Determination of the Antifungal Agent Voriconazole in Human Plasma Using a Simple Column-Switching High-Performance Liquid Chromatography and Its Application to a Pharmacokinetic Study

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A simple column-switching high performance liquid chromatographic (HPLC) method that does not require any complicated pretreatment has been developed to determine voriconazole in human plasma samples. An internal standard (IS) and borate buffer (pH 9.0) were added to plasma samples, which were then injected directly into the column-switching HPLC system using MAYI-ODS as a pre-column. The calibration curve for voriconazole showed good linearity in the range of 0.2—10 μg/ml in human plasma. The mean RSD (%) value of intra-day ($n=6$) and inter-day ($n=5$) precision were less than 5.4% and 8.2%, respectively. This system could make more than three hundred successive, accurate measurements when a washing step with ammonium acetate solution was added. This method was successfully applied to measure the therapeutic voriconazole level in patients' plasma, and was used in a study of voriconazole pharmacokinetics after oral administration.

Key words voriconazole; column-switching; HPLC; therapeutic drug monitoring

Voriconazole, (2R,3S)-2-(2,4-difluorophenyl)-3-(5-fluoropyrimidin-4-yl)-1-(1,2,4-triazol-1-yl)butan-2-ol, is a wide-spectrum triazole antifungal agent (Fig. 1a). It selectively inhibits cytochrome P450 (CYP) dependent 14α-demethylase, which is a key enzyme in the fungal sterol biosynthesis pathway, specifically the conversion of lanosterol to ergosterol within the membrane component. Compared with other azole compounds, voriconazole shows a broader-spectrum antifungal activity, and it therefore can be used to treat severe or serious fungal infections, *e.g.*, *Candida* spp., *Aspergillus* spp., *Cryptococcus* spp.[2—4] Voriconazole is metabolized hepatically, primarily *via* the CYP isoenzymes CYP2C9, CYP2C19 and CYP 3A4.[4,5] The metabolites of voriconazole do not have antifungal activity. The CYP2C19 isozyme, which acts in the major metabolic pathway for voriconazole, exhibits significant genetic polymorphism, and consequently a number of poor metabolizers (PMs) are found. Potent interactions with voriconazole by CYP isozymes also affect the plasma levels of voriconazole.[5,6] The PMs are found with much higher frequency (18 to 23%) in the Japanese population than in North American and European white populations (3 to 5%).[7,8] Since the PMs result in increased plasma levels of voriconazole,[5,6] it is important to determine the concentration of voriconazole in human plasma to avoid side effects of voriconazole treatment, such as photopsia, skin rashes, liver dysfunction and so on. Studies concerning the safety, tolerability and pharmacokinetics of intravenous and/or oral dosing regimens of voriconazole demonstrated that voriconazole was generally safe and well tolerated, and it exhibited non-linear pharmacokinetics.[9,10] Therapeutic monitoring of voriconazole in plasma, therefore, might be warranted in some patients who are at higher risk, especially due to PMs of the CYP2C19 genotype.

Several methods including high-performance liquid chromatography (HPLC) combined with mass spectrometry,[11] or with UV detection,[12—16] and bioassay,[16] have been reported to determine voriconazole in biological fluids. The previously reported HPLC-UV methods are clinically useful, but sample pretreatment methods such as solid phase extraction[13] or a solvent extraction[15,16] followed by evaporation under nitrogen stream are required for the assay. Such tedious procedures are undesirable for many clinical applications, whereas an on-line HPLC method with direct plasma injection would be much more convenient. Therefore, there have been developed two methods; one is size exclusion chromatography coupled with a reversed-phase HPLC system with column-switching,[12] and another is a direct plasma injection HPLC micro method using an internal surface reversed-phase column.[14]

In this paper, we describe a simple column-switching HPLC system with a direct plasma injection that does not involve any complicated pretreatment. We have used this system to study the pharmacokinetics of voriconazole in healthy volunteers following a single oral administration of a voriconazole tablet, and this new evaluation method was compared with the standard conventional evaluation method.
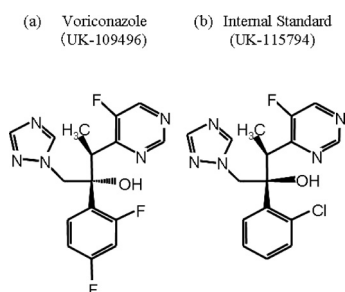

Fig. 1. The Chemical Structures of (a) Voriconazole (UK-109496) and (b) Internal Standard (UK-115794)

Experimental

Chemicals Voriconazole (UK-109496) and UK-115794 (internal stan-

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dard, IS) (Fig. 1) were gifts from Pfizer Global Research & Development (Sandwich, Kent, U.K.). N,N,N',N'-tetramethylethylendiamine (TEMED), acetonitrile and methanol (HPLC grade) were purchased from Kanto Chemical (Tokyo, Japan). All other reagents were analytical reagent grade.

Preparation of Solutions Stock solutions of voriconazole and IS were prepared by dissolving an adequate amount of voriconazole or IS in methanol to final concentrations of 1.0 and 0.1 mg/ml, respectively. These samples were stored at $-20\,°C$. Plasma calibration standards were prepared at final concentrations of 0.2, 0.5, 2.0, 4.0, 8.0, 10.0 μg/ml by spiking adequate amounts of the voriconazole stock solution into drug-free, pooled human plasma. In the same manner, quality control (QC) plasma samples were prepared by spiking with the stock solution of voriconazole to obtain final concentrations of 0.5, 4 and 8 μg/ml. IS is diluted with methanol to 20 μg/ml before use. The sample solutions for assay were prepared as follows: Twenty-five microliters of IS (20 μg/ml) and 150 μl of 0.41 M borate buffer (pH 9.0) were added to 250 μl aliquots of plasma samples, vortex-mixed, and then filtered through a 0.45 μm membrane filter (Millipore, Tokyo).

Instruments The column-switching HPLC system consisted of an on-line degasser (DGU-14A), a low-pressure gradient flow control valve (FCV-10ALvp), two solvent delivery pumps (LC-10ATvp), a high pressure six-port switching valve (FCV-BP), an auto injector (SIL-10A), a column oven (CTO-Avp), a UV detector (SPD-20A) and a system controller (SCL-10Avp) (all from Shimadzu, Kyoto, Japan). A Shim-pack MAYI-ODS (5 μm particle size, 10×4.6 mm i.d., Shimadzu, Kyoto, Japan) was used as a pre-column for on-line sample pretreatment. The chromatographic separations were achieved on a symmetry C_{18} column (5 μm particle size, 250×4.6 mm i.d., Waters, Milford, MA, U.S.A.). The column temperature was maintained at room temperature (25 °C). Detection was carried out at 254 nm with a UV detector.

Column-Switching HPLC System and Analytical Procedure A schematic diagram of an automated column-switching HPLC system to detect and determine voriconazole is shown in Fig. 2. Three individual mobile phases, prepared by mixing two different solutions from three different reservoirs, were pumped by pump 1 through a gradient flow control valve at a flow rate of 2.0 ml/min. Three reservoirs contained 10 mM TEMED phosphate buffer (pH 7.4), acetonitrile and 50 mM ammonium acetate, respectively. Pump 2 always pumped an isocratic mobile phase composed of 10 mM TEMED phosphate buffer (pH 7.4)/acetonitrile (65 : 35, v/v) at a flow rate of 1.0 ml/min. The pumps, the gradient flow control valve and the six-port switching valve were controlled by the system controller. The system was operated according to the following three steps. The switching valve positions and time sequences are shown in parentheses. Step 1 (valve A: 0—1 min and 1—5 min): Filtered plasma sample (50 μl) was directly injected into the pre-column by using the flow path shown in Fig. 2A using a mobile phase of 10 mM TEMED phosphate buffer (pH 7.4)/acetonitrile (95 : 5, v/v) at a flow rate of 2.0 ml/min. After 1 min, the concentration of acetonitrile was increased, and then the mobile phase was changed to 10 mM TEMED phosphate buffer (pH 7.4)/acetonitrile (85 : 15, v/v). Step 2 (valve B: 5—8 min): After 5 min, the flow path was switched from (A) to (B) shown in Fig. 2. The isocratic mobile phase, that is, 10 mM TEMED phosphate buffer (pH 7.4)/acetonitrile (65 : 35, v/v) was pumped at a flow rate of 1.0 ml/min, and then voriconazole and IS onto the pre-column were back-eluted to the analytical column. During the period of analysis, the flow-solvent delivered by pump 1 was changed to acetonitrile/50 mM ammonium acetate (60 : 40, v/v). Step 3 (valve A: 8—20 and 20—32 min): After 8 min, the flow path was switched from (B) to (A) again. Chromatographic analysis was continued, and the pre-column was rinsed with acetonitrile/50 mM ammonium acetate (60 : 40, v/v). After 20 min, the pre-column line was filled with the mobile phase, 10 mM TEMED phosphate buffer (pH 7.4)/acetonitrile (95 : 5, v/v) to prepare for the next analysis.

Assay Validation The linearity of the method was evaluated by analyzing standard voriconazole solutions in the range of 0.2—10.0 μg/ml in drug-free, pooled human plasma. A calibration curve was obtained by plotting the ratio of the peak areas of voriconazole to IS against the concentrations of voriconazole in drug-free plasma. The limit of detection (LOD) was determined as the concentration of voriconazole giving a signal to noise ratio of 3. The intra-day and inter-day precision and accuracy of the method were evaluated by assaying replicates of three QC samples prepared as described above. The intra-day precision of the assay was evaluated by calculating the coefficients of relative standard deviation (RSD) in analyzing six replicates of three different QC samples, and the inter-day precision was determined by analyzing five replicates of three different QC samples within a week. The accuracy is expressed as the mean percentage of the concentration found in plasma divided by the spiked concentration in plasma. The recov-

ery was estimated by dividing the peak area observed after injection of spiked plasma samples by the area observed for spiked samples made using water instead of plasma. The recoveries were determined from five replicates each of low (0.5 μg/ml), medium (4 μg/ml) and high (8 μg/ml) voriconazole concentrations.

Conventional Method The method presented here was compared with a conventional method of measuring voriconazole concentration that includes a solid phase extraction, as reported by Pennick et al.[13] with a slight modification. Briefly, 250 μl of plasma and 25 μl of IS (20 μg/ml) samples were buffered with 700 μl of 0.2 M borate buffer (pH 9.0). The buffered samples were added to Bond Elut C_{18} cartridge (100 mg, 1 ml, Varian Inc., Harbor City, California, U.S.A.), and then washed with 1 ml of 0.2 M borate buffer (pH 9.0), followed by 1 ml of MeOH/H_2O (50 : 50, v/v). The analytes were then eluted with 1 ml of methanol/glacial acetic acid (99 : 1, v/v). The collected eluates were dried under a stream of nitrogen, and then dissolved in 100 μl of mobile phase, which consisted of 0.01 M TEMED phosphate buffer (pH 7.4)/acetonitrile (65 : 35, v/v). Chromatographic analysis was carried out according to the published protocol.[13]

Application The present method was applied to the analysis of voriconazole concentrations in plasma from patients and volunteers after members of each group were orally administered a voriconazole tablet. The subjects were 20 consented patients admitted to Nagano Red Cross Hospital Department of First Internal Medicine (Hematology). Five healthy volunteers also signed the consent form. A single voriconazole tablet (300 mg) was administered to the healthy volunteers after fasting for at least 12 h. Blood samples were drawn at 1, 2, 4, 6 and 8 h after drug administration.

Results and Discussion

Column-Switching HPLC System and Operation In this study, we report an alternative, simple column-switching HPLC method to determine voriconazole, using a fully automated system, as shown in Fig. 2. A Shim-pack MAYI-ODS, comprised of an external surface of porous silica combined with hydrophilic methylcellulose and an internal surface of octadecylsilane, achieved in a highly efficient on-line extraction of drugs in plasma.[17] An aliquot (50 μl) of the filtered sample was introduced to the pre-column via the autosampler using a mobile phase of 10 mM TEMED phosphate buffer (pH 7.4)/acetonitrile (95 : 5, v/v) at a flow rate of 2.0 ml/min. The chromatogram of drug-free plasma is shown in Fig. 3a and that of voriconazole and IS spiked in plasma is shown in Fig. 3b, respectively. Retention times for voriconazole
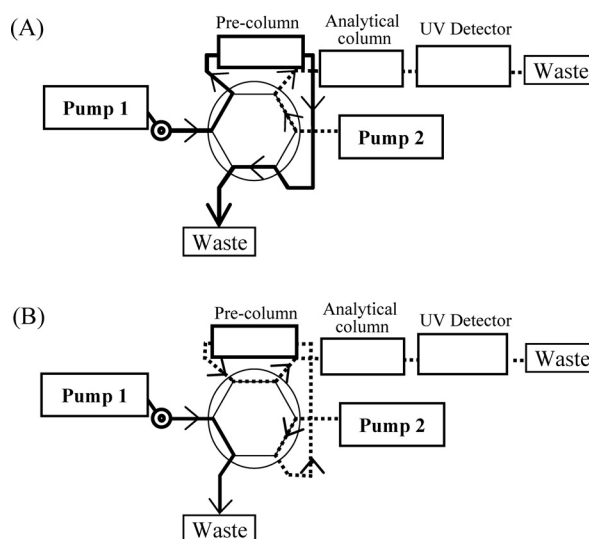

Fig. 2. Schematic Diagram of the Column-Switching HPLC System Using a Six-Port Switching Valve

(A) Sample loading, washing and equilibration position toward the pre-column, and elution and separation position toward the analytical column; (B) back-elution position toward the pre-column.

and IS were approximately 22.9 and 25.9 min, respectively. Voriconazole and IS were retained by the stationary phase of the inner surface, whereas plasma proteins and other matrix compounds were removed. The protein exclusion was evaluated by measuring the absorbance of the collected fractions, as described in the paper.[17] The protein exclusion was nearly 97% during the first minute, and the overall exclusion was estimated to be over 99%. Lower concentrations of acetonitrile prevented protein precipitation in the pre-column. The washing step with 10 mM TEMED phosphate buffer (pH 7.4)/acetonitrile (85 : 15, v/v) for between 1 and 5 min, and that with acetonitrile/50 mM ammonium acetate (60 : 40, v/v) for between 5—20 min were effective in removing matrix elements, and were required to clean the pre-column and to prolong the life of both the pre-column and the analytical column. At 5 min after injection, the switching valve was changed to position (B). Voriconazole and IS were back-eluted to the analytical column with the mobile phase, 10 mM TEMED phosphate buffer (pH 7.4)/acetonitrile (65 : 35, v/v), at a flow rate of 1.0 ml/min for 3 min. One analysis cycle was completed in 32 min.

Few on-line HPLC methods with direct sample injection have been reported for the determination of voriconazole concentrations. One is the multidimensional HPLC method,
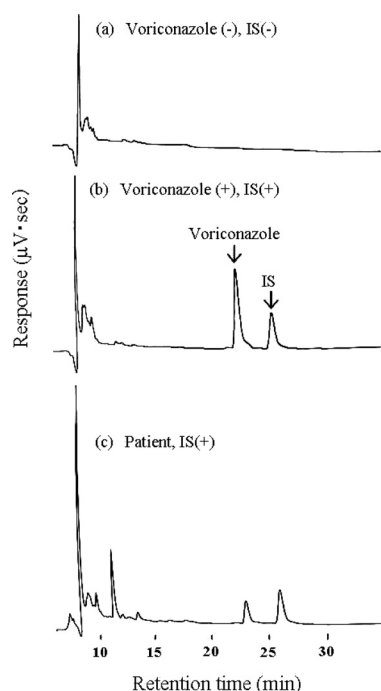

Fig. 3. Chromatograms of Voriconazole and the Internal Standard

(a) Drug-free human plasma, (b) drug-free human plasma spiked with voriconazole and the internal standard and (c) patient plasma and the internal standard.

in which a Sephadex G-25 size-exclusion column is used as the first step to remove plasma proteins, and the separation is coupled with a reversed-phase column-switching HPLC system.[12] This flow system needs three pumps and columns, and two switching valves, which is rather complicated system. Another is the direct plasma injection HPLC method using an internal surface reversed-phase column.[14] The analytical step in this method is very simple, but the method is devoid of sufficient separation of voriconazole from plasma protein. In addition, the column efficiency is extremely influenced by the matrix elements.

The detection sensitivity of voriconazole by the present method was slightly higher than that by the direct plasma injection HPLC method,[14] but was lower than that by the multidimensional HPLC method.[12] However, this system reported here is a simple column-switching HPLC, therefore it should be useful in pharmacokinetic and clinical studies.[18]

Analytical Validation　The calibration curve for voriconazole was linear over the concentration range of 0.2—10 μg/ml in plasma with a correlation coefficient of 0.998 ($y=0.4048x+0.0257$, where $y=$peak area ratio, $x=$concentration). Table 1 shows the results of intra- and inter-day precision and accuracy of voriconazole quantitation. The intra-day precision (RSD) ranged from 3.6 to 5.4%. The inter-day precision (RSD) ranged from 4.7 to 8.2%. The accuracy ranged from 97.1 to 104.9%. Both the precision and accuracy were within acceptable ranges. The limit of detection at a signal to noise ratio of 3 was 0.07 μg/ml. The recovery was estimated by comparing the peak area ratio after injection of voriconazole, dissolved in the mobile phase, with the peak area ratio after injection of the same amount of voriconazole spiked in human plasma. The mean recoveries were 94.8±4.5% (0.5 μg/ml, $n=5$), 99.4±3.6% (4 μg/ml, $n=5$) and 96.8±3.4% (8 μg/ml, $n=5$), respectively. No striking change was observed even after more than 300 plasma samples were analyzed using this column-switching HPLC system.

Application to Pharmacokinetic Studies and Patients' Plasma　The method described here was applied to determine voriconazole concentrations in plasma after an oral administration of voriconazole (300 mg) tablet was given to five healthy volunteers. Figure 4 shows a mean (±S.D.) plasma concentration–time curve of voriconazole. The pharmacokinetic parameters of voriconazole are summarized in Table 2, in which the parameters obtained by the conventional method[13] are tabulated together and compared with our results presented here. The results are in fair agreement with each other. The concentrations of voriconazole in plasma of five healthy volunteers at each point in the time-course were measured by both the present method and the conventional method. As shown in Fig. 5a, there was a good correlation between two methods, with a correlation coefficient (r^2) of

Table 1. Precision and Accuracy of HPLC Analysis of Voriconazole in Human Plasma

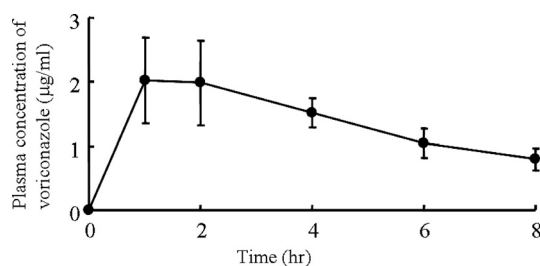| Spiked (μg/ml)	Intra-day ($n=6$)			Inter-day ($n=5$)		
	Found (mean±S.D.; μg/ml)	Precision (RSD, %)	Accuracy (%)	Found (mean±S.D.; μg/ml)	Precision (RSD, %)	Accuracy (%)
0.5	0.52±0.02	4.6	104.9	0.48±0.02	4.7	95.6
4	3.93±0.14	3.6	98.2	4.06±0.31	7.6	101.4
8	7.77±0.42	5.4	97.1	8.21±0.68	8.2	102.6

Fig. 4. Mean (±S.D.) Plasma Concentration–Time Curve of Voriconazole Followed by a Single Oral Administration of Voriconazole Tablet (300 mg) to Five Human Healthy Volunteers

Table 2. Pharmacokinetic Parameters of Voriconazole in Human Plasma

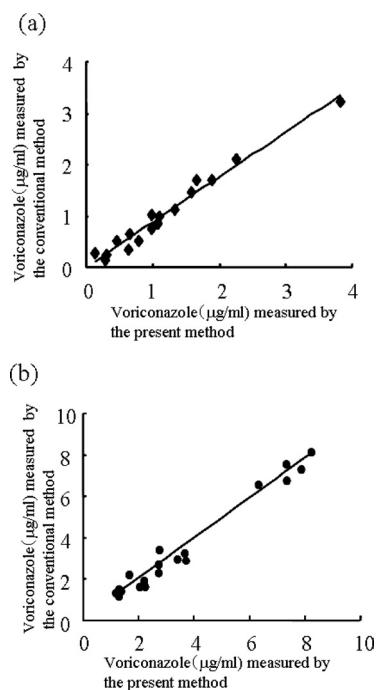|	Present method	Conventional method
T_{max} (h)	1.4±0.5	1.6±0.5
C_{max} (μg/ml)	2.1±0.7	2.0±0.6
AUC_{0-8} (μg·h/ml)	10.9±2.1	10.5±2.0
$t_{1/2}$ (h)	5.6±1.9	5.8±2.3

Fig. 5. Relationship between the Plasma Concentration of Voriconazole Measured by the Method Presented Here and That by the Conventional Method, Applied to (a) 25 Samples from Five Human Healthy Volunteers and to (b) 20 Patient Samples

0.9532 (n=25, y=0.9439x+0.0821, where y=the conventional method, x=the present method). In addition, the present method was applied to measure the voriconazole concen-

tration in plasma of 20 patients received voriconazole treatment. These results also showed a good correlation between the two methods, with a correlation coefficient (r^2) of 0.9747 (n=20, y=0.9589x−0.0742, where y=the conventional method, x=the present method) (Fig. 5b).

Conclusion

A simple column-switching HPLC method to determine voriconazole in human plasma samples has been developed. This method does not require any tedious pre-purification procedures. The method has been fully validated and shown to be specific, accurate, precise and reproducible. It was successfully applied to pharmacokinetic studies of voriconazole, and its usefulness was evaluated using plasma from healthy human volunteers and from patient volunteers. Because it proved easier to use while providing results comparable in quality to the more involved conventional method, we hope that this analytical method will be adopted for clinical use.

References and Notes
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