Development and Validation of HPTLC method for Determination of Voriconazole in Human Plasma

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**ABSTRACT**

Voriconazole is a new antifungal agent that is a derivative of fluconazole, having wide-spectrum activity and a fungicidal action against Candida and Aspergillus species. Voriconazole displays non-linear pharmacokinetics in adults but has linear pharmacokinetics in children. Interindividual variability is generally high; both in children and adults, and diverse manifestations of toxicity are possibly attributed to high drug concentrations. This indicates the need to monitor voriconazole concentration in plasma after oral dose. A simple, selective and sensitive high performance thin layer chromatographic method for the determination of voriconazole in human plasma is developed and validated. After precipitation of plasma proteins with acetonitrile, the protein-free supernatant was spotted on plates precoated with silica gel 60 \(F_{254}\). Cephalexin was used as an internal standard. The mobile phase consisted of a mixture of toluene : methanol : triethylamine in the ratio of 6:4:0.1 v/v/v. The drug showed considerable absorbance at 254 nm. The method was found to be linear over the concentration range of 50–400 ng/band. Mean drug recovery was found to be 98.82%. Voriconazole in plasma samples was stable when stored for different stability conditions. The method was found to be precise and accurate and can further be extended for pharmacokinetic studies for therapeutic drug monitoring of voriconazole in routine clinical practice.

**Keywords:** Voriconazole, HPTLC, human plasma, method validation.

**INTRODUCTION**

Voriconazole [(\(2R,3S\))-2-(2,4-difluorophenyl)-3-(5-fluoro-4-pyrimidinyl)-1-(1H-1,2,4-triazol-1-yl)-2-butanol] is a new second generation antifungal agent, structurally derived from fluconazole, having one triazole moiety replaced by a fluoropyrimidine ring and a methyl group...
added to the propanol backbone. It has an extended spectrum of activity against a wide variety of yeasts and molds. Developed for the treatment of life-threatening fungal infections, it appears to be an effective therapy option for invasive aspergillosis, fluconazole-resistant candidiasis and refractory or less-common invasive fungal infections. Its primary mode of action is inhibition of fungal cytochrome P450-dependent 14α-sterol demethylase, an essential enzyme in ergosterol biosynthesis [1-3]. Voriconazole (VRC) is metabolized by the cytochrome P450 system, with < 2% of the dose excreted unchanged [4].

VRC displays non-linear pharmacokinetics in adults but has linear pharmacokinetics in children. Interindividual variability is generally high; both in children and adults, and diverse manifestations of toxicity are possibly attributed to high VRC concentrations [1]. C\textsubscript{max} for voriconazole in patients is reported to be 3.79 µg/ml within 1-2 hrs for 200 mg dose [4]. Clinical studies suggest that VRC plasma concentration should be between 0.5-8.0 µg/ml. However, plasma concentration of > 6 µg/ml is associated with occasional liver function abnormalities [5]. Therefore, therapeutic drug monitoring may be helpful to individually optimize therapy with Voriconazole [1].

Several methods are reported to determine the levels of voriconazole in biological fluids by using LC-MS-MS [6-7], LC-ES-MS [8], HPLC with fluorescence detection [1], HPLC with UV detection [3-5, 9-15], and by microbiological methods [9, 10]. Few methods have also been reported to determine the levels of voriconazole in biological fluids in combination with itraconazole [5] and posaconazole [16, 17]. HPTLC method has been reported for determination of voriconazole in bulk and pharmaceutical dosage form [18]. To the best of our knowledge, no High Performance Thin Layer Chromatographic (HPTLC) method has been reported for determination of voriconazole in human plasma.

The present method describes a simple, selective and sensitive HPTLC method with a calibration range of 50-400 ng/band for voriconazole in human plasma. The method utilizes protein precipitation with acetonitrile as the sample preparation technique. Cephalexin was used as an internal standard. The mobile phase employed was toluene: methanol: triethyl amine (6:4:0.1 v/v/v). The method was validated as per CDER Guidelines for Industry, Bioanalytical method validation [19].

MATERIALS AND METHODS

Chemicals
Voriconazole working standard was kindly supplied by Jubilant Organosys Ltd., Delhi and Cephalexin by Maxim Pharmaceuticals, Pune. The drugs were used as such without further purification. The structures for voriconazole and cephalexin are described in Figure 1. AR grade methanol, toluene, tri-ethylamine and acetonitrile were purchased from S. D. fine chemical Laboratories, Mumbai, India.

Instrumentation
Chromatographic separation of drugs was performed on aluminium plates precoated with silica gel 60 F\textsubscript{254}, purchased from E-Merck, Germany. Samples were applied on the plate as a band
with 6 mm width using Camag 100 µl sample syringe (Hamilton, Switzerland) with a Linomat 5 applicator (Camag, Switzerland). Linear ascending development was carried out in a twin trough glass chamber (10 x 10 cm) at room temperature and a densitometric scanning was performed using Camag TLC scanner 3 in the range of 400-200 nm, operated by winCATS software (Version 1.4.3, Camag). Deuterium lamp was used as a radiation source. All weighing was done on Shimadzu balance (Model AY-120).

Figure 1: Chemical structures of voriconazole and cephalexin

**Chromatographic conditions**
Samples were applied on the 10 cm x 10 cm plate as band with a width of 6 mm and slit dimensions were kept as 5.00 x 0.45 mm. 50 µl volume of each sample was applied on TLC plate. Chamber saturation time was 15 minutes and the migration distance was 80 mm.

**Sample preparation**
Stock solutions for voriconazole and Cephalexin (Internal Standard, IS) were prepared by separately dissolving in methanol, to obtain a concentration of 1 mg/ml. Working solution for VRC was prepared by diluting suitably with methanol to get the concentration of 4 µg/ml. The stock solutions were stored at 4°C. Working solution for IS was prepared by diluting suitably stock solution of IS with methanol to get the concentration of 20 µg/ml.

Q.C. (Quality Control) samples for VRC were prepared at concentration levels so as to get 150, 200 and 300 ng/band. 1.5, 2.0 and 3.0 ml of working stock solution of VRC (4 µg/ml) was separately taken into glass tubes and to each tube was added 0.2 ml of IS (20 µg/ml). The contents were evaporated to dryness on water bath at 70°C. The dry residue was then reconstituted with 1 ml of drug free human plasma and the samples were vortex mixed for 10 min. Proteins were precipitated using 1 ml of acetonitrile. The contents of the tubes were further vortexed for 3 min, followed by centrifugation for 5 minutes at 2500 rpm. After centrifugation, 50 µl of supernatant from each concentration were applied on the TLC plate.

The calibration curve for VRC was obtained using seven calibration standard levels (50, 100, 150, 200, 250, 300 and 400 ng/band). Sample preparation technique for calibration standard levels was same as that for Q.C. samples. For all calibration curves, a linear regression was used, considering the ratio of the peak area of analyte to internal standard versus concentration applied. A correlation coefficient of more than 0.99 was obtained for each calibration curve.
Method Validation

Selectivity
Selectivity is the ability of an analytical method to differentiate and quantify the analytes in the presence of other components in the sample. The selectivity of the method was evaluated by analyzing pooled plasma samples obtained from two different sources spiked at LLOQ (Lower Limit of Quantification - 50 ng/band).

Calibration/standard curve
Linearity was tested for the range of concentrations 50-400 ng/band. Each sample in five replicates was analyzed and peak areas were recorded. The response factor for each concentration was calculated by taking ratio of peak area of VRC and IS. The response factors were then plotted against the corresponding concentrations to obtain the calibration graphs.

Accuracy, precision and lower limit of quantification
The accuracy and precision of the method were evaluated using the Q.C. samples. Intra-day accuracy and precision was measured by consecutively analyzing Q.C. samples in one single day. The procedure was repeated for three different days to test the inter-day accuracy and precision. Accuracy was calculated as percentage accuracy, whereas precision was measured in terms of relative standard precision (R.S.D.) of each calculated concentration. Lower limit of quantification (LLOQ) was found to be 50 ng/band, since the response obtained was five times the response compared to blank.

Recovery
Recovery for VRC was evaluated at three concentration levels corresponding to three routine Q.C. samples (150, 200 and 300 ng/band) analyzed in triplicate. Recovery was determined by comparing the ratio of the peak area of VRC with IS obtained after the application of the processed plasma calibration samples with those achieved by working standard solution in the methanol.

Stability
Freeze-thaw stability of voriconazole was determined by assaying low and high Q.C. samples (150 and 300 ng/band) in triplicate over three freeze-thaw cycles. First freeze-thaw cycle consisted of 24 hrs freezing at -50° C followed by a complete thaw at a room temperature. The next two freeze-thaw cycles were of 12 hrs each frozen state at -50° C followed by a complete thaw at a room temperature. Short term stability consisted of two Q.C. samples stored for 4 hrs at room temperature and long term stability involved storage of two Q.C. samples for 14 days at 4° C. For stock solution stability, the stock solutions of the drug and IS were stored for period of 5 days in refrigerator at 4° C and then for 6 hrs at room temperature. Post preparative stability, where Stability of the spiked samples for 200 ng/band of VRC and 100 ng/band of IS were determined after the storage for 5 hrs at room temperature. All these Q.C. samples were then evaluated in triplicate and the results were compared with the freshly prepared samples of same concentrations.
RESULTS

Chromatographic characteristics
Retention factor for VRC and IS were found to be $0.72 \pm 0.03$ and $0.50 \pm 0.03$ respectively. Representative densitograms of blank human plasma, blank plasma spiked with IS (100 ng/band) and IS (100 ng/band) with VRC (200 ng/band) are shown in figure 2, 3 and 4 respectively.

![Figure 2: Densitogram of blank human plasma](image)

![Figure 3: Densitogram of blank human plasma spiked with IS, 100 ng/band (Rf-0.50±0.03)](image)
Selectivity
The selectivity of the method was evaluated by analyzing pooled plasma samples obtained from two different sources spiked at LLOQ (50 ng/band) in which no interference by endogenous components was noted. % RSD (Relative standard deviation) for 6 replicates spiked at LLOQ was found to be 6.02%.

Calibration/standard curve
All five calibration curves analyzed during the course of validation were found to be linear for the concentrations ranging from 50-400 ng/band and best fitted by linear equation $y = mx + c$. The correlation coefficient was 0.997, with mean slope of 0.002 and a mean y-intercept of 0.354.

Table 1: Intra-day, inter-day precision & accuracy of voriconazole in human plasma QC samples

<table>
<thead>
<tr>
<th>Theoretical (ng/band)</th>
<th>Observed (mean ng/band ± SD)</th>
<th>Precision (% R.S.D.)</th>
<th>Accuracy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intra-day</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>150</td>
<td>150.75 ± 4.3702</td>
<td>2.90</td>
<td>100.5</td>
</tr>
<tr>
<td>200</td>
<td>201.40 ± 5.4181</td>
<td>2.69</td>
<td>100.7</td>
</tr>
<tr>
<td>300</td>
<td>301.65 ± 4.0638</td>
<td>1.35</td>
<td>100.55</td>
</tr>
<tr>
<td>All</td>
<td>2.31 ± 0.8408</td>
<td>100.58 ± 0.1041</td>
<td></td>
</tr>
<tr>
<td>Inter-day</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>150</td>
<td>149.90 ± 4.3322</td>
<td>2.89</td>
<td>99.93</td>
</tr>
<tr>
<td>200</td>
<td>200.39 ± 5.3315</td>
<td>2.66</td>
<td>100.19</td>
</tr>
<tr>
<td>300</td>
<td>301.30 ± 4.2658</td>
<td>1.42</td>
<td>100.43</td>
</tr>
<tr>
<td>All</td>
<td>2.32 ± 0.7907</td>
<td>100.18 ± 0.2501</td>
<td></td>
</tr>
</tbody>
</table>

% R.S.D. = SD/mean x 100, accuracy = observed/theoretical x 100
Accuracy, precision and lower limit of quantification

The method showed good accuracy and precision in plasma samples. Table 1 shows the results for intra- and inter-day precision and accuracy for VRC in plasma samples. Intra- and inter-day (R.S.D.) precisions were 2.31 ± 0.8408 and 2.32 ± 0.7907 respectively. Intra- and inter-day accuracies were 100.58 ± 0.1041 and 100.18 ± 0.2501 respectively. LLOQ was found to be 50 ng/band.

Recovery

Table 2 shows the results of the recovery tests for the three Q.C. levels of VRC tested (150, 200 and 300 ng/band) and IS (100 ng/band). The mean recovery for VRC and IS was found to be 98.82% and 99.11% respectively.

Table 2: Recovery of voriconazole and IS in human plasma Q.C. samples

<table>
<thead>
<tr>
<th>Added (ng/band)</th>
<th>% R.S.D.</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>voriconazole</td>
<td>IS</td>
</tr>
<tr>
<td>150</td>
<td>3.5</td>
<td>5.62</td>
</tr>
<tr>
<td>200</td>
<td>3.0</td>
<td>3.66</td>
</tr>
<tr>
<td>300</td>
<td>2.59</td>
<td>3.31</td>
</tr>
<tr>
<td>All</td>
<td>3.03%</td>
<td>4.20%</td>
</tr>
</tbody>
</table>

% R.S.D. = SD/mean x 100

Table 3: Stability of voriconazole and IS in human plasma Q.C. samples

<table>
<thead>
<tr>
<th>Stability</th>
<th>Conc. (ng/band)</th>
<th>Mean Stability (%)</th>
<th>% R.S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freeze thaw stability (three cycles)</td>
<td>150</td>
<td>98.92</td>
<td>2.48</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>98.84</td>
<td>1.41</td>
</tr>
<tr>
<td>Short term stability (for 4h at RT)</td>
<td>150</td>
<td>97.92</td>
<td>2.89</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>98.71</td>
<td>1.01</td>
</tr>
<tr>
<td>Long term stability (for 14 days at 4°C)</td>
<td>150</td>
<td>98.53</td>
<td>1.47</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>99.24</td>
<td>1.43</td>
</tr>
<tr>
<td>Stock solution stability (for 5 days at 4°C, 6hrs at RT)</td>
<td>200</td>
<td>97.43</td>
<td>1.68</td>
</tr>
<tr>
<td></td>
<td>100 (IS)</td>
<td>97.99</td>
<td>3.27</td>
</tr>
<tr>
<td>Post preparative stability (for 5hrs RT)</td>
<td>200</td>
<td>98.47</td>
<td>1.13</td>
</tr>
<tr>
<td></td>
<td>100 (IS)</td>
<td>99.39</td>
<td>1.37</td>
</tr>
</tbody>
</table>

% RSD = SD/mean x 100, RT (room temperature)

Stability

Plasma Q.C. at two concentrations (150 and 300 ng/band) was used for freeze-thaw, short term and long term stability studies. Stock solution and post preparative stability was also performed for the drug (200 ng/band) and IS (100 ng/band). It was performed to evaluate the influence of storage conditions from the sample collection to analysis. Table 3 represents the results of stability studies. Results indicated that VRC is stable in human plasma for the given stability conditions. The deviation of the mean test responses to the freshly prepared solutions was less than 5% at any of the stability conditions.
DISCUSSION AND CONCLUSION

In the study, rapid and sensitive HPTLC method has been developed for the determination of voriconazole in human plasma by protein precipitation technique. Validation results proved that the developed method performs well with selectivity, precision, accuracy, stability and linearity for the concentration range of voriconazole to be found in human plasma. The validated method covers the wide range of linearity over 50-400 ng/band (1-8 µg/ml) and is therefore suitable for the determination of voriconazole in human plasma at different therapeutic dose levels. The present method involves minimal sample pretreatment, resulting in fast analysis. Also it utilizes protein precipitation as the sample preparation technique, which eliminates the drawbacks of less recovery due to liquid-liquid extraction or the use of solid phase extraction cartridges which is relatively costly. The mean recovery of voriconazole and IS was found to be 98.82% and 99.11% respectively. HPTLC technique, offers advantage of high throughput. As compared to LC-MS-MS and HPLC methods, the present method is economical, simple and fast. The proposed method can be used for therapeutic drug monitoring in order to optimize drug dosage on an individual basis.

Acknowledgement
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