Plasma Voriconazole Estimation by HPLC

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Abstract Voriconazole, an antifungal drug exhibiting wide inter-individual variability, is an ideal candidate for therapeutic drug monitoring (TDM). The aim of the present study was to standardize a simple, sensitive and rapid high performance liquid chromatography (HPLC) method with ultraviolet detection to determine plasma voriconazole concentration. The HPLC method consisted of a combination of acetonitrile and water (7:3) as mobile phase with 1 ml/min flow rate and detection at 255 nm. Plasma protein precipitation was carried out using perchloric acid and the filtered supernatant was passed through C18 column (250 × 4.6 mm, 5 µm) for the separation of voriconazole. The limit of quantification of voriconazole was 0.2 mg/L. The assay was validated with a linearity of 0.2–15 mg/L and used clinically for TDM in patient samples. The inter-assay precision was below 15 % for routine quality control samples. Weight based voriconazole doses were prescribed to 26 patients for empirical treatment of invasive fungal infections. Voriconazole therapy was managed from the baseline drug levels and follow up analysis reflected achievement in clinical efficacy. Routine TDM of voriconazole may reduce adverse events and improve the treatment response in invasive fungal infections.

Keywords Voriconazole · HPLC · Therapeutic drug monitoring

Abbreviations

BMI	Body mass index
CV	Coefficient of variation
HPLC	High performance liquid chromatography
IFI	Invasive fungal infection
IPA	Invasive pulmonary aspergillosis
LC–MS	Liquid chromatography–mass spectrometry
LOQ	Limit of quantification
QC	Quality control
TDM	Therapeutic drug monitoring
Vrc	Voriconazole

Introduction

Increasing frequency of immunocompromised conditions like cancer, organ transplantation, HIV infection etc. have resulted in a rise in life threatening co-conditions like invasive fungal infections (IFIs). Several triazole anti-fungal agents such as fluconazole, ketonazole, itraconazole, voriconazole and posaconazole are used for treatment to improve the therapeutic efficacy [1]. Voriconazole (Vrc), a structural derivative of fluconazole, is a second-generation triazole with broad-spectrum antifungal activity. It has an enhanced potency against *Aspergillus* species, candidemia in nonneutropenic patients, esophageal candidiasis, disseminated candidiasis, and is also used as a salvage therapy for fungal infections caused by *Scedosporium apiospermum* and *Fusarium* species [2, 3]. Voriconazole acts as an enzyme inhibitor by blocking the synthesis of ergosterol, a major component in the fungal cell membrane, and thereby the growth of the micro-organism [4, 5].

Vrc is metabolized by hepatic cytochrome P450 (CYP) isozymes predominantly by CYP2C19 while CYP2C9 and

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CYP3A4 contribute to a minor extent [5]. These isozymes are polymorphic thus affecting the enzyme activity and influencing Vrc metabolism [6, 7]. The elimination half-life is approximately 6–9 h, and it increases with increasing dosage. Steady-state plasma concentrations are attained after 5–7 days of treatment. Comprising a narrow therapeutic index, pharmacokinetics of Vrc is profoundly influenced by food and drug interactions and inter-individual variability regardless of route of administration [8–11]. Factors well documented to be associated with inter-individual variability of voriconazole exposure include liver dysfunction, gastrointestinal abnormalities (e.g. mucositis or diarrhea) impairing drug absorption, intake with or without food, alcohol abuse in the past, concomitant use of potent CYP450 inducers or inhibitors, *CYP2C19* genetic polymorphism; thus rationalizing the necessity for therapeutic drug monitoring (TDM) of voriconazole [6–13].

Quantification of azoles by various analytical techniques such as high performance liquid chromatography (HPLC) [5, 9, 14], liquid chromatography-tandem mass spectrometry methods (LC-MS) [15] and microbiological methods [16] have been reported. HPLC methods are specific and sensitive for plasma voriconazole concentrations however, special columns and sample preparation with liquid phase extraction and/or tedious precipitation steps are involved [5, 17]. We aimed to optimize an HPLC based method for estimation of voriconazole in plasma. The method detailed below is a simple, selective, and sensitive HPLC method with UV detection and linearity of 0.2–15 mg/L. We also present data of therapeutic drug monitoring in twenty six patients on voriconazole therapy.

Material and Methods

The study was initiated as a part of standardization for a laboratory diagnostic test. The ethical approval for the data analysis of the patient samples was obtained from the Institutional Review Board.

Chemicals and Instrumentation

Voriconazole (C₁₆H₁₄F₃N₅O; CAS# 137234-62-9) powder was obtained from Sigma Aldrich (St Louis, MO, USA), HPLC grade Acetonitrile, Methanol and Perchloric acid were obtained from Merck (Darmstadt, Germany) while Milli-Q water from MiliporeElix 10[®] water purification system. Waters 1525 multisolvent delivery system pump and Waters 2487 variable wavelength UV–Vis detector with Empower Version 2 software were used for estimation. The mobile phase consisted of Acetonitrile:MilliQ water (7:3 v/v) filtered through a 0.45 µm hydrophilic polypropylene filter. The separation was performed in

isocratic mode at 1 ml/min flow rate using Waters Symmetry C18 column (250 mm × 4.6 mm, 5 µm) maintained at 25 °C.

Sample Collection and Processing

Matrix based calibrator and controls were prepared using appropriate dilutions of voriconazole stock solution (1 g/l) prepared in methanol. A series of working standards with a concentration of 0.6, 1, 3, 5, and 7 mg/L and three level quality control (QC) samples with a concentration of 0.8, 2 and 4 mg/L were prepared. Plasma proteins were precipitated using perchloric acid. The samples were vortexed and centrifuged at 14,000 rpm for 15 min. Hundred microliters of filtered clear supernatant was injected into the chromatographic system [18, 19].

Validation Parameters

The method was validated for linearity, accuracy, precision, recovery and inter-equipment variability as per guidelines for bioanalytical method validation [20]. Three level controls were processed in every batch for accuracy and calibrator was injected both at the start and end of the batch to assess precision and ensure minimal carry over during the analysis. An acceptable coefficient of variation (CV) for QC samples was ±20 % [20]. The method was also validated using an external commercially available HPLC based Reagent kit for determination of antifungal azoles (Catalogue No—27037; Chromsystems, GmbH, Munich, Germany).

Therapeutic Drug Monitoring of Voriconazole in Patient Samples

Voriconazole drug analysis was performed in 26 patients (14 males and 12 females) either suffering from invasive fungal infections or were on empirical treatment for at least 4 days. Demographic and clinical details of each patient were collected in a predetermined proforma at the time of sample collection. Plasma separated from EDTA whole blood was used for drug analysis. An invasive fungal infection was defined as per the invasive fungal infection group of European organization for research and treatment of cancer (EORTC) and the Mycoses Study Group of the National Institute of Allergy and Infectious Disease (MSG) [21]. Vrc level for each patient was correlated with time of sample collection, drug dose and clinical response.

Statistical Analysis

The method validation parameters were assessed based on correlation coefficient of the calibration curve for the linear

range and %CV of quality control samples. The demographic details and voriconazole levels in the patients were expressed as median (range). Statistical analysis and multiple regression analysis of drug levels was calculated using GraphPad Quickcalc statistical software (GraphPad Software Inc., San Diego, CA, USA) with a statistical significance of less than 0.05.

Results

Peak Identification

Vrc standards with increasing concentrations were injected serially to identify the retention time. Proportionate increase in peak height and area under the curve were assessed. Voriconazole eluted at 4.4 min (Fig. 1).

Linearity and Limit of Quantification (LOQ)

Matrix based calibrators from 0.0 to 50.0 mg/L (0.00–143 $\mu\text{mol/L}$) were assayed to obtain method specifications. Limit of Quantification (LOQ) as per European Pharmacopeia standards was 0.2 mg/L (0.57 $\mu\text{mol/L}$) while method linearity was from 0.2 to 15 mg/L (0.57–43.0 $\mu\text{mol/L}$) with an average correlation coefficient higher than 0.998 (Fig. 2). Voriconazole concentrations as high as 50 mg/L showed minimal carry over.

Precision, Accuracy and Recovery

As a part of analytical validation the parameters such as intra-day and inter-day precision were assessed with 5 replicates for 3 controls 0.8, 2 and 4 mg/L on separate occasions. Accuracy was checked by the recovery of controls i.e. within 80–120 % (Table 1).

Method Comparison with Commercially Available Kit

Trough Vrc levels for 11 patients were estimated by both in house method and the commercially available kit. A correlation coefficient of ~ 0.99 (Fig. 3) was obtained for these methods qualifying them for suitability.

Voriconazole Drug Level in Patients

The method was effectively used to determine drug levels in patients on prophylactic ($n = 4$) or anaphylactic ($n = 22$) voriconazole therapy. The patients either had proven aspergillosis ($n = 4$), candidiasis ($n = 2$) or cladothialophora bantiana infection ($n = 1$) or probable

invasive pulmonary aspergillosis (IPA) ($n = 15$) in addition to their primary immunocompromised condition such as post-operative complications, cancer, other infections and solid organ transplant.

We received 45 plasma samples from 26 patients on ongoing voriconazole therapy. Baseline trough levels which is the ideal time for TDM of Vrc were performed in 20 out of 26 patients (77 %) while in the remaining 6 patients, blood collection was done at random time points in view of toxicity or drug interactions. Follow-up analysis was performed in 13 patients wherein 5 patients required dose adjustments so as to obtain desired drug level with optimal clinical efficacy. The laboratory used literature based reference range of 2–6 mg/L for plasma voriconazole levels [7, 22]. Demographic details and trough Vrc levels of these patients are described in Table 2. It is evident from Table 2 that despite all patients achieving their steady state concentrations only 13 (65 %) attained therapeutic level while 6 patients (30 %) were in the sub-therapeutic range. One patient in this group remained in the sub-therapeutic range even after 455 days of therapy while one patient experienced toxic level & side-effects within 7 days of treatment.

Blood collection and Vrc level estimation in 6 patients was performed at random time points in view of their toxicity or drug interactions. Three of these reflected side-effects like visual disturbances and hallucinations within median 11 days of therapy and had a median Vrc of 9.1 mg/L even after 36 h of drug discontinuation.

Two patients were in therapeutic range after ~ 8 h of voriconazole dose while the remaining 1 patient was on co-medication with an interacting drug (rifampicin) and hence had peak Vrc level in the sub-therapeutic range i.e. 1.1 mg/L. Discontinuation of Rifampicin showed an increase in trough Vrc level to 2.1 mg/L on follow up analysis.

Multivariate regression analysis of plasma voriconazole levels with age ($p = 0.2504$), gender ($p = 0.095$), BMI ($p = 0.7680$), dose ($p = 0.5075$) and duration of therapy ($p = 0.2907$) did not show any statistical significance.

Discussion

Voriconazole has an unpredictable drug dose-exposure relationship and its serum concentration has been linked to both efficacy and toxicity serving it as an ideal candidate for TDM. Several methods using different mobile phase composition (both buffers and organic solvents) and sample preparation procedure such as liquid: liquid extraction, precipitations etc. have been reported [5, 14, 23]. In this study we have used single step perchloric acid protein precipitation and a mixture of acetonitrile and water as

Fig. 1 Chromatograms of Voriconazole, **a** Blank plasma, **b** voriconazole standard (7 mg/L) spiked in plasma

mobile phase. This reduces an inter-assay variability caused due to sample extraction and buffer preparation. The validation parameters obtained in our study qualify this method to be robust & sensitive for estimation of plasma voriconazole level using HPLC.

Voriconazole therapy is initiated in patients with invasive fungal infections both for therapeutic as well as prophylactic management. Usually these patients have an underlying condition such as malignancy, immunosuppressed or

immunocompromised post-transplant/surgical status etc. These clinical conditions may contribute to an altered therapeutic efficacy of voriconazole. Genetic variants of the metabolizing enzyme i.e. *CYP2C19* have also been reported to influence voriconazole metabolism [24] suggesting a large inter-individual variability. Our data also shows high variability in voriconazole level ranging from sub-therapeutic i.e. < 2 mg/L (30 %) to toxic i.e. > 6 mg/L (5 %) in patients receiving recommended doses. These variations could be

Fig. 2 Linearity of the assay. The plot represents standard concentrations of voriconazole (0.0–15 mg/L) versus area under curve observed with a correlation coefficient of 0.993. The plot in the *inset* represents the standard concentrations of voriconazole (0.0–50 mg/L) versus area under curve observed with a correlation coefficient of 0.974

Table 1 Precision and accuracy of voriconazole

Intra-assay (within run) (n = 5)			
Concentration (mg/L)	0.8	2	4
Mean (mg/L)	0.92	2.32	4.76
SD (mg/L)	0.044	0.028	0.056
%CV	4.7	1.2	1.05
% Recovery (between 80 and 120 %)	115	116	119
Inter-assay (between run) (n = 5)			
Concentration (mg/L)	0.8	2	4
Mean (mg/L)	0.85	2.08	4.3
SD (mg/L)	0.11	0.25	0.50
%CV	12.8	12.01	11.59
% Recovery (between 80 and 120 %)	106	104	107

attributed to the above mentioned factors wherein sub therapeutic level was influenced in individual cases either by low dosage or drug interactions with co-interacting drug (rifampicin) while high levels could be attributed to high drug dose and obesity of the patient. A similar influence of high body weight and dose has been reported by Leveque et al. and Koselke et al. [4, 12]. Voriconazole toxicity, mainly neurotoxicity and abnormal liver function test results have been associated with elevated trough concentrations of 5–6 mg/L [4]. A strong correlation between CYP2C19 genetic variant and voriconazole level was also observed in our laboratory (data not shown). These factors play a vital role in inter-individual

Fig. 3 Comparison of the two methods. Observed concentration of voriconazole from inhouse method v/s concentration obtained from commercial kit

variability and reiterate the need of therapeutic drug monitoring of voriconazole.

A follow up drug analysis subsequent to appropriate management (dose adjustments/withdrawal of interacting drug etc.) showed an optimal therapeutic efficacy in our study.

In conclusion, we report a robust validated method for detection and quantification of plasma voriconazole level with no interferences, rapid analysis and minimal sample requirement enhancing the advantages of the assay. A fixed dosing regimen is not optimal for voriconazole therapy and drug levels would serve as a useful guide for dosing in patients with several influencing factors. Thus,

Table 2 Demographic details and trough Vrc levels of patients on Vrc therapy

	Sub-therapeutic median (range)	Therapeutic median (range)	Toxic median (range)
No. of patients (n = 20)	6	13	1
Age (years)	43 (8–54)	46 (14–77)	68
Weight (kg)	52.5 (21–83)	64.5 (44–72)	65
Vrc dose (mg/day)	400 (200–400)	400 (200–600)	500
Duration of therapy (days)	37 (6–455)	5 (4–90)	7
Vrc levels (mg/L)	0.8 (0.2–1.3)	3.2 (2.3–4.7)	7.9

voriconazole drug monitoring and clinical history correlation play a vital role in accomplishing antifungal therapeutic efficacy.

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Conflict of interest All authors declare that they have no conflict of interest.

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