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THERAPEUTIC DRUG MONITORING OF VORICONAZOLE IN HUMAN SERUM USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

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ABSTRACT

A fast, simple, selective, and specific high-performance liquid chromatographic (HPLC) method for the measurement of voriconazole in human serum has been developed. Sample preparation involved a single dilution step, solvent extraction, protein precipitation, and ultra-centrifugation. A commercially available compound micafungin was used as internal standard. Voriconazole and micafungin were extracted from serum samples using methanol and analyzed on a Zorbax Eclipse Plus C18 column with fluorescence detection set at excitation and emission wavelengths of 254 and 372 nm, respectively. The calibration curve was linear through the range of 0.2-10 μg/ml using a 0.1 ml sample volume. Both the within-run and between-run precisions were <5%. Accuracies ranged from 97 to 105%. The method has been applied to monitor and optimize voriconazole therapy in clinical practice.

KEYWORDS: Voriconazole, HPLC, fluorescence.

INTRODUCTION

Voriconazole (VRC), an antifungal drug, is used frequently to treat invasive fungal diseases, particularly invasive aspergillosis. In addition, it has also been approved for the treatment of invasive candidosis, as well as for less frequent fungal infections such as fusariosis and scedosporiosis. VRC exhibits its fungicidal action by inhibiting fungal cytochrome P450-

dependent 14α-sterol demethylase, a key enzyme of ergosterol biosynthesis, which leads to a disruption of the integrity and the function of the fungal membrane.

Studies of VRC pharmacokinetics in human have been reported by Purkins *et al.*[1], in which VRC displays non-linear pharmacokinetics in adults and linear pharmacokinetics in children. According to previous studies, VRC serum concentrations were unpredictable and varied.[2] High variability has also been observed in serum VRC concentrations within and between patients.[3-5] Although serum VRC concentration is unpredictable, therapeutic drug monitoring (TDM) could be most useful in managing dosage regimen, ascertaining clinical efficacy, and minimizing adverse effects.

For measuring VRC concentrations in biological samples, high-performance liquid chromatography (HPLC) has been the primary technique in modern clinical and analytical laboratories. Scrutinizing all reported detection technologies, mass (MS) detector[6-22] is generally superior in sensitivity and specificity to both ultraviolet (UV) and fluorescence (FL) detectors.[23-31] However, the purchase, maintenance and running costs of HPLC coupled with MS system (LC/MS or LC/MS/MS) are high. From an economic standpoint, both HPLC/FL and HPLC/UV methods allow cost-effective analysis. Also, both methods provide adequate sensitivity, selectivity and specificity for the determination of clinically relevant VRC concentrations (1-5.5 μg/ml) in patient samples.

TDM of VRC use in patients ordered by the clinicians here at the Cincinnati Children's Hospital Medical Center (CCHMC) is currently performed using a HPLC/UV method[23] at the TDM Laboratory. Rapid turnaround time of test result via the in-house service of TDM has always been satisfied, and as such demands of TDM services are continuously increasing. To allow more flexibility in managing schedules and efficient usage of HPLC instruments, an alternative method is very desirable. In this article, a rapid and simple HPLC/FL method for routine TDM of VRC is described. A commercially available compound micafungin is used as the internal standard. The current method shows simplicity, selectivity, specificity, and sufficient sensitivity for the measurement of VRC concentrations in samples. Simple and fast sample preparation involves a single dilution step, precipitation of proteins, and ultracentrifugation.

MATERIALS AND METHODS

Chemicals and reagents: VRC (VFEND®, Pfizer, NY) for injection and micafungin (Mycamine®, Astellas Pharma US, Northbrook, IL) for injection were obtained from the

Department of Pharmacy at CCHMC. Micafungin was used as the internal standard (IS). Trifluoroacetic acid (TFA) and ammonium acetate were analytical grade from Sigma (St. Louis, MO). HPLC-grade acetonitrile (Fisher Scientific, Fair Lawn, NJ) and HPLC-grade methanol (Fisher) were used without further purification. Deionized water from Millipore's Milli-Q System (Bedford, MA) was used throughout the analysis.

Instrumentation and chromatographic conditions

The Hitachi Chromaster™ HPLC system was equipped with Model 5110 quaternary pump, Model 5210 autosampler, Model 5310 column oven, and Model 5440 FL detector. The output signal was monitored and processed using the EZChrom Elite® software. Analytical column was a 250-mm x 4.6-mm Zorbax Eclipse Plus C18 column (Agilent) with 5-µm spherical particles. An Upchurch precolumn filter equipped with 0.5 µm frit, SS, was used to protect the analytical column. The column was maintained at 45°C and the autosampler was cooled to 4°C. A programmable FL detector set at excitation and emission wavelengths of 254 and 372 nm, respectively.

Details of chromatographic and detection conditions are described in Table 1. Ammonium acetate buffer (0.1 M) was prepared by dissolving 7.7g of ammonium acetate in 1000 ml of deionized water containing 0.1% TFA. The mobile phase consisted of a mixture of 40% ammonium acetate buffer, 40% acetonitrile and 20% methanol (v/v/v), which was degassed via sonication for 10 minutes and then filtered through a 0.2-µm (47 mm diameter) MAGNA nylon filter (GE Water & Process Technologies, Minnetonka, MN) under reduced pressure. The flow rate was 1 ml/min. The chromatographic run time was 8 min per injection.

Standard solutions and controls

VFEND® in a 30 ml Type I clear glass vial containing nominally 200 mg VRC was reconstituted with deionized water to produce a solution I containing 10 mg/ml. A small portion of the resultant solution was further diluted with methanol to produce a solution II containing 100 μg/ml. A series of six calibrator solutions were prepared using the solution II with the appropriate volume of methanol to final concentrations ranged from 0.2–10 μg/ml of VRC.

Mycamine® in a 10 ml Type I clear glass vial containing nominally 100 mg micafungin was reconstituted with deionized water to produce a solution I containing 20 mg/ml. A small portion of the resultant solution was further diluted with methanol to produce a solution II

containing 100 μg/ml. A small portion of solution II was further diluted with methanol to produce an IS working solution containing 10 μg/ml of micafungin.

Serum free of VRC and IS were used as blank sample that were pooled and centrifuged to remove clots. Three quality controls (QCs) were prepared in blank sample to final concentrations of 1, 4, and 8 μg/ml VRC.

Single dilution step, solvent extraction and protein precipitation

All procedures were performed at room temperature, while all serum sample, IS working solution, calibrator solutions and methanol were kept on ice throughout the experiments. Aliquots of blank sample (100 μl), IS working solution (100 μl), calibrator solutions (100 μl) and methanol (100 μl) were pipetted into respective microcentrifuge tubes and capped. The tubes were vortex-mixed for 1 min and then ultracentrifuged at 4°C 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. A 100 μl patient sample or QC along with a 100 μl of IS working solution were pipetted into a microcentrifuge tube. The serum samples were extracted with addition of 200 μl of methanol. These tubes were capped and processed in the same manner as calibrators.

Method validation

The selectivity of the HPLC/FL method was tested by analyzing different human serum samples from patients not treating with VRC and/or IS but with commonly prescribed drugs (Figure 1a). The chromatograms of these serum samples were compared with the chromatograms obtained by serum spiked with VRC and IS. Furthermore, 30 de-identified samples submitted for the testing of other drugs were also checked. Drugs assayed at therapeutic concentrations included acetaminophen, carbamazepine, carbamazepine-epoxide, chloramphenicol, diazepam, ethosuximide, felbamate, fluconazole, gabapentin, ibuprofen, itraconazole, ketoconazole, lacosamide, lamotrigine, levetiracetam, lorazepam, methsuximide, normethsuximide, milrinone, oxcarbazepine and its monohydroxy metabolite, phenacetin, phenobarbital, phenytoin, posaconazole, primidone, rufinamide, salicylate, topiramate, valproate, vigabatrin, and zonisamide.

Calibration curves were established by plotting the peak height ratio of VRC to that of IS against VRC concentration. The linear regression equation was generated by analyzing blank samples to which six calibrators were added in the range of 0.2-10 μg/ml and by least-squares

linear regression analysis. The equation was used to calculate the concentrations of QC and unknown sample. The sensitivity was determined based on signal-to-noise ratio by measuring the lowest limit of quantitative (LLOQ) concentration of VRC that could be measured with acceptable accuracy and precision. The LLOQ was established using 10 samples and determined the CV (Table 2).

The within-run accuracy and precision were evaluated using six determinations per QC (Table 3), while between-run accuracy and precision were evaluated by carrying out 12 independent determinations for each of three QCs over 7 days. Each control was analyzed in duplicate. The deviation of the mean from the nominal value served as the measure of accuracy and expressed as percentage of the relative error. Precision was expressed as coefficient of variation (CV) calculated with the formula: [CV% = (standard deviation/mean of measured values) x 100].

The recoveries of VRC and IS were evaluated by comparing the detector responses obtained from three QCs of VRC and IS added to and extracted from serum to the detector responses obtained for the true concentrations of VRC and IS added to water.

Stability experiments for VRC and IS were performed on three QCs under a variety of storage and handling conditions: freeze-thaw cycles at room temperature (under chemical/biological hood); bench-top stability (experiments was conducted at room temperature under normal laboratory light for up to 24 h); long-term stability (freeze samples for 7 days at –20°C and then thaw for 30 min at room temperature); stability of stock solutions of VRC and IS were evaluated; stability of processed samples sitting on bench-top for up to 24 h were tested. Reproducibility of the method was assessed by repeated measurements using QCs and serum samples.

Comparison to HPLC/UV method

Patient serum samples were separated into paired sets and stored at -20°C until analysis. One set of serum samples was submitted to analysis by HPLC/UV as previously described and reported. Thirty samples ranged 0.2-9 µg/ml were selected for reanalysis via the current method. To confirm the reliability of this method, paired results from two different methods were compared (Figure 2).

RESULTS AND DISCUSSION

Sample preparation

A single dilution step involving organic solvent extraction and protein precipitation has been proved to be a rapid, simple and efficient procedure. Quantitative extraction of VRC has been obtained previously by using acetonitrile[8, 10, 12, 15, 28], methanol[11, 18, 19] or a mixture of both solvents[16, 20] as the only sample preparation. In current study, three different agents (acetonitrile, methanol and a mixture of both) were investigated. All three different agents showed comparable efficiency. However, the methanol extracts produced best chromatographic behaviors of VRC and IS in current study. Methanol was therefore chosen and used for sample preparation.

Chromatography

Figure 1 represents two typical chromatograms obtained from two patients without VRC and IS therapy (a) and with only VRC therapy (b). These chromatograms show no interfering peaks with VRC or IS. The retention time of VRC and IS were ~5.8 and ~7.3 min, respectively. These two compounds resolved without any overlapping of their peaks or ambiguity in identification. Optimal chromatography, well-resolved and satisfactory separation of VRC and IS were produced using the optimized chromatographic conditions and an optimal column oven temperature set at 45°C. Furthermore, no interference was observed in thirty patient samples containing endogenous matrix components, metabolites, decomposition products, concomitant medication, and other xenobiotics.

Linearity, limits of detection and quantitation

The calibration curve was linear over a concentration range of 0.2-10 μg/ml with a correlation coefficient (r) >0.995. The slope was 9.0558 ± 4.16% (mean ± SD) and the intercept was 0.0225 ± 5.2% (mean ± SD). The limit of detection (LOD) was found to be ~0.07 μg/ml, which was determined at a signal-to-noise ratio of 3:1, and the LLOQ was determined to be 0.2 μg/ml. A maximal level of 9 μg/ml has been previously observed in >1000 patient samples for routine VRC tests. The validated linearity up to the upper limit of quantitation (ULOQ, 10 μg/ml) makes this method applicable across the wide range of serum concentrations for patients receiving VRC. Table 2 presents the coefficients of variation (CV) averaged 15% and 4.1% for VRC at LLOQ and ULOQ, respectively. The accuracy of LLOQ and ULOQ samples were ranged from 85 to 120% and from 95 to 108%, respectively. Based on the Guidance for Industry Bioanalytical Method Validation[32] published by the Food and

Drug Administration, the following criteria were met in developing a calibration curve: accuracy within 80-120% of the target value for the LLOQ and 15% deviation of standards other than LLOQ from nominal concentration.

Method reproducibility, precision and accuracy

Reproducibility of the method was assessed by repeated measurements using the method, including three patient samples (Table 2) and three QCs (Table 3). Reproducibility data for three patient samples containing VRC concentrations ranged 1.25-3.91 μg/ml are summarized in Tables 2. All CVs for patient samples were <6% indicating the reproducibility of the method. Experimental results of the within- and between-run for spiked samples are provided in Table 3. All CVs were <5% confirming good precision of the method. The minimal deviation of the mean from the true value indicated excellent accuracy of the method with accuracy ranging from 97% to 104.6%. Excellent recovery of VRC (100.7 ± 3.7%, mean ± SD) indicated the method was accurate, precise, and reproducible.

The absolute recoveries were determined at concentrations of 1, 4 and 8 μg/ml VRC and 10μg/ml IS. Recovery was determined by comparing the average peak height for six extracted serum samples spiked with VRC and IS with that for six aqueous solutions of VRC and IS of identical concentrations in which serum was replaced by water. The recoveries of VRC and IS were 94.9 ± 1.6% (mean ± SD) and 91.6 ± 2.3%, respectively.

Stability of VRC and micafungin

According to the package insert (Pfizer Laboratories), a slight degradation of VRC in infusion solution is observed after 24 h storage at room temperature when the drug is diluted with 4.2% sodium bicarbonate infusion, due to the alkaline nature of this diluent. However, VRC is quite stable in acidic and neutral media and its degradation could only occur by heating and/or long exposure periods. Stability experiments have also been previously conducted by other authors.[6-31] Data showed that VRC was stable in all storage and handling conditions between sample collection and analysis.

Stability of micafungin has also been previously reported[33] that this compound was stable in neutral medium and moderately unstable in acidic and alkaline media at room temperature. In the current method, VRC and micafungin were prepared in neutral medium and their stability were evaluated under the designed storage and handling conditions as described in the method section. At -20°C, no significant change in concentrations could be observed during

24 h for both compounds. Stored at room temperature for 24 h, VRC concentration remained constant while micafungin concentration declined for 8%. However, micafungin was stable in prepared samples on the autosampler (at 4°C) of the HPLC-system for 24 h. Table 4 presents the results for 7 days stability at -20°C, no significant difference in VRC and micafungin concentrations was observed during repeated freeze-thaw cycles. The experiment was carried out by six technicians using 6 patient samples that were stored in freezer (at -20°C) for 7 days. During this period, the freeze-and-thaw cycles had been repeated six times. Method precision (CVs <6%) proved also relevant for rugged analysis of VRC.

Analytical methods comparison

Up to date, only two HPLC/FL methods have been previously published[30, 31] for measuring VRC in biological samples. The first HPLC/FL method was developed by Michael *et al.*,[30] in which IS (UK-115 794) was not readily available and sample volume was 0.3 ml. Sample preparation was tedious and labor-intensive. The second HPLC/FL method was reported by Heng *et al.*,[31] in which ketoconazole was used as IS and sample volume was 0.2 ml. Their chromatographic run required 13.5 min per injection. In comparison, the current method used simpler sample preparation, a smaller sample volume (0.1 ml), and a shorter chromatographic run time (8 min).

Accuracy of the current method was further examined by measuring the concentrations of VRC in 30 de-identified patient samples. The results obtained by this method were compared well with results from the HPLC-UV method (Fig 2). The correlation between the two methods was good; the linear regression statistics indicated an r^2 value of 0.989 ($P < 0.0001$). The linear regression equation for correlation was $y = 1.018\ x - 0.043$ with a standard error value of 0.16; where y, the current method and x, the HPLC/UV method.

Table 1. Instrumental and chromatographic conditions.

Instrumental parameters	Conditions
Instrument used:	Hitachi Chromaster™ HPLC system
Analytical column:	Zorbax Eclipse Plus C18, 5-µm, 250 x 4.6 mm
	Ammonium acetate buffer:acetonitrile:methanol (40/40/20 v/v/v)
Elution mode	Isocratic
Temperature of the column	45° C
Temperature of the autosampler	4° C
Flow rate	1.0 ml/min
Volume of injection	20 µl
FL Detection	254 nm (Ex) and 372 nm (Em)
Run time	8 min

Table 2. Method reproducibility.

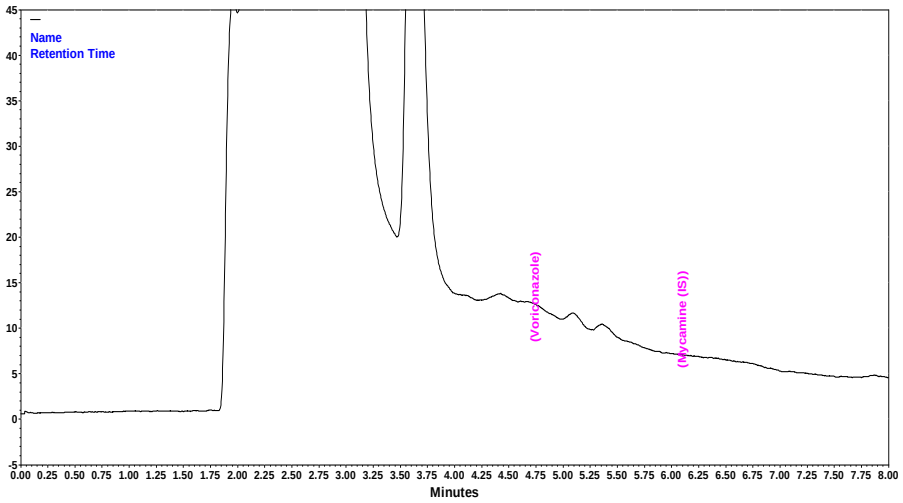
	Mean Concentration (μg/ml)	Concentration Range (μg/ml)	Standard Deviation (μg/ml)	CV (%)
LLOQ (*n* = 10)	0.20	0.17 – 0.24	0.03	15.0
ULOQ (*n* = 10)	10.22	9.51 – 10.79	0.42	4.1
Patient #1 (*n* = 6)	1.35	1.25 – 1.44	0.08	5.9
Patient #2 (*n* = 6)	2.88	2.75 – 3.02	0.14	4.9
Patient #3 (*n* = 6)	3.69	3.45 – 3.91	0.17	4.6

Table 3. Method precision and accuracy.

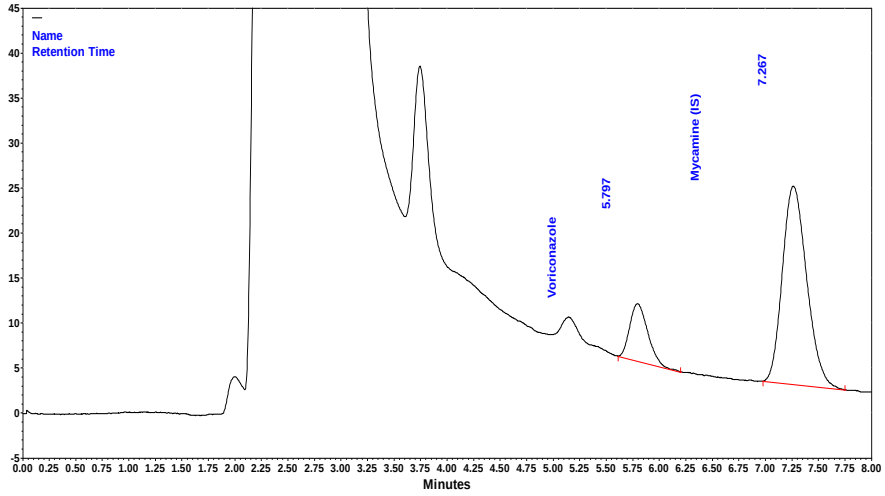
	True Concentration (μg/ml)	Found concentration (μg/ml)	Recovery (%)	CV (%)
Spiked sample:	1	0.97	97.0	4.7
Within-run	4	4.18	104.6	3.4
(*n* = 6)	8	7.94	99.3	2.9
Between-run	1	0.99	99.0	4.8
(*n* = 12)	4	4.08	101.9	3.9
	8	8.21	102.6	3.5

Table 4. Method precision performed by six technicians over a period of seven days.

Patient Sample #	*n*	Mean Concentration (μg/ml)	Concentration Range (μg/ml)	Standard Deviation (μg/ml)	CV (%)
1	6	6.28	5.96 – 6.51	0.20	3.1
2	6	3.36	3.12 – 3.58	0.15	4.6
3	6	1.29	1.17 – 1.36	0.07	5.3
4	6	4.34	4.14 – 4.69	0.21	4.8
5	6	5.39	5.13 – 5.77	0.22	4.1
6	6	2.75	2.65 – 2.93	0.11	4.0



(a)



(b)

Figure 1. Chromatograms of two serum samples obtained from two representative patients without VRC and IS therapy (a) and with VRC therapy (b). The VRC concentration in sample was 3.1 μg/ml. The retention times of VRC and IS were ~5.8 and ~7.3 min, respectively.

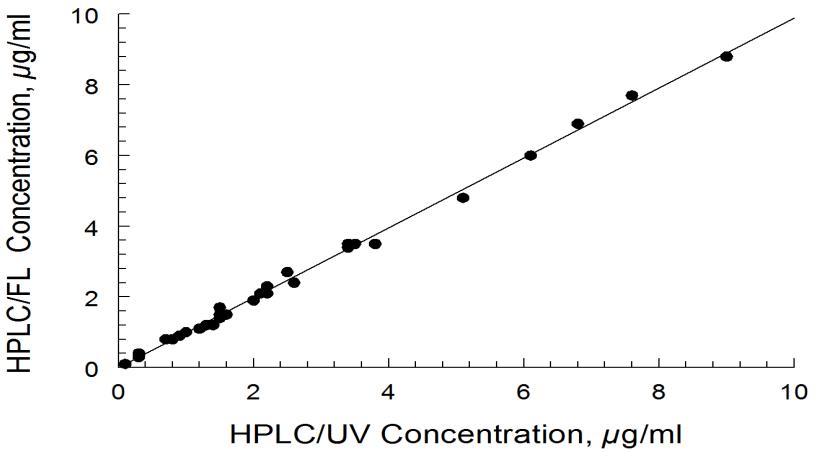


Figure 2. Method comparison between HPLC/FL and HPLC/UV.

CONCLUSION

A simple, accurate, and reproducible method for the measurement of VRC in human serum has been developed. The current method uses an efficient procedure consisting of a single dilution step, solvent extraction, protein precipitation and ultracentrifugation which avoids the use of more complex liquid-liquid extraction or solid-phase extraction procedure and substantially decreases set-up time. The method meets the requirements of high sample throughput in clinical practice. The method has potential cost savings lying in the use of single dilution step process and a quick chromatographic run to be completed within 8 min.

Furthermore, the method employs a commercially available micafungin to serve as IS for VRC. It should be excluded that both VRC and micafungin are administered at the same time, so no errors in quantitation should be expected. This method can be readily adopted by laboratories to support monitoring of VRC concentrations in serum sample, optimize efficacy of VRC therapy and minimize adverse effects.

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