Development and Validation of Voriconazole Concentration by LC-MS-MS: Applied in Clinical Implementation

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Background: Voriconazole (VRZ) is a triazole antifungal used for treatment of invasive fungal infection, which is a life-threatening condition. Therapeutic drug monitoring is recommended for identifying the optimal dose in patients who have hepatic/renal impairment or reduced function of the CYP2C19 metabolizing enzyme. **Methods:** One hundred microliters of sample plasma was extracted by protein precipitated with 200 μ l of acetonitrile containing fluconazole as internal standard (IS). After vortexing and centrifugation, supernatant was dried and reconstituted with 100 μ l of mobile phase (ACN: 0.1% formic acid in 10 mM Ammonium acetate) (50:50 v/v) before injected. The column was C18,

2.7 μ m, 3.0 $\times$ 50 mm at flow rate of 0.5 ml/min with retention time of 0.5 and 0.75 min for VRZ and IS, respectively. The tandem mass spectrometer was set in multiple reactions monitoring (MRM) mode with the following transition; VRZ m/z 350.10 $\rightarrow$ 281.10 and 307.20 $\rightarrow$ 220.20 (IS). **Results:** The accuracy and precision inter- and intra-day were less than 9%, over the range 0.05–10 μ g/ml. The linearity was consistent ($r^2 = 0.9987$) and recovery was more than 85.0% for both analyses. **Conclusion:** This method is applicable for routine monitoring of patients' VRZ plasma level with fast and accurate runtime to assess CYP2C19 genotype. J. Clin. Lab. Anal. 31:e22011, 2017. © 2016 Wiley Periodicals, Inc.

Key words: antifungal; applications; genetics; LC-MS/MS; quantitative

INTRODUCTION

Voriconazole (VRZ) is a triazole antifungal drug used for treatment of invasive Aspergillosis, a life-threatening condition in immunocompromised patients as well as Candidiasis. The pharmacokinetics of VRZ is highly variable due to many factors including gender, age, genetic variation, liver function, fatty food, and drugs interaction. Previous studies showed that not only is VRZ AUC and $C_{\max}$ were different between children aged 2–12 years and adults, due to linear and non-linear kinetics, but the VRZ plasma clearance was almost three times greater in children than in adults, which suggests saturation of VRZ clearance in adults (1–3). These findings were supported by the study of population pharmacokinetics which showed that the VRZ intravenous dose of 7 mg/kg in young children gave similar results to a 3–4 mg/kg dose given to adults. Oral

bioavailability of VRZ was also lower in the young (44.6%) compared to adults (~96%) (4). VRZ is mainly metabolized by hepatic CYP2C19. Polymorphism of CYP2C19 and the saturation of metabolism may elevate the plasma concentration and cause hepatotoxicity in a dose-dependent manner (5). CYP2C19 polymorphisms contribute greatly to VRZ metabolism either increasing VRZ plasma level in poor metabolizers (CYP2C19*2,*3) or decreasing VRZ plasma level in ultra-rapid metabolizers (CYP2C19*17) (6). To

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optimize the outcome and avoid adverse drug reactions with VRZ, an ideal 1-week-trough concentration of VRZ was suggested to be 1.0–5.5 µg/ml for systemic fungal infection (7, 8).

Many methods for determining the human plasma level of VRZ have been introduced with HPLC-UV (9, 10), HPLC-FLR (11), immunoassay (12), and LC-MS/MS (6, 13–20). To our knowledge, the fastest run time recorded is 1.5 min (20), whereas the others report 3–5 min. For routine daily monitoring, fast run time is desirable. The purpose of this study is to validate routine analysis of VRZ in human plasma with fast run time and high sensitivity to monitor drug level in poor metabolizer or extensive metabolizer CYP patients in a hospital setting.

MATERIALS AND METHODS

Chemical and Reagents

Voriconazole and Fluconazole (as internal standard; IS) were purchased from Sigma (Sigma-Aldrich Co. LLC, Singapore). Acetonitrile (ACN) and methanol were HPLC grade (RCI Labscan limited, Bangkok, Thailand) and other reagents, including ammonium acetate and formic acid, were analytical grade (Carlo Erba reagent SAS, Val de Reuil, France). Blank human plasma used in this study was obtained from the blood bank at Ramathibodi Hospital (Mahidol University, Bangkok, Thailand). The standard stock solution of VRC (100 µg/ml) in methanol was prepared then diluted into 50 µg/ml working solution and stored at –20°C. The calibration standard final concentrations were prepared at 0.05, 0.1, 0.4, 0.8, 1.2, 2.5, 5, and 10 µg/ml in human plasma. LLOQ and QC samples were prepared at 0.05, 0.15, 2.0, and 6.4 µg/ml in human plasma. The IS stock solution was also prepared at 100 µg/ml before dilution to 50 µg/ml for the working solution. The final concentration IS solution was prepared at 0.5 µg/ml in human plasma. All working solutions were aliquoted and stored at –20°C before analysis and freshly prepared by spiking blank human plasma with the appropriate working solutions.

Chromatographic Apparatus and Condition

The analysis was performed by Agilent 1260 infinity LC (Agilent Technologies, Foster City, CA) with a Poroshell 120 EC-C18, 2.7 µm, 3.0 × 50 mm column (Agilent Technologies) coupled with an atmospheric pressure ionization source (API-3200) triple quadrupole mass spectrometer (Applied Biosystem/MDS SCIEX, Foster City, CA). The mobile phase consisted of acetonitrile and 0.1% formic acid in 10 mM

Ammonium acetate (50:50 v/v) and the flow rate was set at 0.5 ml/min. Total run time was 3.5 min. The retention time (Rt) of IS and VRZ was 0.5 and 0.75 min, respectively, with no carry-over from the previous injection. The confirmation multiple reaction-monitoring (MRM) mode was set at 350.10 → 281.10 and 127.00 with the expected ion ratio with range from 1.89 to 2.83. Analysis was performed in quantitative MRM modes by monitoring the ion transitions from m/z 350.10 → 281.10 (VRZ) and m/z 307.20 → 220.20 (IS). The MS/MS conditions were as follows: spray needle voltage, 5.5 kV; source temperature, 500°C; auxiliary gas flow, 71 l/min; equalizer gas pressure, 80 psi. The instrument was controlled and the data integration was performed with Analyst software (v 1.5.2; SCIEX).

Sample Preparation

One hundred microliters of plasma was used for analysis with 10 µl of IS (0.5 µg/ml) added before being precipitated by addition of 200 µl of 100% cold ACN. The sample was re-suspended then centrifugation was performed at 15,000 × g for 5 min. Three hundred microliters of supernatant was collected into a new tube. Samples were evaporated by speed vacuum at 50°C until dry and reconstituted with 100 µl mobile phase, then vortex mixed. Centrifugation was repeated at 15,000 × g for 5 min and 100 µl of supernatant transferred into glass microvials before the sample was injected 1.0 µl into LC-MS/MS system.

Method Validation

This method was fully validated for specificity, linearity, precision, accuracy, recovery, and stability according to the United States Food and Drug Administration bioanalytical method validation guidance 2011 (21).

Specificity and selectivity

Specificity was assessed by using pooled blank human plasma samples from six different sources. Samples were prepared at concentration of 150 ng/ml in six replicates and handled according to the “Sample preparation” to confirm the lack of interference of endogenous compound with analyte or IS.

Linearity of calibration curve

To determine the linearity of the calibration curve, eight concentrations of calibration standard (range 0.05–10.0 µg/ml) were prepared in six replicates sets. The linearity was calculated by weighted (1/x)

least-squares linear regression (quadratic model) of peak area response ratio against concentrations.

Limit of quantitation

The lower limit of quantification (LLOQ) was defined as the lowest concentration of VRZ yielding an assay precision with $CV \leq 20\%$ and accuracy within the range 80–120% of the actual value. VRZ response at the LLOQ should be at least five times the response compared to blank response ($S/N \leq 5$).

Precision and accuracy

Three sets of LLOQ and QC samples at 0.05, 0.15, 2.0, and 64 $\mu\text{g/ml}$ (LLOQ, QCL, QCM, and QCH respectively) were prepared in six replicates according to the “Sample preparation” protocol. The precision and accuracy were assessed within acceptable range of 20% for LLOQ and 15% for the others. For intra-day precision and accuracy, each concentration of QC sample was assayed in six replicates, although the inter-day accuracy and precision were determined by the evaluated of six replicates of each QC sets for three consecutive days. The accuracy of these QC samples should be within the range of $100 \pm 15\%$ of the actual value with $CV \leq 15\%$.

Recovery of extraction

Three sets of QC samples at 0.15, 2.0 and 64 $\mu\text{g/ml}$ (QCL, QCM, and QCH, respectively) were prepared in six replicates each by spiking VRZ and IS into human plasma.

The extraction was performed as described in “Sample preparation”. The percentage recovery of VRZ and IS was assessed by determining the mean peak area ratio of VRZ and IS from the extracted QC samples with the mobile phase spike containing the same concentration of analyte.

Stability

To measure consistency of the analysis, VRZ stability was assessed for the effect of freezing and thawing, holding for extended periods in the autosampler and long-term storage. The assays were performed at low and high QC samples (0.15 and 64 $\mu\text{g/ml}$) with six replicates for each storage condition. In brief, the freeze-thaw stability was determined after three complete freeze-thaw cycles from either -20°C or -80°C to room temperature. The autosampler stability was measured with the extracted sample stored in the injector at room temperature for up to 18 hr. Long-term

stability was measured after the spiked plasma samples were stored at each condition (4, -20 , and -80°C) for 7, 30, 120, and 365 days. The acceptable range for stability was considered less than 15% for precision and within 85–115% for accuracy.

Application for Routine VRZ Determination in Human Plasma

This project was approved by the Committee on Human Rights Related to Research Involving Human Subjects, Faculty of Medicine Ramathibodi Hospital, Mahidol University (Protocol number; ID 04-56-01). Sixty-seven human plasma samples were obtained from patients with invasive fungal infections and who received a daily VRZ regimen of 400–500 mg. Blood collected at trough level CYP2C19 phenotype was categorized as poor metabolizer (PM; $*2/*2$, $*2/*3$, and $*3/*3$) ($n = 7$), intermediate metabolizer (IM; $*1/*2$, $*1/*3$) ($n = 29$), and extensive metabolizer (EM; $*1/*1$) ($n = 31$) phenotypes. No rapid metabolizers (UM; $*17$) were found in this study. EDTA blood was centrifuged to recover plasma immediately after collection, aliquoted and stored at -20°C for analysis in the same day. The buffy coat was used for CYP2C19 genotyping. DNA extraction and genotyping was performed as mentioned in our previous study using Magna Pure Compact (Roche Diagnostics Asia Pacific Pte. Ltd, Singapore) and real-time PCR technique (ViiATM 7; Thermo Fisher Scientific, Singapore) (22).

RESULTS

Specificity

The fragmentation spectrum of VRZ was shown to transition from protonated VRZ to degraded ion product in which triazole ring is lost (Fig. 1). The analysis confirmed that there were no endogenous compounds interfering in the analyte or IS. After being spiked with 150 ng/ml of VRZ and 0.15 $\mu\text{g/ml}$ with IS 0.05 $\mu\text{g/ml}$ (Fig. 2), the retention time (Rt) of VRZ and IS was 0.5 and 0.75 min, respectively. The total run time was 3.5 min.

Linearity of Calibration Curve

The peak area response ratio of VRZ and IS against concentrations showed good correlation in the range 0.05–10.0 $\mu\text{g/ml}$ in human plasma. The linearity of quadratic model equations was $y = -8.61 \times 10^{-8}x^2 + 0.00349x + 0.124$, with $r^2 = 0.9987$ as shown in Table 1.

Fig. 1. The spectrum shows the protonated VRZ; Parent ions; and the fragmentation ions; daughter ions; m/z transition from 350.2 > 281.3, resulting from loss of the triazole ring.

Precision and Accuracy

The limit of quantitation (LLOQ) of VRZ was 0.05 µg/ml and intra-day and inter-day accuracy of the LLOQ was $95.30 \pm 3.41\%$ and $87.80 \pm 6.58\%$, whereas the intra-day and inter-day precision (%CV) were 7.2% and 7.9%, respectively. Six replicates of three sets of QC samples at low (0.15 µg/ml), medium (2.0 µg/ml) and high concentration of VRZ (64 µg/ml) were determined during a 3-day validation period. The intra-day and inter-day accuracy were within $100 \pm 15\%$, whereas the intra-day and inter-day precisions (%CV) were less than 15% as shown in Table 2.

Recovery of Extraction

Voriconazole plasma samples were extracted and analyzed as described. Percentage recovery of VRZ and IS was evaluated by comparing the peak areas obtained from six replicates of extracted samples at each concentration and six replicates of spiked standard after blank extraction at each concentration. The mean percent recovery of VRZ was $85.95 \pm 9.03\%$,

$87.30 \pm 6.55\%$, and $84.63 \pm 8.19\%$ at QCL, QCM and QCH, respectively. The percent recovery of IS was $85.38 \pm 7.52\%$, as shown in Table 3.

Stability of VRZ

The error in precision of the VRZ concentration was less than 15% and the accuracy was also within 85–115% in freeze-thaw cycles (-20 and -80°C) at room temperature, autosampler stability (25°C), and long-term stability (4, -20 and -80°C) assays. The stability of freeze-thaw accuracy at -20 and -80°C for QCL was 92.89% and 105.67% with %CV 7.2% and 2.5%, whereas QCH was 98.80% and 104.92% with %CV 5.4% and 6.5%, respectively. For the autosampler assay, the measurement was stable up to 18 hr with %accuracy for QCL and QCH of 90.33% and 98.22%, whereas %CV was 2.6% and 6.9%, respectively. When assessing the long-term stability of samples stored at 4, -20 , and -80°C for both QCL and QCH, %accuracy was more than 92.0% and %CV less than 7.4% for each condition. In summary, VRZ was highly stable in human plasma and can be kept at 4°C for up to a year.

VRZ Plasma Level Monitoring

Sixty-seven human plasma samples were evaluated for plasma VRZ level with the described method. Voriconazole plasma was used to determine all phenotype groups; PM group ($n = 7$), IM group ($n = 29$) and EM ($n = 31$); without going below LLOQ or over the ULOQ. Moreover, even in the same phenotype the plasma VRZ concentrations were highly variable, as shown in Figure 3.

DISCUSSION

LC-MS/MS method was developed and validated to measure the concentration of VRZ in human plasma. This method demonstrated high accuracy and high precision and was successfully applied to routine drug monitoring for patients. Variations in extraction processes were introduced, including direct injection (13), solid-phase extraction (18), in-line extraction (19), or protein precipitate with organic solvent (10, 14, 20). Direct injection is the fastest procedure possible, but the purity and sensitivity is quite low (98.0 ng/ml), which lowers the column lifespan. SPE and in-line extraction give high reproducibility at high financial cost. Protein precipitation is simple, with no need for highly complicated techniques; however this procedure takes time to evaporate the organic solvent which may interfere with the analysis. It has been suggested that

Fig. 2. Representative chromatograms (a) 0.15 µg/ml of VRZ and (b) 0.5 µg/ml of IS spiked in blank human plasma ($n = 6$).

TABLE 1. The Linearity of Calibration Curves of VRZ

Nominal concentration (ng/ml)	Day 1		Day 2		Day 3		Mean	SD	%CV
	Actual concentration (ng/ml)	Accuracy (%)	Actual concentration (ng/ml)	Accuracy (%)	Actual concentration (ng/ml)	Accuracy (%)			
50	41.29	82.6	45.04	90.1	51.85	103.7	46.1	5.35	11.62
100	106.64	106.6	98.49	98.5	94.71	94.7	99.9	6.10	6.10
400	437.79	109.4	461.30	115.3	424.00	106.0	441.0	18.86	4.28
800	798.77	99.8	714.26	89.3	820.44	102.6	777.8	56.10	7.21
1200	1329.98	110.8	1316.7	109.7	1064.01	88.7	1236.9	149.87	12.12
2500	2188.67	87.5	2471.99	98.9	2616.16	104.6	2425.6	217.49	8.97
5000	5180.70	103.6	4881.02	97.6	5005.49	100.1	5022.4	150.55	3.00
10000	9970.84	99.7	10062.50	100.6	9958.08	99.6	9997.1	56.96	0.57
r^2	0.9979		0.9990		0.9991		0.9987		
Equation	$y = -9.14e^{-008}x^2 + 0.00305x + 0.127$		$y = -2.89e^{-008}x^2 + 0.00342x + 0.046$		$y = -1.38e^{-007}x^2 + 0.004x + 0.198$				

the organic phase can be introduced into the LC-MS/MS without evaporation or reconstitution and yield a high sensitivity (39 ng/ml) (14). This study presents a highly sensitive method with LLOQ at 50 ng/ml, close to the previously reported values (14, 20) and may be

even faster than the method suggested by Xiong and colleagues (20), in which VRZ retention time was 0.9 min with flow rate 1.0 ml/min. In this study, VRZ retention time was only 0.75 min with flow rate of 0.5 ml/min. The recovery of this assays for both VRZ

TABLE 2. Precision and Accuracy of the Method for Determination of VRZ

Analyte	Concentration (ng/ml)	Intra-day (<i>n</i> = 6) (mean ± SD)	%Accuracy	%CV	Inter-day (<i>n</i> = 18) (mean ± SD)	%Accuracy	%CV
VRZ	50	47.65 ± 3.41	95.30	7.2	43.90 ± 3.47	87.80	7.9
	150	133.17 ± 5.49	88.78	5.5	132.67 ± 5.60	88.44	4.2
	2,000	1,873.30 ± 84.54	97.72	5.4	1,893.30 ± 152.41	94.67	8.0
	6,400	6,498.33 ± 562.54	101.54	8.7	6468.89 ± 552.89	101.08	8.5

TABLE 3. The Percent Recovery of VRZ and IS (*n* = 6)

Analyte	Concentration (ng/ml)	Recovery (% Mean ± SD)	%CV
VRZ	150	85.95 ± 9.03	10.5
	2,000	87.30 ± 6.55	8.1
	6,400	84.63 ± 8.19	9.6
IS	500	85.38 ± 7.52	8.8

and IS was higher than 84% and 85%, respectively, due to the similar structure of VRZ and IS, fluconazole. No endogenous compounds with signal-to-noise ratio (*S/N*) of more than 5 were seen at the retention times of VRZ and IS. The intra-day and between-day accuracy and precision of the assay were within the acceptance limits according to the US FDA Guidance for Industry on Bioanalytical Method Validation (21). Stability of VRZ plasma samples was very stable: in a cool environment and following storage at 4°C for up to a year, the recovery was over 94%. In daily drug monitoring for VRZ in patients, 19 samples were analyzed and all were found to be within the calibration concentrations range regardless of CYP2C19 phenotypes. Even though the same regimen was used, large variability was still observable, which may be due to other factors like age. It has been reported that children have higher clearance than adults (1–3).

Fig. 3. The box plot of VRZ plasma concentration per dose for each CYP2C19 phenotype (*n* = 67).

Metabolism via a minor pathway by CYP3A4 or CYP2C9 may also affect enzyme activity (23, 24).

There are three limitations in our study which may influence the interpretation of analysis. Firstly, use of fluconazole as internal standard is contentious because VRZ may be used after fluconazole in a clinical setting, especially in hematology patients who are treated with fluconazole for prophylaxis of invasive *Candida* infection before progressing into invasive Aspergillosis, for which VRZ is the drug of choice (25, 26). Thus, the background level of fluconazole in these cases is likely to be higher and add to the IS concentration used in analysis. Due to the long half-life of fluconazole (~30 hr), samples collected from patients with the past medication of fluconazole should be compared against a different IS. Many IS were suggested for VRZ determination, e.g., dimethyl diquinoxaline (14), ketoconazole (17), deuterated VRZ (VORI-d₃) (6, 16), and fentanyl (20). Fluconazole was selected because of the related structure to VRZ, good stability, and also eluted fast as closely as VRZ (*R*_t 0.5 and 0.75 min, respectively). Secondly, only small sample size of PM (7/67; 10.44%) was collected into this study. Thirdly, VRZ levels were not adjusted to account for co-medication or underlying disease which might alter VRZ plasma level.

In conclusion, an assay method for quantitation of VRZ in human plasma was developed which is simple, accurate, and reliable and can be applied for routine work in a hospital setting to evaluate the patient VRZ plasma level in cases of both systemic fungal infection or in bone marrow transplant.

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