The limit of detection (LOD) was 15 ng/mL and 0.02 µg/mL respectively for PSC and VRC.

Reproducibility

A series of QC samples were prepared at 5 different concentrations in the range of 100-1000 ng/mL and 1-10 µg/mL for PSC and VRC respectively. Intra-day accuracy was calculated after 3 replicate runs of the same extracted sample; inter-day accuracy was calculated after analysis on 5 consecutive days (Table 2).

Stability

PSC and VRC methanolic stock solutions showed no significant degradation between solutions kept at room temperature for 24 h, at -20°C for 3 months. QC samples stored at -80°C showed no significant degradation when analysed after 3 and 6 months. We also evaluated freeze-thaw stability of both analytes by assaying QC samples over three freeze-thawing cycles.

Ion suppression

The presence of ion suppression was evaluated comparing the instrument response for QC samples injected directly in mobile phase, the same amount of compound added to preextracted samples and the same amount of compound added to matrix before extraction. No significant decrease (< 4%) of signal was observed.

Patient samples analysis

Posaconazole: Time (T max) to reach peak serum concentrations (C max) ranged from 4 to 6 hours (Table 3) reports C max (mean ± SD and range) of PSC reached on day 1 (after a single oral dose of 200 mg), on day 4 (after 10 oral dose of 200 mg) and on day 8 (after 22 oral dose of 200 mg).

Figure 6 shows PSC serum concentrations (mean ± SD) on day 1, 4 and 8. The mean (± SD) PSC Area Under the Curve (AUC) (calculated, from 0 to 8 hours, by means trapezoidal role) were, 660.37 (± 372.44), 3451.5 (± 1607.8) and 6236.5 (± 4098.0) ng h/mL on day 1, 4 and 8 respectively.

Voriconazole

The mean (± SD) IV and OS VRC daily dose were 266.66 (± 76.13) and 222.5 (±40.99) mg respectively. Table 4 reports serum concentrations immediately before administration (C max) and 30 minutes and 2 hours after after IV and OS administration respectively (C max).

Discussion and Conclusion

A simple, specific and sensitive HPLC assay was developed to quantify PSC and VRC in serum samples using solid phase extraction (SPE) and MS spectrometry detection. Three HPLC methods [3,14,17] for simultaneous quantitation of PSC and VRC in human serum have been previously described in the literature. In our procedure we use PSC and VRC as IS for quantitation of VRC and PSC respectively because PSC and VRC are never co-administered, they present similar lipophilicity and different molecular weight (PSC 700.2, VRC 349.3 a.m.u). Diazepam [3] and linezolid [17] represent not a good choice as IS because they could be co-administered with antifungal therapy. The presence of diazepam and/or linezolid in patient samples due to drug intake would compromise PSC and VRC quantitation. To obtain clean and reproducible chromatograms, efficient sample recoveries, SPE represent an optimal choice. Protein deproteinization [16,20] is a simple procedure with good recoveries but this procedure is not specific and many endogenous and exogenous compounds appear on chromatograms as interfering peaks; analytes could be absorbed at the precipitate and moreover presents some inconveniences because organic solvent use for precipitation (methanol, acetonitrile or acid solution) not always represents the optimum for chromatographic column and detection. Liquid-liquid extraction (LLE) [3,14] is more specific than protein precipitation but requires large solvent volume and longer time. SPE procedure present some advantages: permit to prolong columns’ life because sample extracted are cleaner; the major part of interferences are eliminated and solvent consume is smaller than LLE.

The bioanalytical assay we proposed presents the advantage that...
extraction procedure, HPLC apparatus and detection are the same for both analytes: PSC and VRC samples can be extracted and analyzed in the same analytical session with time and material saving.

The proposed method, thanks to its sensitivity (PSC: LOQ 40 ng/mL and LOD 20 ng/mL; VRC: LOQ 0.05 µg/mL and LOD 0.03 µg/mL) was successfully applied to a pharmokinetic variability study. The method, thanks to its rapidity (30 minutes for extraction procedure and 7 minutes for chromatographic analysis) and specificity, has been demonstrated to be of great usefulness in our laboratory for therapeutic drug monitoring (TDM) too. With this procedure we analyzed, for over 1 year, about 600 patient samples of PSC and VRC without HPLC apparatus deterioration.

References