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Quantification of Antifungal Drug Voriconazole in Serum and Plasma by HPLC-UV

Peter H Tang*

Divisions of Pathology and Laboratory Medicine, Departments of Pathology and Pediatrics, Cincinnati Children's Hospital Medical Center, University of Cincinnati College of Medicine, 3333 Burnet Avenue, Cincinnati, Ohio 45229-3039, USA

Abstract

A simple, sensitive and rapid high-performance liquid chromatographic (HPLC) method for the determination of voriconazole in human serum or plasma was developed. Voriconazole and internal standard clonazepam were extracted from plasma or serum with methanol and analyzed on a Microsorb-MV C18 column with ultraviolet (UV) detection set at wavelengths of 256 and 310 nm, respectively. The calibration curve was linear through the range of 0.1-10 mg/L using a 0.1 mL sample volume. The within-run and between-run precisions were all less than 6%. Accuracies ranged from 97 to 106%. Absolute recovery was 96.4 ± 1.3% for voriconazole. The method has been applied to monitor voriconazole use in order to ascertain clinical efficacy and minimize toxic effects.

Keywords: Voriconazole; Clonazepam; HPLC

Introduction

Voriconazole is a broad-spectrum, second-generation triazole antifungal agent, designated chemically as (2R, 3S)-2-(2,4-difluorophenyl)-3-(5-fluoro-4-pyrimidinyl)-1-(1*H*-1,2,4-triazol-1-yl)-2-butanol, that is a derivative of fluconazole. Voriconazole has been widely used for the treatment of invasive fungal diseases, particularly invasive aspergillosis. In addition, voriconazole is also approved for the treatment of invasive candidosis, as well as for less frequent fungal infections such as fusariosis and scedosporiosis. Its fungicidal action is due to inhibition of fungal cytochrome P450-dependent 14α-sterol demethylase, a key enzyme of ergosterol biosynthesis. Inhibition of ergosterol biosynthetic pathway leads to a disruption of the integrity and the function of the fungal membrane.

Purkins et al. reported human pharmacokinetic data for voriconazole which displayed linear pharmacokinetics in children and non-linear pharmacokinetics in adults [1]. According to previous studies, voriconazole serum concentrations were varied and unpredictable [2]. High variability had also been observed in serum voriconazole concentration within and between individuals [3-5]. Although serum voriconazole concentration is unpredictable, therapeutic drug monitoring (TDM) could be most useful in order to ascertain clinical efficacy and to minimize toxic effects.

For the determination of voriconazole concentrations in the biological fluids, the main methods presently used are high-performance liquid chromatographic (HPLC) techniques coupled with mass spectrometry (MS), fluorimetry or spectrophotometry. The MS methods require small sample volumes [6-22] and are generally superior in sensitivity and specificity to both fluorimetry [23] and spectrophotometry [24-45]. However, the purchase and running costs of LC-MS and LC-MS/MS instruments are high. Fluorescent and ultraviolet (UV) detectors are sensitive enough to measure clinically relevant concentrations (1-5.5 µg/mL) of voriconazole in serum or plasma, allowing the use of common HPLC instrumentation. The majority of published methods used internal standard, however, a few analytical protocols were associated to external standard calibration [6,9,39-45].

Previously, TDM of voriconazole ordered by the physicians here at the Cincinnati Children's Hospital Medical Center (CCHMC) was carried out by the reference laboratory. However, providing clinicians quicker test results for better clinical service is highly desirable. For this purpose, an analytical procedure was developed for measuring voriconazole concentrations in serum and plasma. In this article, a simple and rapid HPLC method with UV detection for routine TDM of voriconazole is described. The current method provides sufficient specificity and sensitivity to meet the clinical needs. It is of particular importance that the current method requires as little as 0.1 mL of sample for voriconazole measurement in pediatric practice. Sample preparation involves precipitation of proteins with a single step methanol extraction and centrifugation.

Experimental

Voriconazole and clonazepam were obtained from Sigma (St. Louis, MO). Other chemicals used were analytical grade or HPLC grade from Sigma. The stock solution of voriconazole (100 µg/mL) was prepared in methanol. A series of six calibrator solutions, ranged from 0.1–10 µg/mL, were prepared using the voriconazole stock solution with the appropriate volume of methanol to final concentrations of 0.1, 0.5, 1.25, 2.5, 5 and 10 µg/mL. The stock solution of internal standard clonazepam (100 µg/mL) was prepared in methanol. A working solution internal standard clonazepam (5 µg/mL) was prepared using the stock solution with the appropriate volume of methanol to final concentration of 5 µg/mL. Serum free of voriconazole was pooled and centrifuged to remove clots. Three quality controls were prepared in the pooled serum to final concentrations of 1, 4 and 8 µg/mL. An aliquot of 100 µL of pooled serum or controls was transferred into a microcentrifuge tube and capped and stored at –20°C until analysis.

***Corresponding author:** Peter H Tang, PhD, Divisions of Pathology and Laboratory Medicine, Departments of Pathology and Pediatrics, Cincinnati Children's Hospital Medical Center, University of Cincinnati College of Medicine, MLC 1010, 3333 Burnet Ave, Cincinnati, OH 45229, USA, Tel: (513) 636-7328; Fax: (513) 636-8850; E-mail: peter.tang@cchmc.org

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In this study, 30 de-identified samples ordered for concentrations of voriconazole were included for comparison between the current method and reference method. Thirty-two de-identified samples submitted for the testing of other drugs were also analyzed to identify potential interference.

Apparatus

The Agilent 1260 Infinity HPLC system equipped with Model 1260 quaternary pump, Model 1260 Hip ALS autosampler, Model 1290 thermostat, Model 1290 TCC control module, and Model 1260 diode array detector (DAD). The output signal was monitored and processed using the EZChrom Elite® software. Analytical column was a 250-mm x 4.6-mm Microsorb-MV C18 column (Agilent) with 5-µm spherical particles. An Upchurch precolumn filter equipped with 0.5 µm frit, SS, is used to protect the analytical column. The mobile phase consisted of 0.05 M ammonium acetate/acetonitrile/methanol at 40:20:40 (v/v/v). The mobile phase was filtered before use through a 0.2-µm (47 mm diameter) MAGNA nylon filter (GE Water & Process Technologies, Minnetonka, MN) under reduced pressure.

Methods

After pooled serum was thawed at room temperature, internal standard working solution (100 µL), calibrator solutions (100 µL) and methanol (100 µL) were pipetted into respective microcentrifuge tubes and capped. The tubes were capped and vortex-mixed for 1 min and then centrifuged for 10 min at 10,000 rpm. The supernatant was transferred to an autosampler vial, capped, and 20 µL was then injected onto the column. Peak height measurements were analyzed to obtain the ratio of voriconazole versus clonazepam. Ratios of voriconazole versus clonazepam were used to establish a calibration curve and to quantify voriconazole from the calibration curve. Patient or controls were prepared by transferring 100 µL of patient or controls into respective microcentrifuge tubes; 100 µL of internal standard solution was then added to each tube followed by 200 µL of methanol. The tubes were capped and processed in the same manner as calibrators.

A calibration curve was established by adding 6 calibrators to the pooled sera, and the linearity of calibration curve was evaluated by least-squares linear regression analysis. The calibration was performed 3 more times over 2 months. Each time 6 calibrators were carried out in duplicate and concentrations were calculated from the calibration curve.

The absolute analytical recovery was determined at a concentration for voriconazole (4 µg/mL) and for clonazepam at the concentration which was used during the analysis of the serum samples (5 µg/mL). Absolute analytical recovery was determined by comparing the average peak height for five extracted serum samples spiked with voriconazole and clonazepam with that for five aqueous solutions of voriconazole and clonazepam of identical concentrations in which serum was replaced by water.

Standard solutions of several commonly prescribed drugs were injected onto the HPLC in order to check for their retention times. Blank serum from 6 pooled sera was tested for endogenous interferences. Furthermore, serum samples from patients not taking voriconazole and treated with commonly prescribed drugs were analyzed to check for potential interferences.

The within-run accuracy and precision were evaluated using six determinations per control. The deviation of the mean from the true value served as the measure of accuracy. Between-run accuracy and precision were evaluated by carrying out 12 independent determinations for each of four controls over a period of 2 weeks. Each control was analyzed in duplicate.

Further method validation was carried out by 3 different analysts using 6 patient samples. Patient samples were stored at 4°C over a period of 2 weeks; each sample was analyzed in duplicate. The stability of voriconazole in serum sample was also assessed by storage of six patient samples at 4°C over a period of 2 weeks and at room temperature in the light for 4 days and 7 days; values obtained were compared with fresh samples.

Accuracy of the method was further examined by measuring the concentrations of voriconazole in 30 de-identified patient samples. The results obtained by this method were compared with results from the reference laboratory.

Results and Discussion

An internal standard is not required in the external standard method. The disadvantage of the external standard method is the loss of analytes by adsorption at the precipitate. However, the addition of an internal standard is used to correct the loss of analytes due to adsorption. Several commercially available compounds were investigated and clonazepam was found to be best fit for an internal standard. Protein precipitation by organic solvent is a simple and rapid procedure. Quantitative extraction of voriconazole was obtained previously by using protein precipitation with acetonitrile [8,10,12,15,32,39,42,43,45], methanol [11,18,19] or a mixture of both solvents [16,20] as the only sample preparation. In the preliminary study, three different protein precipitation agents (acetonitrile, methanol and a mixture of both) were investigated. All three different protein precipitation agents showed comparable extraction efficiency (by measuring the peak-areas of voriconazole and clonazepam). However, the methanol extracts produced shaper peaks of voriconazole and clonazepam which translated into higher peak-heights for both compounds. Methanol was chosen as the precipitation solvent, and the recoveries of clonazepam and voriconazole were 93.1 ± 0.7% (mean ± SD) and 96.4 ± 1.2%, respectively.

For this study, the effects of column operating temperature were evaluated at various temperatures such as 25, 30, 35, 40, 45 and 50°C. A better operating temperature was found to be at 40°C for producing sharper peaks, earlier elution and better separation. Using the proposed sample preparation protocol and optimized chromatographic conditions, a well-resolved and satisfactory separation of internal standard clonazepam (retention time: ~5.76 min) and voriconazole (retention time: ~7.45 min) was obtained. Typical chromatograms monitored at wavelengths of 256 and 310 nm are presented in figure 1. These two compounds resolved without any overlapping of their peaks or ambiguity in identification.

A maximal level of 9 µg/mL has been observed in >250 patient specimens for routine voriconazole test, which was reported by the Laboratory Administration at CCHMC. The validated linearity up to 10 µg/mL for the method described here makes this method applicable across the wide range of serum concentrations found for patients receiving voriconazole. The current method validation was based on the Guidance for Industry Bioanalytical Method Validation [46] published by the Food and Drug Administration. The current method met all the requirements for developing a calibration curve. The current method was linear between the concentrations of LLOQ (0.1 µg/mL) and the upper limit of quantitation (ULOQ, 10 µg/mL). The coefficients of variation (CV) averaged 4% and 15% for voriconazole at ULOQ and LLOQ, respectively. Analytical performance at LLOQ met criteria of

precision of <20% and accuracy within 80-120% of the target value. The limit of detection (LOD) was estimated at a signal-to-noise ratio of 3:1, which was found to be ~0.06 μg/mL.

The assay results for spiked and pooled samples are shown in table 1. No significant difference was observed between results of spiked and pooled serum. The accuracy was <6% for all concentrations. The minimal deviation of the mean from the true value indicates the excellent accuracy of the method. The within-run precision was <5% of CV, confirming good precision of the method. Between-run precision of voriconazole demonstrated CV values of <6%. Overall the percentage recovery of voriconazole ranged from 97 to 106%, indicating the consistent, precise, and reproducible extraction efficiency of the method. The current method can be performed by any trained person. Table 2 gives details of the method precision of these samples analyzed by 3 analysts who are medical technologists. All precisions were similar, with none of the CVs being >6%.

Further method validation was performed; patient samples containing voriconazole were diluted with pooled serum and assayed. These results are shown in table 3. All the results were within 97-106% of anticipated concentrations, and similar results were observed upon serial dilutions of the high control from 8 to 2 μg/mL. Thus, there did not appear to be matrix interferences compromising the accuracy of the method. The method is optimized for 100 μL of serum sample, but it can be done using a sample as small as 25 μL without compromising its accuracy. There was no evidence for loss of voriconazole stored at 4°C for at least 2 weeks (P<0.0001). Voriconazole appeared stable at room temperature in the light for at least 7 days (P<0.0001), enabling postal samples to be accepted for analysis.

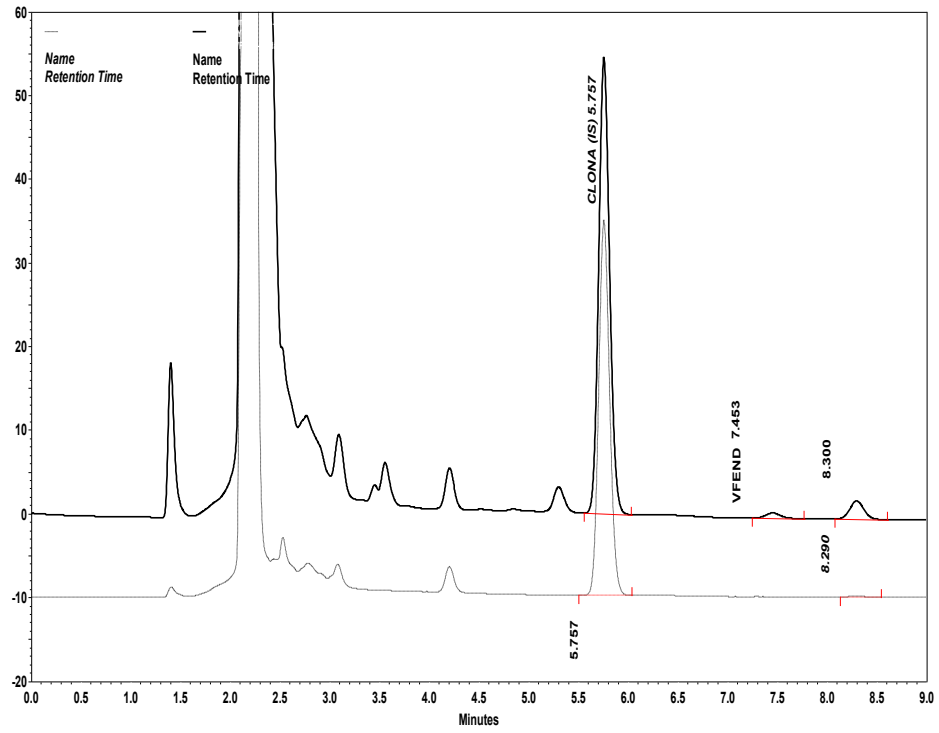

Figure 1: Chromatograms obtained from a representative patient on voriconazole therapy. The peaks of voriconazole (trough level of 0.2 μg/mL) and internal standard clonazepam were monitored at 256 nm (solid line) and 310 nm (dot line), respectively. The retention times of internal standard clonazepam and voriconazole were ~5.76 and ~7.45 min, respectively.

	True concentration (μg/L)	Found concentration (μg/L)	Recovery (%)	CV (%)
Spiked sample:				
Within-run (*n* = 6)	1 4 8	1.04 4.17 8.25	104.0 104.3 103.1	3.1 2.4 1.8
Between-run (*n* = 12)	1 4 8	1.06 4.06 8.21	106.0 101.5 102.6	4.5 3.7 2.9
Pooled sample:				
Within-run (*n* = 6)	1 4 8	0.97 4.01 8.14	97.0 100.3 101.7	4.4 3.7 3.2
Between-run (*n* = 12)	1 4 8	1.03 4.11 8.12	103.0 102.8 101.5	5.6 4.8 4.3

Table 1: Method precision.

Figure 2 illustrates a comparison between the current method and reference method on 30 de-identified patient samples. The correlation between the two methods was good; the linear regression statistics indicated an r^2 value of 0.992 (P<0.0001). The linear regression equation for correlation was y=1.014 x-0.022 with a standard error value of 0.14; where y, the current method and x, the reference laboratory method.

The effects of potential interference from other commonly used drugs were evaluated. To study potential interference, 32 de-identified patient samples submitted for the testing of various therapeutic drugs were analyzed. No apparent interference was observed. Drugs assayed at therapeutic concentrations include: acetaminophen; carbamazepine, carbamazepine-epoxide, and hydroxy metabolites; chloramphenicol; diazepam; ethosuximide; felbamate; fluconazole; gabapentin; ibuprofen; itraconazole; ketoconazole; lacosamide; lamotrigine; levetiracetam; lorazepam; methsuximide and normethsuximide; milrinone; oxcarbazepine and its monohydroxy metabolite; phenacetin; phenobarbital; phenytoin and metabolites; posaconazole; primidone; rufinamide; salicylate; topiramate; valproate; vigabatrin, and zonisamide.

Patient Sample #	*n*	Mean Concentration (μg/L)	Concentration Range (μg/L)	Standard Deviation (μg/L)	CV (%)
1	6	1.53	1.46-1.60	0.07	4.5
2	6	2.24	2.12-2.36	0.12	5.4
3	6	2.40	2.29-2.51	0.11	4.6
4	6	3.08	2.93-3.23	0.15	4.9
5	6	4.67	4.48-4.87	0.20	4.3
6	6	5.95	5.62-6.28	0.33	5.5

Table 2: Method precision performed by three different analysts over a period of two weeks.

Patient #/ dilution	Found* Concentration (μg/L)	Calculated Concentration (μg/L)	Percentage of expected (%)
1/neat	1.26		100
1/x2	0.64	1.28	101.6
1/x4	0.33	1.32	104.8
2/neat	6.47		100
2/x2	3.29	6.58	101.7
2/x4	1.60	6.40	98.9
3/neat	4.09		100
3/x2	2.12	4.24	103.7
3/x4	1.03	4.12	100.7
4/neat	5.34		100
4/x2	2.61	5.22	97.8
4/x4	1.37	5.48	102.6
5/neat	2.09		100
5/x2	1.07	2.14	102.4
5/x4	0.55	2.20	105.3
6/neat	3.41		100
6/x2	1.74	3.48	102.1
6/x4	0.89	3.56	104.4
Control/neat	8.16		102.0
Control/x2	4.12	8.24	103.0
Control/x4	2.09	8.36	104.5

*each sample was measured once.

Table 3: Effects of diluting patient sample and control with pooled serum on the concentrations of voriconazole.

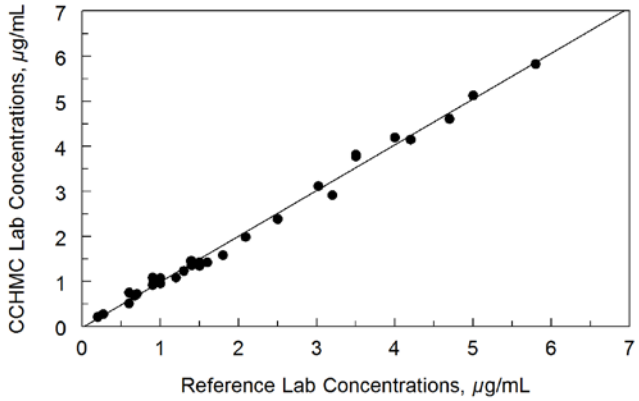

Figure 2: Method comparison data for serum voriconazole levels.

Conclusion

This paper describes a simple, accurate, and reproducible method for the measurement of voriconazole in human serum or plasma. Sample preparation is rapid and efficient, and voriconazole recovery is excellent. The current method uses a single step methanol extraction and avoids the use of more complex liquid-liquid extraction or solid-phase extraction procedure, which substantially decreases set-up time. The method meets the requirements of high sample throughput in clinical analysis. The method uses a commercially available internal standard and has potential cost savings lying in the use of single extraction process. The method can be used to measure voriconazole in as small as 25 μL of sample, which is idea for pediatric sample.

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