Rapid High Performance Liquid Chromatographic Assay for Determination of Voriconazole Concentration in Human Plasma

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Background: *Blood voriconazole level is affected by several factors and concentration of voriconazole in patient's plasma is crucial for treatment response.*
Objective: *To develop and validate a method of high performance liquid chromatography (HPLC) for determination of plasma voriconazole level.*
Material and Method: *One-hundred and twenty microlitres of plasma sample was extracted with 6% perchloric acid. The extracted plasma was separated on a reversed-phase C18 column with isocratic phase which consists of acetonitrile and Milli-Q-water (68:32, vol/vol). The limits of quantitation, accuracy, precision, stability and recovery were validated. Plasma samples from 10 patients receiving voriconazole during treatment of invasive fungal diseases were collected for voriconazole assays.*
Results: *The lower limit of quantitation was 0.2 μg/mL. Linearity was demonstrated from 0.2 μg/mL to 20 μg/mL with 0.98 correlation coefficient value. Intra-day and inter-day variability of the voriconazole plasma concentration ranged from 0.78% to 3.01% and 1.52% to 4%, respectively. Accuracy ranges from 99.3%-101%. The extraction recovery ranges from 99.2%-101%. Plasma voriconazole level in patients showed significantly variable from patient to patient and the levels were higher during the first 2 weeks of voriconazole treatment.*
Conclusion: *The present method is simple, accurate, and precise for measurement of voriconazole plasma concentrations and can be applied for routine laboratory. Significant variability of voriconazole level in patient plasma highlights the importance of therapeutic antifungal drug monitoring in patients receiving voriconazole.*

Keywords: *High performance liquid chromatography, HPLC, Voriconazole, Validation, Therapeutic drug monitoring, Antifungal agent*

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Voriconazole is a triazole antifungal agent which inhibits ergosterol synthesis, an important component in fungal cell membrane[1]. Voriconazole has been approved for treatment of invasive fungal diseases, especially aspergillosis[2]. This antifungal agent is metabolized via the human hepatic cytochrome P450 (CYP) enzyme, in which CYP2C19 is the most commonly involved isoenzyme, followed by CYP2C9 and CYP3A4. Inhibitors or inducers of these isoenzymes may increase or decrease voriconazole plasma concentration, respectively. Previous data indicates that CYP2C19 exhibits genetic polymorphism and the prevalence of a poor metabolizer is higher among in Asian population compared with Caucasians[3,4]. Several studies have demonstrated a correlation between voriconazole level and clinical efficacy and toxicity[5-7]. In addition to CYP2C19 genetic polymorphisms, many factors affect voriconazole plasma level including liver dysfunction and drug-drug interactions[7]. Therefore, monitoring of plasma voriconazole levels is important for effective antifungal treatment with voriconazole.

Determination of plasma voriconazole level is not available in Thailand, although several methods

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using high performance liquid chromatography (HPLC) for this purpose have been validated[8-11]. The present study describes an easy, fast and sensitive HPLC method with ultraviolet (UV) detection for quantifying the voriconazole plasma level. The present study was approved by Siriraj Institutional Review Board (SIRB).

Material and Method

Patients

The present study is part of the larger study of voriconazole level in Thai patients in correlation with clinical response to voriconazole treatment. Patients who received voriconazole therapy for invasive fungal diseases were enrolled and 5-mL blood samples in EDTA were collected at day 0 (before voriconazole administration), 3, 7, 14, and 28. Each plasma sample was sent for voriconazole assay within 2 hours. All patients received 400 mg voriconazole twice a day for one day followed by 200 mg voriconazole twice a day thereafter. There was no dose adjustment for voriconazole during antifungal treatment.

HPLC Methods

1. Chemicals

Voriconazole pure substance (USP Reference Standards) was purchased from US Pharmacopeia (Rockville, MD, US). HPLC grade super gradient acetonitrile, methanol and perchloric acid were purchased from LABSCAN (Bangkok, Thailand).

2. Instruments and HPLC conditions

The HPLC system consisted of a Waters Alliance liquid chromatography system (Waters Corporation, Milford, MA, USA), including a Model 2695 Separate Module and a Model 2487 Dual Wavelength UV detector. The analytical column was a Symmetry C18 analytical column, 5 µm (250 x 4.6 mm I.D.) protected with sentry guard column C18 (5 µm, 20 x 3.9 mm ID) (Waters Corporation, Milford, MA, USA). Separation was performed at 37°C using a column heater (temperature control system, Waters Corporation, Milford, MA, USA). The mobile phase was consisted of acetonitrile and Milli-Q-water (68:32, vol/vol). The mobile phase was filtered through a 0.2-µm membrane prior to use. The UV detection wavelength was 255 nm. The flow-rate was 0.8 mL/min. The analysis time was set at 10 min per sample and the injection volume was 30 µL.

3. Standard and quality control preparation

The stock solution of voriconazole was prepared at a concentration of 1 mg/mL in methanol. Five milligrams of voriconazole pure substance was dissolved in 5 mL volumetric flask to give a 1 mg/mL drug concentration.

For preparation of standard curve, stock solution was serially 10-fold diluted in Milli-Q-water to a final concentration of 10 µg/mL. The standard curve covering the concentration ranges between 0.2 and 20 µg/mL (0.2, 0.5, 1, 2, 3, 5,10, 15 and 20 µg/mL) was prepared by adding appropriate volumes of these diluted solutions to 120 µL of drug-free human plasma. The quality control (QC) samples at the concentration of 0.5, 2, 5 and 17 µg/mL were prepared in the same way with standard curve preparation. Stock and working solutions were liquored and stored at -80°C until used.

4. Sample extraction

One hundred and twenty µL of each plasma sample (standard and QC standard) including a sample of voriconazole-free plasma (blank) was added to 1.5 ml microcentifuge tubes. A 60 µL solution of 6% perchloric acid was added to all tubes and mixed for 5 min with votex. The samples were then centrifuged at 13,000 rpm for 5 min. After centrifugation, the supernatants were filtrated through a 0.2-µm membrane and 30 µL of the supernatant was injected into HPLC machine.

5. Extraction recovery

Extraction recoveries were measured in triplicate at voriconazole concentration of 0.5, 2, 5 and 17 µg/mL by comparing the amount of drug of interest from extracted standard sample with non-extracted standard sample at these same concentrations.

6. Accuracy and precision

Accuracy, intra-day and inter-day precision for each sample were determined by analyzing 5 replicates of QC samples at 4 different concentrations (0.5, 2, 5 and 17 µg/mL) for 5 separate days.

7. Selectivity

Selectivity was determined by comparing the chromatogram of spiked plasma with blank plasma to ensure that no interfering peaks were found in the chromatogram of the drug of interest.

8. Stability

Stability testing was determined by analyzing QC samples under various conditions. The QC samples at 0.5 and 5 µg/mL of voriconazole were separated into

2 sets. The first set was stored at room temperature for 24 hrs. The second set was subjected to 3 freeze-thaw cycles. Each QC sample was analyzed by comparing with the same concentration of freshly thawed QC samples.

9. Standard curve and statistical analysis

The EmpowerPro software (Waters Corporation, Milford, MA, USA) was used to generate the standard curve by plotting areas under the curve of the extracted spike plasma versus various concentrations of voriconazole. The correlation coefficient and coefficient variation in percent (CV%) were evaluated in the present study. Finally, the calibration graph was used for calculation of voriconazole concentrations in the samples from their areas under curve.

Results

The chromatogram of drug-free plasma and spiked plasma are shown in Fig. 1. The assay run time was 10 minutes. The mean retention time was 4.502 minutes. At a detection wavelength of 255 nm, assays performed on drug-free human plasma have no presence of any interfering peak at the retention times of the drug of interest (Fig. 1A).

Standard curve using human plasma ranging from 0.2 to 20 µg/mL was analyzed. A least-squares linear regression was used to calculate the equation relating the peak area and the concentration of voriconazole. The standard curve was linear within the range between 0.2 and 20 µg/mL. The constant coefficient was 0.33 and the correlation coefficient was 0.97. The limit of detection (LOD) of this assay is as same as the lower limit of quantitation (LOQ) (0.2 µg/mL).

Intra-day and inter-day precision data for plasma analysis of the drugs of interest were evaluated for 4 concentrations. Precision, accuracy and extraction recovery of our HPLC method are shown in Table 1. All variability is expressed as CV%. The CV% calculated for the intra-day and inter-day variability of the HPLC were less than 3.1% and 4%, respectively. The accuracies ranged from 99.3-101% and the drug recoveries from plasma ranged from 99.2-101%.

The stability of drug under various conditions at 2 concentrations of QC standard is shown in Table 2. All assayed samples were stable for 24 hours at room temperature. No degradation was observed at -80°C after three freeze-thaw cycles. At least 98% of the initial concentrations were recovered. Therefore, all of these

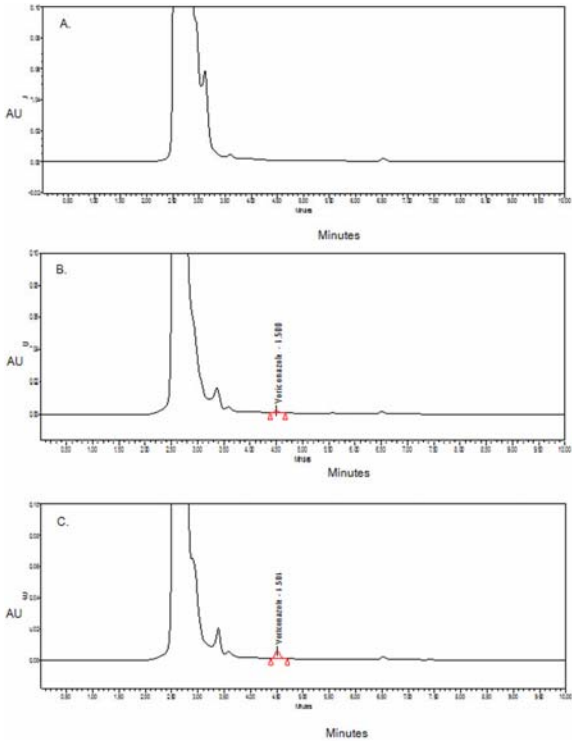

Fig. 1 Representative chromatograms of (A) blank plasma; (B) 0.5 µg/mL of voriconazole; (C) 2 µg/mL of voriconazole. (AU; Absorption Units)

analyses have shown that plasma voriconazole was considered to be stable under the tested conditions.

A preliminary evaluation of this HPLC assay was performed by measuring the voriconazole trough level in plasma samples collected from 10 patients receiving voriconazole for treatment of invasive fungal diseases. The mean plasma concentrations of voriconazole over time after voriconazole administration (day 3, 7, 14 and 28) are shown in Fig. 2. All patients received a fixed dose of voriconazole and no dose adjustment was done. There was a wide variation of plasma voriconazole concentration in patients with invasive fungal diseases who were receiving voriconazole treatment. However, the plasma voriconazole level was higher during the first two weeks of treatment in most patients.

Discussion

Voriconazole has been approved and used in Thailand since 2005 and recent data supported the use of therapeutic drug monitoring for voriconazole dose adjustment to ensure a better clinical outcome and to avoid the potential toxicities[12]. However, voriconazole

Table 1. Intra-day and Inter-day precision and accuracy of HPLC analysis at 4 different spikes of voriconazole concentration (CV; coefficient variation)

Expected (μg/mL)	Intra-day (n = 5)		Inter-day (n = 25)		Accuracy (%)	Recovery (%)
	Measured (μg/mL)	CV (%)	Measured (μg/mL)	CV (%)		
0.5	0.490 ± 0.015	3.01	0.488 ± 0.019	3.94	99.6	98.4
1	0.992 ± 0.026	2.61	0.998 ± 0.024	2.41	99.3	99.2
2	2.000 ± 0.029	1.48	1.974 ± 0.038	1.95	99.8	99.3
5	5.044 ± 0.071	1.41	5.050 ± 0.113	2.24	100.9	100.9
17	17.040 ± 0.132	0.78	17.070 ± 0.259	1.52	99.9	100.2

Table 2. Stability for the voriconazole in drug-free plasma (CV; coefficient variation)

Concentration (μg/mL)	Conditions	Recovery (%)	CV (%)
0.5	24 hours at room temperature	98.0	4.08
	3 freeze-thaw cycles	98.3	3.27
5.0	24 hours at room temperature	102	2.77
	3 freeze-thaw cycles	99.0	4.59

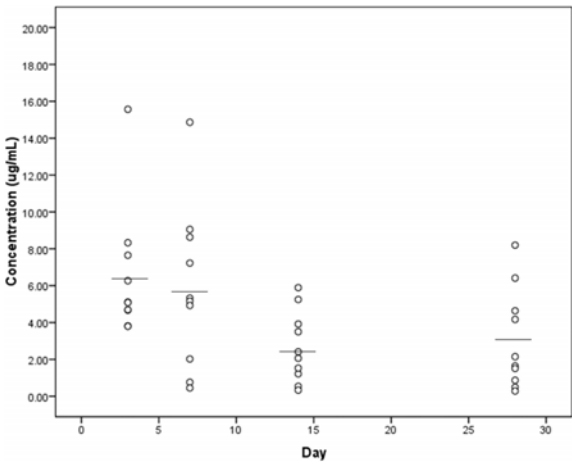

Fig. 2 The plasma concentrations of voriconazole in 10 patients following voriconazole administration (day 3, 7, 14 and 28). Horizontal lines indicate mean values

assay in plasma is not available in Thailand. The suggested dosing of voriconazole was made based on studies in Caucasians and was applied unchanged for Asian populations. Voriconazole is metabolized mainly via CYP2C19 which determined by genetic polymorphisms[13]. The difference in CYP2C19 polymorphism between Asians and Caucasians is well known in that Asians tend to exhibit a relatively greater proportion of poor metabolizers[14]. Therefore, determining the level of voriconazole in plasma of patients receiving voriconazole will be helpful in dose adjustment among Asian population. The present study has shown a method for voriconazole level assay in Thai patients using rapid HPLC. The chromatographic run time of 10 min allows the analysis to be used for a large number of samples. The liquid-liquid extraction used as sample preparation method is simple and rapid, and can be used with a small volume of plasma sample (120 uL). Therefore, this method allows the analysis to be able to use even in pediatric plasma samples in which blood volume may be limited.

As part of our larger study, the authors determined voriconazole level in plasma of 10 patients who received fixed dose voriconazole treatment and the results showed that voriconazole concentrations were quite variable in different patients. These findings suggested that therapeutic drug monitoring is therefore essential in each patient receiving voriconazole treatment, as the level of this antifungal agent can be affected by several factors. Although most of the patients in the present study are CYP2C19 intermediate or extensive metabolizers (data not shown), the levels of voriconazole during the first 2 weeks of treatment were significantly higher than those at and after 2 weeks of treatment (Fig. 2) despite no dose adjustment. This phenomenon may be explained by the relatively lower

activity of CYP2C19 enzyme during the critically ill condition of these patients. Once the patient had improved, the more active CYP2C19 activity metabolized more substrate and this may have contributed to a lower level of plasma voriconazole. However, other drug interactions may also affect voriconazole levels. This is a preliminary report of our voriconazole level study in Thai patients. Our larger study to determine association between voriconazole level and treatment response is currently ongoing.

Conclusion

A simple, rapid and sensitive HPLC assay was developed for determination of plasma voriconazole level and it has been completely validated for precision, accuracy, stability, LOQ, recovery and linearity. The quality of the assay was determined by both inter-day and intra-day reports. The stability and effectiveness of the method were reported by QC study and drug recoveries were consistent. Finally, this HPLC method is a sensitive, accurate and precise method for monitoring plasma concentrations of voriconazole in both pediatric and adult patients and can be applied as part of routine laboratory service.

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Potential conflicts of interest

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การวัดระดับยาวอริโคนาโซลในพลาสมาผู้ป่วยด้วยวิธีโครมาโทกราฟีของเหลวสมรรถนะสูงแบบรวดเร็ว

กันทิมา แสงศิริวุฒิ, เมธี ชยะกุลคีรี

ภูมิหลัง: *ปัจจุบันมีการใช้ยาวอริโคนาโซล (voriconazole) ในการรักษาโรคติดเชื้อราชนิดลุกลาม (invasive fungal) อย่างกว้างขวาง เนื่องจากผู้ป่วยแต่ละคนที่ได้รับยาในขนาดเท่ากันมีระดับยาในเลือดที่แตกต่างกัน และความเข้มข้นของระดับยาวอริโคนาโซลในเลือดยังมีความสำคัญต่อการตอบสนองต่อการรักษาด้วย การศึกษานี้มีวัตถุประสงค์เพื่อพัฒนาและตรวจสอบความถูกต้องของวิธีวิเคราะห์ความเข้มข้น ของยาวอริโคนาโซลในพลาสม่าผู้ป่วยด้วยวิธีโครมาโทกราฟีของเหลวสมรรถนะสูงแบบรวดเร็ว (high performance liquid chromatography; HPLC)*

วัสดุและวิธีการ: *เก็บพลาสมาจากผู้ป่วย 10 ราย ที่ได้รับ voriconazole ในการรักษาโรคเชื้อราชนิดลุกลามที่มารับการรักษาที่โรงพยาบาลศิริราช มาวิเคราห์ระดับยา voriconazole สกัดยาวอริโคนาโซล ออกจากพลาสม่าจำนวน 120 ไมโครลิตร โดยใช้กรด perchloric 6% นำพลาสม่าที่สกัดแล้วมาแยกออกจากกันบน HPLC คอลัมน์ชนิด C18 โดยเฟสเคลื่อนที่ (mobile phase) ประกอบด้วย acetonitrile 68% และ น้ำ Milli-Q 32% การตรวจสอบความถูกต้องของวิธี HPLC ที่พัฒนาขึ้น ประกอบด้วยการตรวจหาระดับความเข้มข้นที่น้อยที่สุด ที่สามารถวัดปริมาณได้ (limits of quantitation), การตรวจสอบความถูกต้อง (accuracy), ความแม่นยำ (precision), ความคงสภาพ (stability) และ ความสามารถของวิธีสกัดยา (recovery)*

ผลการศึกษา: *วิธี HPLC สามารถวิเคราะห์ปริมาณยา voriconazole ในระดับต่ำสุดถึง 0.2 มคก./มล. คือตั้งแต่ 0.2 มคก./มล. ถึง 20 มคก./มล. โดยมีค่าความแปรปรวนของการวิเคราะห์ภายในวัน อยู่ระหว่างร้อยละ 0.78-3.01, ความแปรปรวนระหว่างวัน อยู่ระหว่างร้อยละ 1.52-4, ค่าความถูกต้อง อยู่ระหว่างร้อยละ 99.3-101 และความสามารถของวิธีสกัดยาอยู่ระหว่างร้อยละ 99.2-101 ความเข้มข้นของระดับยาวอริโคนาโซลในเลือดผู้ป่วยแต่ละคนมีระดับยาที่แตกต่างกันมาก และตรวจพบระดับยาสูงในช่วงสองสัปดาห์แรกของการรักษา*

สรุป: *ได้วิธี HPLC ที่ง่าย, ถูกต้องและแม่นยำเหมาะสำหรับการวิเคราะห์หาความเข้มข้นของ voriconazole ในพลาสมาในห้องปฏิบัติการประจำ นอกจากนี้ความแปรผันของระดับ voriconazole ในพลาสมาอย่างมีนัยสำคัญของผู้ป่วยแต่ละราย แสดงให้เห็นถึงความสำคัญของการตรวจวัดระดับยา voriconazole ในเลือดเพื่อการรักษาในผู้ป่วยแต่ละรายที่ได้รับยา voriconazole*