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Research Article

Developing a Ultra-High-Performance Liquid Chromatographic-Diode Array Method to Quantify Voriconazole in Human Plasma

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ABSTRACT

A fast and selective Reversed-Phase (RP) liquid chromatography-Diode Array (DAD) method was developed and validated to determine Voriconazole in human plasma. Thanks to the high throughput and sensitivity of Ultra-High-Performance Liquid Chromatographic (UHPLC) Chromatography, there were only 7.5 minutes of run time, and no buffers were used. 200 µl serum and 200 µl internal standard fluconazole were added. After simple protein precipitation with 400 µl methanol, 10 µl of the clear supernatant separated on Agilent Zorbax Eclipse Plus® C18 Column (50*2.1 mm, 1.8 µm). The column temperature was maintained at 40°C. The flow rate was 0.4 ml/min using a gradient elution of methanol and water. The retention time of Fluconazole and Voriconazole were 4.2 and 6.2 min, respectively. Detection was performed using a diode array detector set at a wavelength of 261 nm. The method was optimized and validated per FDA Guidance for Industry, Bioanalytical method validation. System suitability, in terms of theoretical plates, resolution, tailing and injection precision in terms of retention time and area, was performed to ensure the suitability and effectiveness of the chromatographic system as per ICH guidelines. The developed method provided outstanding recovery, accuracy, precision, selectivity, stability and reproducibility results. The calibration curve was linear over a range of 0.2-12 µg/ml with a correlation coefficient 0.9997c, adequately covering the therapeutic range for appropriate drug monitoring. The limit of detection and quantification were 0.2 µg/ml and 0.5 µg/ml, respectively. The intra-day accuracy and precision CVs were 91.2%-120% and 0.8%-6.8%, respectively, and the inter-day accuracy and precision were 92.6%-115% and 2.5% to 7.5%, respectively. Absolute recovery was 100% ± 15% for Voriconazole.

Keywords: Voriconazole; Fluconazole; UHPLC; Diode array; Reversed phase

INTRODUCTION

Invasive Fungal Infections (IFIs) have become an emerging clinical entity over the past few decades. Several triazole antifungal agents such as fluconazole, ketonazole, itraconazole, voriconazole and posaconazole are used to treat fungal infections [1]. Voriconazole is a broad-spectrum second-generation triazole antifungal agent. It is synthesized from fluconazole. Voriconazole commonly treats invasive aspergillosis and candidiasis fungal infections [2]. It inhibits the action of fungal cytochrome P450-dependent 14α-sterol demethylase, which is considered a key enzyme of ergo sterol biosynthesis, destroying the function of the fungal membrane [3] (Figure 1).

Voriconazole exhibits non-linear pharmacokinetics and is mainly metabolized in the liver by the cytochrome P450 system. Previous studies proved that voriconazole serum concentrations are different and unpredictable [3]. Serum levels vary according to age, genetic factors, and interactions with other drugs. Therefore, it is crucial to know more about the pharmacological properties, metabolism, interactions, and side effects. Therapeutic drug monitoring is crucial to maximize the efficacy and minimize the risk of toxicity.

High-performance liquid chromatography is considered one of the most predominant technologies used in analytical laboratories for analyzing drugs worldwide during the past 30- plus years due to its robustness and lower instrumental and running costs [2]. UHPLC was first introduced in 2004. LC separation in UHPLC is done *via* smaller particle size columns (typically sub-2 µm) and an internal diameter of 2.1 mm [4]. In addition to that, instrument hardware

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has been modified in concert with low particle size columns. The system volume was reduced to 350 µl to decrease band spreading and improve peak shape and sensitivity. UHPLC separation keeps the same analytical separation as HPLC, but it drastically changed in resolution, speed and sensitivity [4]. Today most laboratories are concerned with reducing cost and time in the development of new drugs and, at the same time, keeping the quality parameters of their products. Speed has a great impact on increasing sample throughput and lab productivity [5]. A novel and fast UPLC-DAD assay for TDM of Voriconazole in human plasma after simple protein precipitation is developed and then validated as per FDA guidelines.

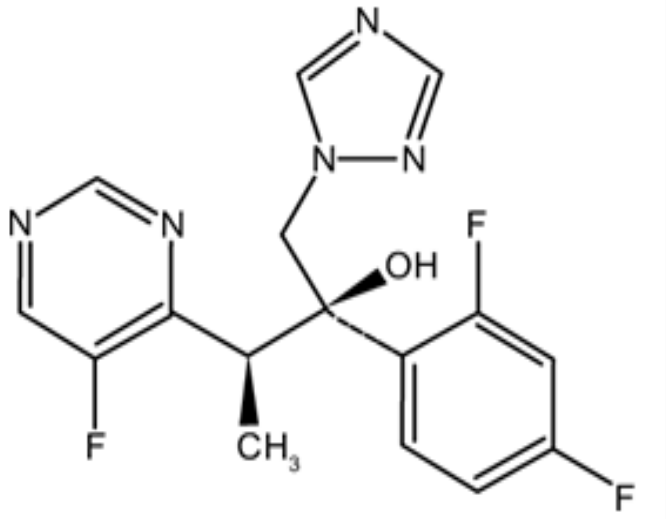


Figure 1: Chemical Structure of Voriconazole.

MATERIALS AND METHODS

Voriconazole and Fluconazole powders were purchased from Sigma Aldrich (St Louis, MO, USA) with purity ≥ 99.9%. High-Performance Liquid Chromatography grade Methanol was obtained from Merck (Darmstadt, Germany), and water for injection was used.

Instrumentation and chromatographic conditions

Chromatography was performed on Agilent®1290 Infinity UHPLC system equipped with 1290 quaternary pump,1290 thermostated Autosampler, 1290 temperature control with injection precision <0.15RSD and 1290 diode array detector. The output signal was monitored and processed using open lab software. Separation was done via Zorbax Eclipse plus C18 2.1*50 mm. 1.8 µm column with a cooling Autosampler at 10°C, and column oven maintained at 40°C. Gradient Elution of water and Methanol at a flow rate of 0.4 ml/min. The analysis started with an initial flow of 10% methanol for 0.4 min, increased to 60% to 6.2 min, and then back to the initial condition of 10% till 7.5 min. 10 µl was injected into the column. Voriconazole and Fluconazole were detected using an Agilent Diode Array detector at 261 nm wavelength. The retention time of Fluconazole and Voriconazole is 4.2 and 6.2 minutes, respectively. Quantitate detection methodology is peak area, and Qualitative detection methodology is retention time and spectrum. All chromatographic conditions are illustrated in table 1 (Figures 2 and 3).

Table 1: Chromatography and optimized parameters.

Instrumental Parameters	Conditions
Flow rate	0.4 ml/min
Injection volume	10 µl
Wavelength	261 nm
Column	Zorbax Eclipse Plus C18 2.150 mm 1.8 µm
Column temperature	40°C
Auto sampler temperature	10°C
Mobile phase	CH3OH:H2O (Gradient Elution)
Retention time	-
Voriconazole	6.2 min
Fluconazole	4.2 min

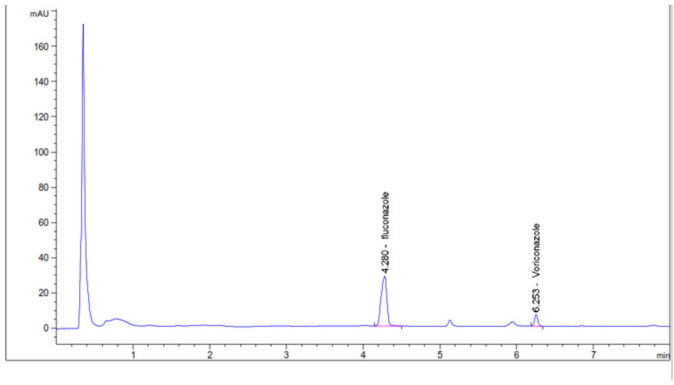


Figure 2: Representative UHPLC Chromatogram (Voriconazole Standard (4 µg/ml) at 6.2 min spiked in plasma and 250 µg/ml Fluconazole used as internal standard at 4.2 min).

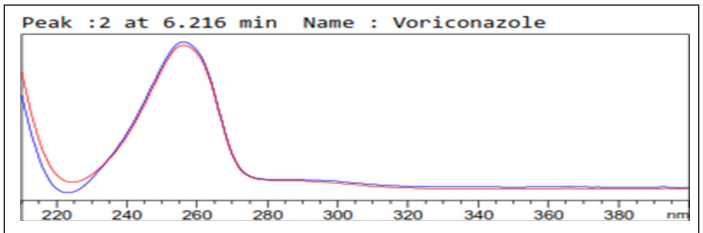


Figure 3: UV spectrum of Voriconazole. Note: ▬ : Voriconazole; ▬ : Fluconazole.

Preparation of stock solutions and standard working solution

10 mg/ml stock solution of Voriconazole was prepared in methanol. Serial dilutions of Voriconazole were prepared as working solutions for the calibration curves. They were as follows: 0.2, 0.5, 1, 2, 4, 6, 8, and 12 µg/ml. Quality control samples at 0.2, 0.5,4, and 6 µg/mL were prepared in the same way as the calibration standards. A working internal standard solution was prepared at a concentration of 250 µg/ml in methanol: water (50:50). QC samples were refrigerated at 2°C-8°C all over the analysis procedure.

Extraction procedure

200 µl internal standard and 200 µl Voriconazole working solutions were added to 200 µl plasma with 400 µl methanol in respective micro centrifuge tubes. The tubes are capped and vortexed for at least 20 seconds. After centrifugation at 10,000 rpm for 10 minutes

at ambient temperature, clear supernatant was transferred into an autosampler vial and injected onto the system.

RESULTS AND DISCUSSION

UHPLC method validation

The validation has been performed according to FDA Guidance for Industry: Bioanalytical method validation [6]. The described method was validated with respect to system suitability, linearity, the Limit of Detection (LOD) and Limit of Quantitation (LOQ), precision, accuracy, and selectivity.

Linearity, Limit of Detection and Limit of Quantitation

Linearity was determined using standard plasma samples with concentrations ranging from 0.2-12 µg/mL by plotting the peak area ratio of Voriconazole to IS against the nominal concentration of Voriconazole in plasma. For calibration, all levels were extracted and analysed in duplicates. The calibration curves were constructed by weighted (1/x2) least-squares linear regression. LOD and LOQ were found to be 0.2 µg/ml and 0.5 µg/ml respectively (Figure 4).

Accuracy and precision

Four concentrations of the QC samples were prepared for each concentration in 3 different days (LLOQ, LOQ, MOQ and UOQ). The intra-day accuracy and precision CVs were 91.2%-120% and 0.8%-6.8%, respectively, and the inter-day accuracy and precision were 92.6%-115% and 2.5% to 7.5%, respectively. Accuracy was expressed as a percentage of the mean deviation from the actual value, determined with the formula [(mean measured concentration-true concentration)/true concentration] x 100. Precision was expressed as Relative Standard Deviation(RSD) calculated as follows: [RSD%=(standard deviation/mean of measured values) x 100]. Criteria for accuracy is within ± 15% deviation from the actual value except at the Limit of Detection (LOD), where it should not deviate by more than 20%. A criterion for precision is within 15% of the RSD except for the LOD, where it should not exceed 20% of the RSD% [7] (Table 2).

Extraction Recovery

The recovery was calculated by comparing the relative peak areas of VRC/Flu added to the blank plasma and extracted using the protein precipitation procedure (A) with those obtained from the compound spiked into the post-extraction supernatant at three QCs (B) concentration levels (0.7, 6 and 12 µg/ml). The ratio (A/B x 100) was used to evaluate the extraction recovery. The extraction recoveries of Voriconazole from human plasma for three were 114.3%, 87.5% and 99.3%, respectively (Table 3).

Selectivity

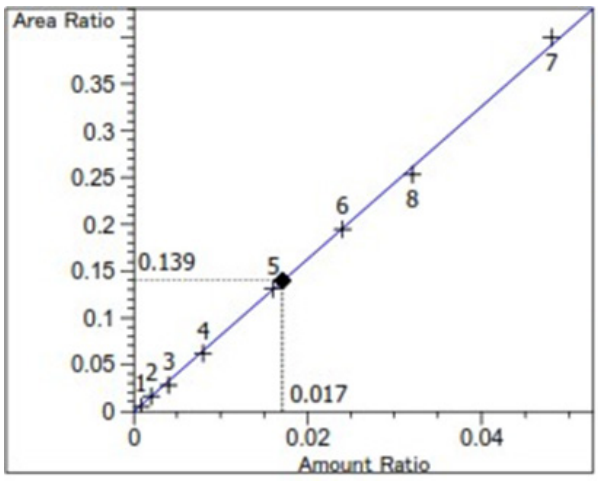
The selectivity of the method was evaluated by analysing plasma samples from six normal volunteers in which no interference was noted at the retention times of Voriconazole and Fluconazole (Figure 5).

Stability

Long-term stability was not performed for this method. The outcomes of stability studies of the Voriconazole/Fluconazole standards and QC samples reveal, regarding the VRC/Flu working solution, they are stable for 48 hours at room temperature and one month at 2-8°C (refrigeration temperature), while the QC samples were stable at 2-8°C for the whole duration of the analysis.

System suitability

System-suitability test was an integral part of method development and has been used to ensure the adequate performance of the chromatographic system suitability was done on the system to ensure the accuracy and precision of the system by injecting six successive injections of both Voriconazole and Fluconazole standard solution. The following parameters were determined: plate count, tailing factor, resolution, %RSD of retention time and peak area. System suitability results and acceptance criteria in accordance with the ICH guidelines (ICH guideline Q2 (R1), 2005) in table 4.

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Voriconazole at exp. RT: 6.164
DAD1 B, Sig=261,4 Ref=off
Correlation:          0.99970
Residual Std. Dev.:   0.00490
Formula: y = mx
      m:      8.16573
      x: Amount
      y: Area
Calibration Level Weights:
    Level 1    : 1
    Level 2    : 1
    Level 3    : 1
    Level 4    : 1
    Level 5    : 1
    Level 6    : 1
    Level 8    : 1
    Level 7    : 1
```

Figure 4: Voriconazole bioassay linearity and calibration curve (R2= 0.9997).

Table 2: Intra- and interday accuracy and precision of the method.

Nominal µg/ml	Found µg/ml	%Accuracy	Precision %RSD
Intra-day precision and accuracy			
Within run variability(n=6 at each concentration)			
0.2	0.24	20	6.8
0.5	0.46	8	4
4	3.65	8.8	0.82
6	5.94	1	3.4
Inter-day precision and accuracy			
Between run variability(n=18 at each concentration)			
0.2	0.21	5	7.5
0.5	0.463	7.4	5.4
4	3.93	1.8	2.5
6	5.65	5.8	2.5

Note: Nominal value: Plasma concentration of Voriconazole quality control; RSD: Relative Standard Deviation.

Table 3: Extraction recovery of Voriconazole.

Conc µg/ml	Relative AUC post spiked plasma(A)	Relative AUC post spiked plasma(B)	% Recovery
0.7	0.08	0.07	114.3
6	0.63	0.72	87.5
12	1.149	1.154	99.6

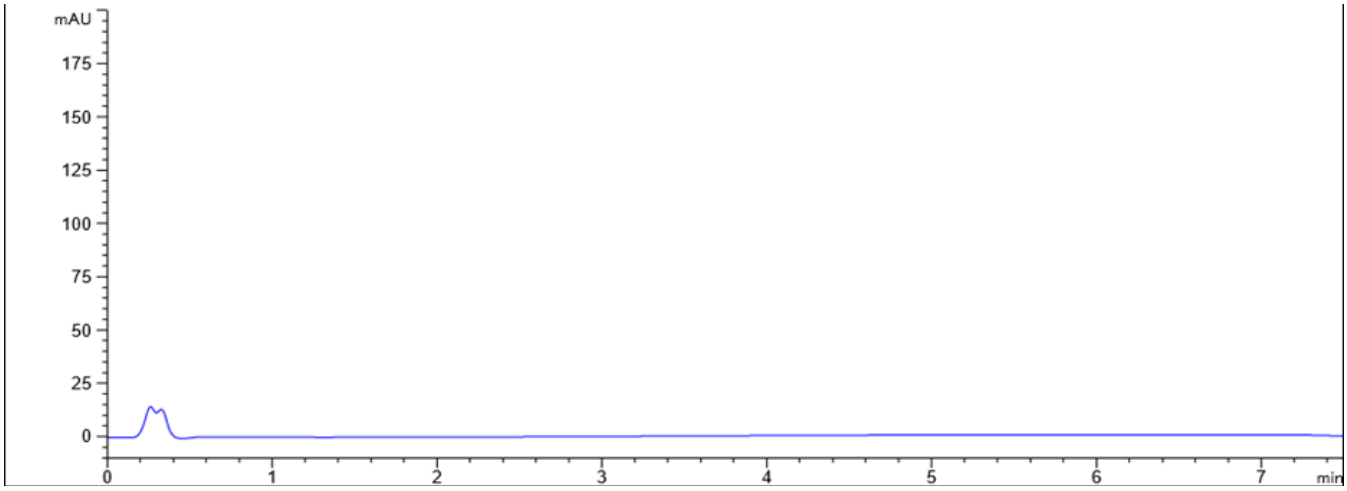

Figure 5: Representative UHPLC chromatogram of Blank Plasma.

Table 4: System suitability results and acceptance criteria.

System suitability parameters	Acceptance criteria	Results	0.72
		Voriconazole	Fluconazole
Injection precision, RT(min)	RSD ≤ 1%	0.31%	0.60%
Injection precision, Peak area	RSD ≤ 1%	0.13%	0.40%
Resolution	Rs= ≥ 2.0	-	18
Tailing	T= ≤ 2.0	1.15	0.95
Efficiency	N= ≥ 2000	87192	18194

CONCLUSION

A simple, precise, accurate, reproducible, highly sensitive and effective stability indicating UHPLC method was developed and validated for the simultaneous quantitative determination of Voriconazole. The method was validated for accuracy, precision, specificity, and linearity. LOD and LOQ values are 0.2 and 0.5 µg/mL, respectively. In this study, the high recovery and low relative standard deviation confirm the method's suitability for determining Voriconazole in human plasma. Based on FDA criteria, the proposed method can be used to monitor and determine the pharmacokinetic parameters of Voriconazole. This method showed good clinical correlation and may be used in routine therapeutic drug monitoring of Voriconazole.

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