Yonago Acta Medica 2023;66(3):365–374 doi: 10.33160/yam.2023.08.009 *Original Article*

Development of a New Method for Simultaneous Quantitation of Plasma Concentrations of Voriconazole and Voriconazole *N*-Oxide Using Column-Switching LC-MS/MS and Its Application in Therapeutic Drug Monitoring

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ABSTRACT

Background Voriconazole therapy for fungal infections usually continues for several years and is often administered on an outpatient basis. Maintaining the voriconazole plasma concentration in the therapeutic range is highly important for effective therapy; however, it is difficult to obtain sufficient information to assess the voriconazole concentration in outpatients. Therefore, we developed a method to simultaneously measure the plasma concentrations of voriconazole and its major metabolite, voriconazole *N*-oxide, to obtain rapid results after outpatient blood collection and before medical consultation and to attain a better understanding of adherence and the drug-drug interactions of voriconazole.

Methods Fifty microliters of patient plasma was deproteinized with methanol, injected into the liquid chromatography-tandem mass spectrometry system, and purified using an online column. Separation was achieved on an InertSustain C18 column (2.1 mm id × 50 mm, 2 μm) with a mobile phase of 30:70 (0.1% formic acid in water:methanol) at a flow rate of 0.2 mL/min. Detection was performed using electrospray ionization in positive ion multiple reaction monitoring mode.

Results The analysis time was 4 min. The calibration curve was linear, in the range of 0.1 μg/mL to 20 μg/mL for voriconazole and 0.05 μg/mL to 10 μg/mL for voriconazole *N*-oxide, with a coefficient of determination at $R^2 > 0.999$.

Conclusion There is no need to dilute the patient's plasma even if the concentration of voriconazole is near the upper limit of measurement. Furthermore, the short measurement-time could immediately inform physicians of the patient's voriconazole concentration during ambulatory medical care. Simultaneous measurement of voriconazole and voriconazole *N*-oxide may also be useful for the immediate adjustment of voriconazole dosage in outpatients and would help us to understand adherence or drug-drug interactions in plasma voriconazole concentrations.

Key words liquid chromatography; pharmacokinetics; tandem mass spectrometry; therapeutic drug monitoring; voriconazole

Voriconazole (VRCZ) is a triazole antifungal agent that inhibits fungal cytochrome P450-mediated 14 alpha-lanosterol demethylation,[1] and is recommended as the first-line therapy for the treatment of invasive pulmonary aspergillosis as well as other severe fungal infections worldwide. VRCZ often causes side effects, such as liver damage and visual impairment, due to an increase in the trough concentration (> 4 μg/mL); however, plasma VRCZ concentrations below the therapeutic range (trough concentration of > 1 μg/mL) do not yield satisfactory clinical results.[2–4] VRCZ plasma concentrations are heavily affected by patient adherence, and as VRCZ is administered between meals, the compliance to VRCZ tends to be poor.[5] VRCZ is metabolized to voriconazole *N*-oxide (VNO) and hydroxyvoriconazole by cytochrome P450 (CYP) 3A4, CYP2C19, CYP2C9 and flavin-containing monooxygenase 3.[6–9] The metabolism of VRCZ is characterized by non-linear pharmacokinetics, and the conversion of the major metabolite to VNO has been reported to be saturated at the clinical dose and no contribution from the CYP2C19 genotype was observed.[10] VRCZ is metabolized by CYP3A4,[9, 11] which is the most abundant CYP enzyme expressed in the human liver and small intestine.[12, 13] CYP3A4 participates in the metabolism of more than 50% of approved drugs and is susceptible to drug-drug interactions.[14] It has been reported that administration of prednisolone, an inducer of CYP3A4, increases the ratio of VNO to VRCZ, emphasizing the importance of CYP3A4 in the metabolism of VRCZ.[15, 16]

Treatment with VRCZ should be continued for a minimum of 6 months for chronic cavitary pulmonary

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Received 2023 March 29
Accepted 2023 July 20
Online published 2023 August 9
Abbreviations: CYP, cytochrome P450; HPLC, high performance liquid chromatography; HQC, high quality control; IS, internal standard; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LLOQ, lower limit of quantification; LQC, low quality control; MQC, medium quality control; MRM, multiple reaction monitoring QC, quality control; RE% relative error; RSD%, relative standard deviation; TDM, therapeutic drug monitoring; VNO, voriconazole *N*-oxide; VRCZ, Voriconazole

aspergillosis and may be continued for a number of years if the disease is responsive and well-tolerated.[17] Most patients treated with VRCZ at the Tottori University Hospital are outpatients as the treatment is long-term. In some patients, treatment with VRCZ is initiated as an outpatient. Therapeutic drug monitoring (TDM) of VRCZ in outpatient's has been reported to be useful for avoiding liver injury and for the continuation of treatment; therefore unintentional deviations in the VRCZ plasma concentration can cause long-term disadvantages.[18] However, TDM of VRCZ in outpatients is difficult due to insufficient information on interactions and adherence. It has been reported that the simultaneous measurement of VRCZ and VNO concentrations gives us valuable information for the assessment of adherence and metabolic capacity of CYP3A4, which is reduced during inflammatory conditions.[19–22] These reports emphasize that TDM of VRCZ and VNO allows physicians to provide safe and effective VRCZ therapy for both hospitalized patients and outpatients.

A number of studies have reported the simultaneous measurement of VRCZ and VNO concentrations in human plasma using high performance liquid chromatography (HPLC) with a UV detector or mass spectrometer.[23–29] However, in most cases the run-time of the measurement method is more than 10 min,[24, 27] except for one study that reported a run-time of 4 min.[26] One disadvantage of this method is the complex and time-consuming pretreatment step which involves evaporative drying.[26] In contrast, the mass spectrometer method does not require a complicated pretreatment step and can measure drugs other than VRCZ and VNO; however, the minimum run-time is 6.9 min, which is too long for immediate consultation to outpatients.[23, 25, 28, 29] Measurement methods for VRCZ and VNO with a low upper limit may require a time-consuming dilution step when its concentration is high. However, in order to rapidly report the VRCZ and VNO concentration of outpatients to a physician, we must drastically reduce the time period between blood collection and medical consultation. Hence, the measurement method needs to comprise a simpler pretreatment step and a shorter measurement time than the previously reported methods.

In this study, we developed a method to simultaneously quantify VRCZ and its metabolite, VNO, in plasma by modifying a previously developed method for the quantification of the VRCZ plasma concentration using liquid chromatography-tandem mass spectrometry (LC-MS/MS) with column switching.[30] Using the developed method, the plasma concentrations of VRCZ and VNO were measured in inpatients and outpatients to evaluate the method's suitability for clinical use.

MATERIALS AND METHODS

Reagents

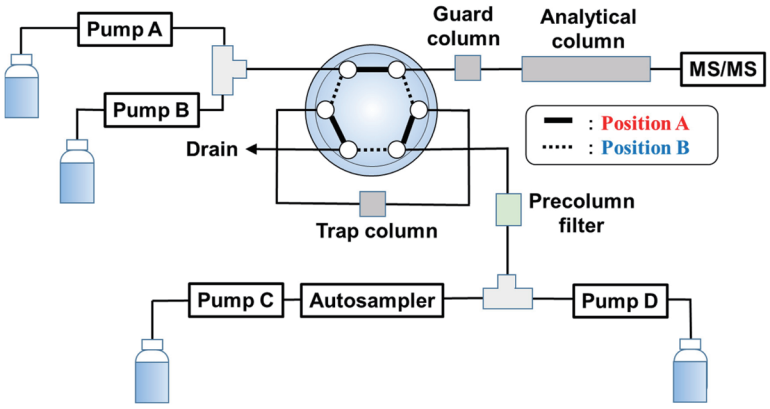
VRCZ was purchased from Tokyo Chemical Industry (Tokyo, Japan) and VNO was purchased from Toronto Research Chemicals (North York, Canada). Paclobutrazol, methanol (HPLC grade), and formic acid (HPLC grade) were purchased from FUJIFILM Wako Pure Chemicals (Osaka, Japan). The Milli-Q Reference-Elix® Essential UV3 water purification system (Merck, Tokyo, Japan) was used to generate ultrapure water. Pooled normal human plasma (in EDTA) was obtained from Cosmo Bio (Tokyo, Japan). Human EDTA plasma from six individuals was obtained from Kohjin Bio (Saitama, Japan).

Preparation of standard solutions and plasma samples

VRCZ, VNO, and paclobutrazol (internal standard; IS) were dissolved in methanol to concentrations of 1000, 334, and 500 μg/mL, respectively, and used as stock standard solutions. A methanol solution containing 200 μg/mL VRCZ and 100 μg/mL VNO was prepared using the VRCZ and VNO stock standard solutions. In addition, standard solutions for the calibration curve (VRCZ; 1, 2, 5, 10, 20, 50, 100, and 200 μg/mL: VNO, 0.5, 1, 2.5, 5, 10, 25, 50, and 100 μg/mL) were prepared by dilution with methanol. The quality control (QC) (VRCZ; 1, 1.5, 15, and 150 μg/mL: VNO, 0.5, 0.75, 7.5, and 75 μg/mL) was prepared from the standard solution by dilution with methanol. The IS stock standard solution was diluted with methanol to a concentration of 0.5 μg/mL, and the preparation was used as the IS working solution. All preparations were stored at −30 °C until further use. Calibration plasma samples containing VRCZ (0.1, 0.2, 0.5, 1, 2, 5, 10, and 20 μg/mL) and VNO (0.05, 0.1, 0.25, 0.5, 1, 2.5, 5, and 10 μg/mL) were prepared via a 10-fold dilution with pooled blank plasma. QC plasma samples containing VRCZ (0.1, 0.15, 1.5, and 15 μg/mL) and VNO (0.05, 0.075, 0.75, and 7.5 μg/mL) were prepared via a 10-fold dilution with blank pooled plasma. The QC plasma samples were labeled in a reverse order of concentration (from low to high): lower limit of quantification (LLOQ), low quality control (LQC), medium quality control (MQC), and high quality control (HQC).

Column-switching LC-MS/MS and its analytical conditions

Column-switching LC-MS/MS was performed using a previously described system with slight modifications.[30] The LC-MS/MS system consisted of a Nexera



Process time (min)	Pump flow rate (mL/min)				Valve position
	A	B	C	D	
0.00–0.05	0.06	0.14	0.10	0.20→2.00	A
0.05–0.50				2.00	
0.50–0.60					B
0.60–0.70				2.00→0.00	
0.70–1.00				0.00	
1.00–4.00					A

Fig. 1. Schematic diagram of the liquid chromatography-tandem mass spectrometry (LC-MS/MS) system, pump flow rate, and valve position used in this study.

X2 LC system (Shimadzu, Kyoto, Japan) and a quadrupole mass spectrometer (LCMS-8040; Shimadzu). Samples were trapped on an InertSustain® AQ-C18 (3 µm, 3.0 mm × 10 mm; GL Sciences, Tokyo, Japan). An InertSustain® C18 (2 µm, 2.1 mm × 50 mm; GL Sciences) and an InertSustain® C18 (3 µm, 1.5 mm ×10 mm; GL Sciences) were used for chromatographic separation. The trap, guard, and analytical columns were maintained at 40 °C in a column oven (CTO-20A; Shimadzu). The mobile phases used are as follows; A = formic acid/water (0.1:100, v/v), B = methanol, C = methanol, D = water. The analytical mobile phase was a mixture of 0.1% aqueous formic acid and methanol (30:70, v/v), and the flow rate was set at 0.2 mL/min. The pretreatment mobile phase consisted of a mixture of methanol and water. The detailed gradient program is illustrated in Fig. 1. A 1 µL aliquot of the sample was injected into the trap column, and pre-concentration was carried out for 30 s. The column switch valve (FCV-32AH; Shimadzu) was then switched so that the analytical column was in line with the trap column and mass spectrometer.

Plasma sample pretreatment

For sample preparation, 50 µL of plasma was mixed with the IS standard solution (50 µL) and methanol (200 µL), and vortexed for 10 s. The mixture was then centrifuged at 9,600 g for 3 min at 20 °C. One microliter of the supernatant was subjected to LC-MS/MS analysis.

MS/MS measurement conditions

Electrospray ionization, which was performed in positive mode monitoring, was selected as the ionization source, and the ionization parameters were as follows: capillary voltage, 4.5 kV; heat block temperature, 400 °C; desolvation line temperature, 250 °C; nebulizer gas (N_2) flow rate, 3.0 L/min; and drying gas (N_2) flow rate, 15.0 L /min. The mass spectrometer was automatically tuned to VRCZ, VNO, and IS using LabSolutions software (Shimadzu). Multiple reaction monitoring (MRM) was used to detect VRCZ (*m/z* 350.00 → 224.05), VNO (*m/z* 366.00 → 224.05), and IS (*m/z* 294.00 → 70.10). The collision energy was set at −19.0 V for VRCZ, −16.0 V for VNO, and −21.0 V for IS.

Full validation of the analytical method

The analytical procedure was validated according to the United States Food and Drug Administration Bioanalytical Method Validation Guidance for Industry.[31] QC samples were pretreated with the same method as regular samples, and 1 µL was injected into the LC-MS/MS for measurement.

Blank human plasma samples from six individuals

and the LLOQ were analyzed to determine the selectivity of the analytical method. To consider a sample without interfering compounds, the response of these compounds should be less than 20% of the LLOQ for the analytes and less than 5% for the IS. Carry-over was investigated by measuring blank plasma samples after the measurement of the highest-concentration calibration sample. The response of the blank sample should not exceed 20% of the LLOQ or 5% of the average IS analytical response.

To obtain a standard curve, the peak area of each analyte to the IS was plotted against the standard concentrations with a weighting factor of 1/*x*. The calculated concentrations of the calibration standards should be ± 15% of the nominal concentration and ± 20% of the LLOQ nominal concentration. Precision and accuracy were evaluated using the LLOQ, LQC, MQC, and HQC. The same calibration curve was used for each analytical unit, and the concentration in the sample was measured five times. Intra- and inter-day precision and accuracy were measured by repeating the same analysis unit three times on the same day and on three different days, respectively. Precision was determined as the relative standard deviation (RSD%), and accuracy as the relative error (RE%) of the nominal versus measured concentrations. The mean concentration obtained from the QC samples should be ± 15% of the nominal concentration and ± 20% of the nominal concentration for the LLOQ to be accurate. The precision should be less than 15% for QC samples and less than 20% for LLOQ.

The recovery of VRCZ, VNO, and IS was evaluated by comparing the mean peak area of four quality controls (LLOQ, LQC, MQC, and HQC) to the mean peak area of the plain standards of the equivalent concentration. The values of three measurements were evaluated. The matrix effect was calculated by comparing the peak area of four QC samples (LLOQ, LQC, MQC, and HQC) and water spiked with amounts equivalent to the respective QC. The values of three measurements were evaluated. The accuracy among the individuals should be within 15%.

Stability of VRCZ and VNO over time in plasma under various conditions

To evaluate the stability in plasma, two concentrations of QC samples (LQC and HQC) were subjected to five freeze–thaw cycles, followed by 6 months or 12 months of frozen storage (−30 °C) or 3 h or 6 h of room-temperature storage (20 °C). To evaluate stability in methanol, two concentrations of QC samples (LQC and HQC) were stored at −30 °C for 12 months. To evaluate the stability of the measured samples after pre-treatment, two concentrations of QC samples (LQC and HQC) were stored at room temperature (20 °C) for 3 h, 6 h, or 24 h. The results are presented as the average of three measurements. Samples were considered stable if the mean concentration obtained at each level was within ± 15% of the nominal concentration.

VRCZ and VNO concentration in patients

The candidates for this single-center retrospective study included patients who were treated with VRCZ orally at Tottori University Hospital between October 2018 and June 2020. Blood samples (n = 152) were collected from 33 patients. Blood samples from patients who met the following criteria were excluded: < 20-year-old; collected less than 5 days from the start of VRCZ medication. To observe the relationship between the plasma concentrations of VRCZ and VNO over time, we analyzed seven individuals (blood samples; n = 53) for whom at least five measurements were obtained at trough concentrations. This study was approved by the Ethical Review Committee of the Tottori University School of Medicine (approval number: 18A102) and was conducted with the patients' opt-out consent.

RESULTS

Analytical validation

The duration of the analysis was set to 4 min because the retention times for VRCZ, VNO, and IS were 2.5, 2.0, and 3.1 min, respectively (Fig. 2). The calibration curves for VRCZ (y = 0.514x + 0.00171) and VNO (y = 0.722x + 0.00014) were linear in the range of 0.1–20 μg/mL and 0.05–10 μg/mL, respectively, with a coefficient of determination at $R^2 > 0.999$. The peak for VRCZ, VNO, and IS were not detected in the MRM chromatograms in blank plasma of the six individuals, indicating high specificity of the method (Fig. 2). The intra-day precisions (n = 5) for VRCZ and VNO were within 4.3% and 3.6%, and the intra-day accuracies were ± 7.7% and ± 7.0%, respectively (Table 1). The inter-day precisions (n = 5) for VRCZ and VNO were within 3.4% and 4.2%, respectively, and the intra-day accuracies were ± 8.3% and ± 8.0%, respectively (Table 1). The recoveries (n = 3) for VRCZ and VNO were 101.4% – 111.4% and 97.0% – 110.6%, respectively, and the recovery for 0.5 μg/mL IS was 100.3% (Table 2). The matrix effects (n = 6) for VRCZ and VNO were 96.5% – 104.5% and 95.5% – 100.8%, respectively, and the matrix effect for 0.5 μg/mL IS was 101.9%, with no significant difference in the accuracy among individuals (Table 2). The carry-over (n = 5) of VRCZ and VNO was 9.6% and 16.6% of the LLOQ, respectively, and the carry-over rate of IS was 0.6% – 0.7%. The stabilities of VRCZ and VNO in

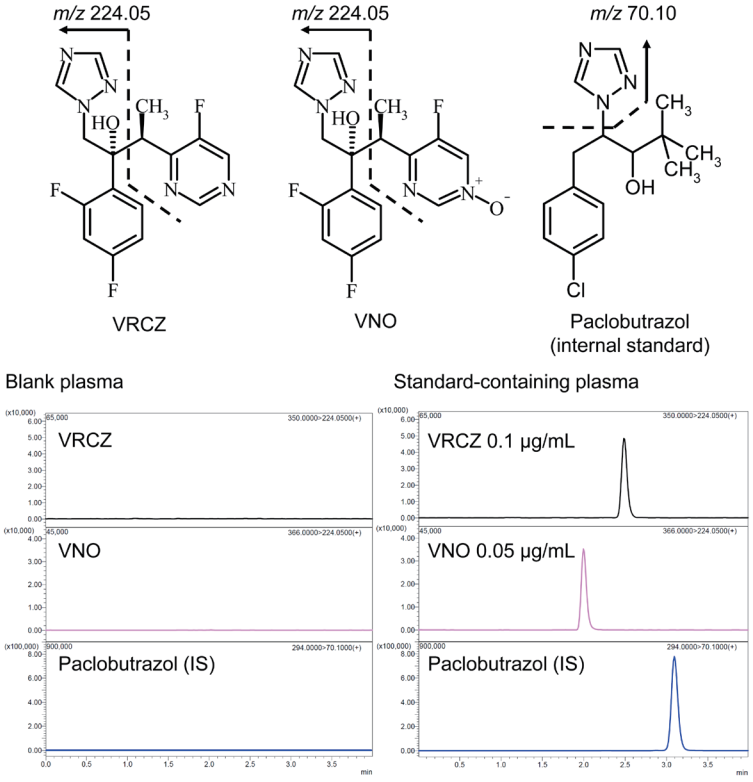


Fig. 2. Chemical structure and multiple reaction monitoring (MRM) chromatogram of voriconazole (VRCZ), voriconazole *N*-oxide (VNO), and paclobutrazol.

Table 1. Intra- and inter-day variations in VRCZ and VNO concentrations in human plasma

Concentration (μg/mL)		Inter-day variation ($n = 5$)		Intra-day variation ($n = 5$)	
		RE (%)	RSD (%)	RE (%)	RSD (%)
VRCZ	0.1	5.1	3.4	–7.7	4.3
	0.15	5.7	3.4	–1.4	3.0
	1.5	8.3	2.5	–0.2	3.2
	15	3.1	2.6	–7.4	2.7
VNO	0.05	8.0	3.2	–2.4	3.6
	0.075	5.6	4.2	–0.3	3.5
	0.75	4.1	3.2	–2.8	3.0
	7.5	3.5	1.7	–7.0	3.0

Precision was expressed as the relative standard deviation (RSD, %), and accuracy was expressed as the relative error of the nominal versus measured concentrations (RE, %). VRCZ, voriconazole; VNO, voriconazole *N*-oxide.

Table 2. Recovery and matrix effects for VRCZ, VNO, and paclobutrazol

Concentration (μg/mL)		Recovery ($n = 3$)	Matrix effect ($n = 6$)
		Mean ± CV (%)	Mean ± CV (%)
VRCZ	0.1	107.3 ± 1.8	96.5 ± 1.7
	0.15	108.3 ± 1.0	99.0 ± 1.3
	1.5	111.4 ± 1.6	104.5 ± 4.1
	15	101.4 ± 1.4	99.5 ± 1.9
VNO	0.05	101.4 ± 0.7	95.9 ± 2.8
	0.075	105.8 ± 2.2	99.5 ± 1.9
	0.75	110.6 ± 1.6	100.8 ± 3.4
	7.5	97.0 ± 1.8	95.5 ± 1.5
Paclobutrazol	0.5	100.3 ± 3.4	101.9 ± 3.2

Each value represents the mean ± coefficient of variation (CV). VNO, voriconazole *N*-oxide; VRCZ, voriconazole.

the plasma, methanol, and measurement solution ($n = 3$) are summarized in Table 3. The VNO concentration was reduced by incubation at room temperature for > 3 h in plasma and 6 h in the measurement solution. No significant degradation was observed for VRCZ.

The relationship between VRCZ and VNO concentration

The background and measurement results for the seven patients in this study are shown in Table 4, and the relationship between the plasma concentrations of VRCZ and VNO is shown in Fig. 3. Plasma concentrations

Table 3. Stability of VRCZ and VNO under different conditions

Matrix	Storage Condition	Time interval	VRCZ concentration (μg/mL)		VNO concentration (μg/mL)	
			0.15	15	0.075	7.5
			mean ± SD (%)	mean ± SD (%)	mean ± SD (%)	mean ± SD (%)
Plasma (n = 3)	–30 °C	6 months	97.1 ± 1.7	103.1 ± 1.9	101.2 ± 0.7	100.3 ± 1.8
		12 months	107.4 ± 3.6	97.4 ± 2.6	101.4 ± 0.3	95.1 ± 3.5
	Room temperature (20 °C)	3 h	110.4 ± 1.2	102.0 ± 2.6	99.0 ± 0.4	89.1 ± 4.0
		6 h	106.1 ± 0.9	102.2 ± 4.9	85.5 ± 2.5	80.5 ± 2.9
	Freeze-thaw	5 cycles	98.1 ± 4.2	103.5 ± 2.0	98.3 ± 4.8	103.9 ± 2.8
Methanol (n = 3)	–30 °C	12 months	109.8 ± 1.0	106.6 ± 4.5	103.2 ± 3.4	107.6 ± 4.3
Measurement solution (n = 3)	Room temperature (20 °C)	3 h	104.0 ± 3.0	102.3 ± 2.2	104.0 ± 2.9	101.0 ± 2.4
		6 h	103.3 ± 2.0	103.7 ± 3.6	101.3 ± 2.7	100.8 ± 3.1
		24 h	100.7 ± 2.1	101.0 ± 2.9	85.3 ± 3.7	81.9 ± 7.9

Values represent the mean ± standard deviation. Measurement solution: 83% aqueous methanol. VNO, voriconazole *N*-oxide; VRCZ, voriconazole.

Table 4. Patient backgrounds and measurements

Patient	Indication	Age (years)	Body weight (kg)	Research period (months)	VRCZ dosage (mg/day)	VRCZ concentration range (μg/mL)	VNO concentration range (μg/mL)
A	Cryptococcal meningitis	53	60.9	2	200–300	1.7–4.5	1.3–2.2
B	Fungal rhinosinusitis	77	70.6	3	500	1.7–2.7	2.8–3.3
C	Fungal rhinosinusitis	70	43.3	2	600	0.4–2.5	2.7–5.4
D	Pulmonary aspergillosis	70	59.5	2	300–400	1.0–4.1	1.6–3.1
E	Deep-seated mycosis	80	43.7	4	200–300	1.1–4.8	1.1–2.4
F	Pulmonary aspergillosis	73	48.5	17	200–300	1.4–3.9	1.4–3.0
G	Fungal rhinosinusitis	67	58.7	17	400	2.7–5.0	3.3–3.8

VRCZ dosage indicates the range of daily VRCZ doses during the study period. VNO, voriconazole *N*-oxide; VRCZ, voriconazole.

of VRCZ and VNO ranged from 0.4 μg/mL to 5.0 μg/mL (median 2.4 μg/mL) and 1.1 μg/mL to 5.4 μg/mL (median 2.7 μg/mL), respectively. We observed a low correlation between VRCZ and VNO concentrations using Pearson's correlation analysis when all plasma data collected from seven patients were included (r = 0.24). In contrast, there was a high correlation between them in almost all cases (patients A, B, C, D, and E) when analyzed individually. We also observed a moderate (patient F) and negligible (patient G) correlation between the VRCZ and VNO concentrations. The slope of the regression line ranged from 0.012 to 1.031 (median 0.423) for the individual cases.

DISCUSSION

In this study, a rapid and robust LC-MS/MS method was developed for the simultaneous quantification of VRCZ and VNO. VNO is a more water-soluble compound than VRCZ, so the trap column was changed from EXP C18 to InertSustain® AQ-C18, which has a strong affinity to the more highly polar compounds. Furthermore, online enrichment on the trap column were improved by slowing the flow rate of ultrapure water, which resulted in the simultaneous measurement of VRCZ and VNO in just 4 minutes.[30] Paclobutrazol has a triazole ring like VRCZ and VNO. The recovery rate of paclobutrazol is close to 100% with a matrix effect of 96.8% and lot-to-lot variation of 0.6%.[30] Retention time of paclobutrazol was close to VRCZ and VNO under the separation condition of this study, and the peak intensity was not affected by VRCZ and VNO concentrations, thus paclobutrazol was judged to be suitable as an internal standard. The selectivity, intra- and inter-day precision and accuracy, and carry-over were within acceptable ranges, and no significant difference was observed in the recovery or matrix effect between VRCZ, VNO, and IS. The

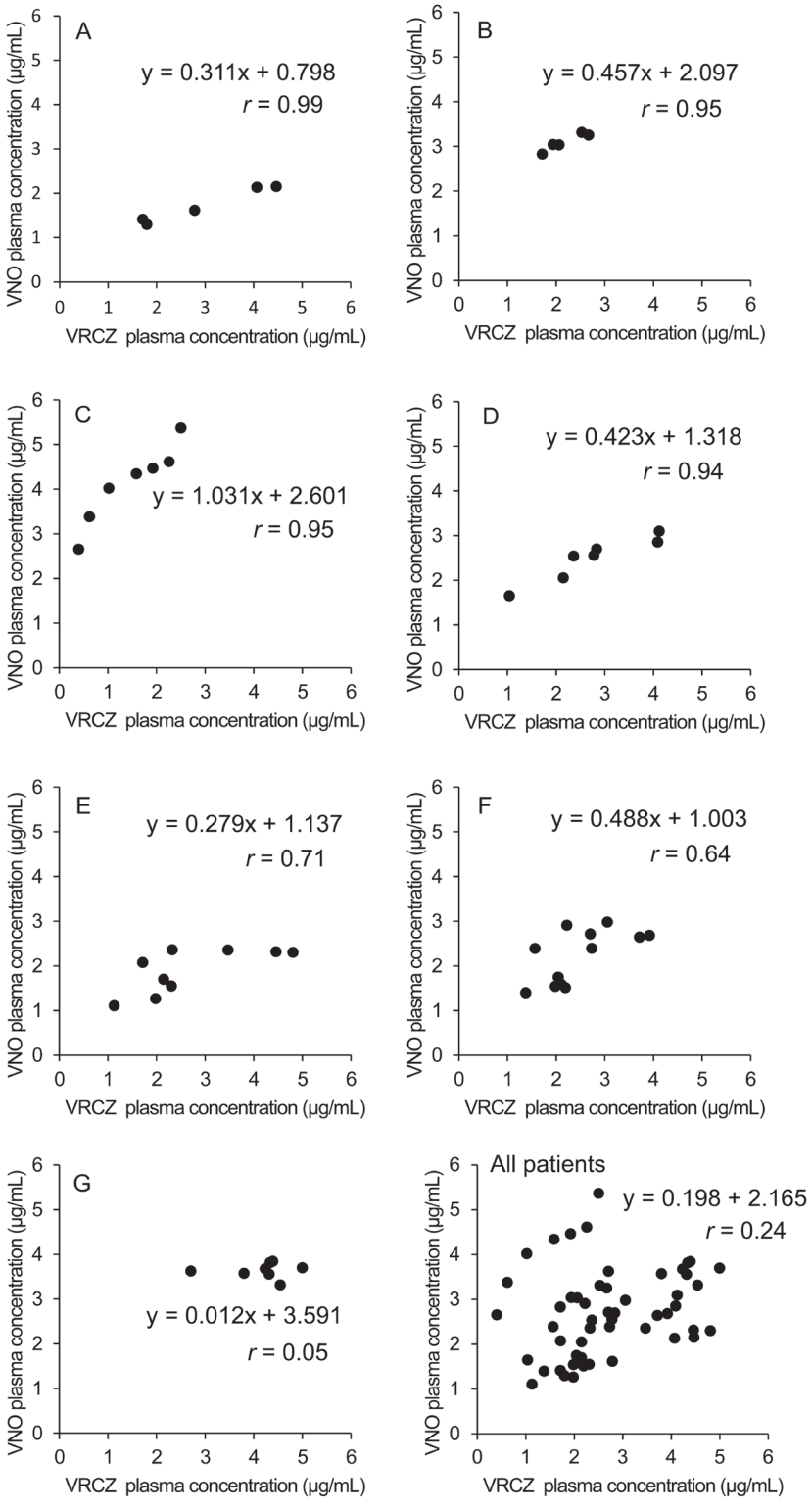


Fig. 3. Relationship between the plasma concentrations of VRCZ and VNO.
The daily VRCZ dosage for each patient: 200–300 mg for patient A, 500 mg for patient B, 600 mg for patient C, 300–400 mg for patient D, 200–300 mg for patient E, 200–300 mg for patient F, and 400 mg for patient G. VNO, voriconazole *N*-oxide; VRCZ, voriconazole.

results met the criteria of the United States Food and Drug Administration Bioanalytical Method Validation Guidance for Industry.[31] The accuracy of VRCZ and VNO was better than the method by Decosterd et al. (4.7% and 6.4%),[25] which had previously reported excellent accuracy using LC-MS/MS. The precision was inferior to other methods without major differences.[23, 25, 29] Consistent with previous reports in stability studies, no degradation was observed for VRCZ under all study conditions, and VNO was reduced in a time-dependent manner through incubation in plasma at room temperature.[25, 26] The stability of VNO in plasma for 3 h and in the measurement solution for 6 h at room temperature indicates the need for prompt preparation and measurement after blood collection. It has been reported that VNO in plasma is stable at 4 °C for at least 12 h.[26] Therefore, blood samples should be stored at 4 °C until preparation, if rapid preparation is not possible.

The total run-time was 4 min, which is significantly shorter than the run-time of 6.9 min that was reported for the similar method using a mass spectrometer,[29] and comparable to that reported by Yamada et al using a UV detector.[26] Although the total run-time is short, the pretreatment is simple, requiring only deproteinization with methanol and no dilution. The centrifugation time after pretreatment was 3 min, which was shorter than the standard time of 10–15 min.[23–29] The solid-phase extraction method has a risk of possible exposure to infectious blood, whereas the column-switching method can wash and concentrate the drugs without exposure. Although the pretreatment and measurement were simple and rapid, there was no clog in the analytical column during the study period, suggesting that the column-switching system played a role in reducing the run-time with good accuracy. The results can be reported within 30 minutes after blood collection due to its short processing- and analysis-time.

The upper limit of detection is 20 μg/mL for VRCZ, which is higher than that of other methods.[23–29] The upper limit of detection for VNO was 10 μg/mL, which was lower than that of a previous report using a UV detector,[27] but higher than other methods using a mass spectrometer.[23, 25, 28, 29] The highest plasma concentrations of VRCZ and VNO in this study were 5.0 and 5.4 μg/mL, respectively, which were within the measurement range. The upper limit of detection for VRCZ in most methods is set at 10 μg/mL and can be measured with normal pretreatment[24, 26, 28]; however, it has been reported that VRCZ plasma concentrations can exceed 10 μg/mL in patients with severe disease or cirrhosis.[32, 33] We experienced the case of VRCZ plasma concentrations exceeding 10 μg/mL,[30] and found that our method can measure the increased concentration without a time-consuming dilution step. The LLOQ is inferior to that of other methods using mass spectrometers[23, 25, 28, 29]; however, the developed method can measure concentrations equal to or lower than those measured by methods using a UV detector.[24, 26, 27] Our method can measure concentrations as low as one-tenth of the trough concentration of ≥ 1 μg/mL, which is the lowest effective concentration of VRCZ, and thus covers a concentration range sufficient for conducting TDM.[34] The developed method has advantages in treatment with VRCZ because the measurement time is short and there is no need for re-measurement at high concentrations, so we can immediately inform physicians of the VRCZ concentration in patients, even during ambulatory medical care. TDM was performed for two of the seven patients (F and G) in the present study during an outpatient visit, and the VRCZ dosage in patient F was altered on the same day as the consultation. Thus, our method can simultaneously measure VRCZ and VNO up to higher concentrations in a shorter-time with better precision and accuracy than previous reports, and can be applied to outpatients.

As shown in Fig. 3, VRCZ and VNO concentrations were closely correlated in six patients, except for patient G; however, we did observe differences in the slopes of the regression lines. Despite no changes in dosage, the fluctuations in VRCZ concentration were observed in patient C and G. There was a fluctuation in the VNO concentration in patient C, but no fluctuation was observed in patient G. The fluctuation in VRCZ concentrations was considered to be caused by changing metabolic activity rather than absorption or adherence, since patient G had an average VNO concentration of 3.6 μg/mL, which is not considered low. In addition, there was no change in concomitant medications in patient G. It has been reported that the metabolism of VRCZ is also affected by hepatic function and inflammation.[21, 22, 32] The study duration was longer in patient G (17 months) than that in patient C (2 months). Although it was not possible to identify the factors affecting metabolism due to the retrospective nature of the study, VRCZ metabolism may have been temporarily altered during the long study period. If dosage changes are made due to temporary changes in VRCZ concentration, outpatients may be disadvantaged for a long time due to the lack of efficacy or adverse reactions when the factors influencing the blood concentration of VRCZ are removed. As it is capable of sensing individual metabolic enzyme activities, including CYP3A4, the developed method could be useful to clarify individual differences in metabolic activity or adherence.

In conclusion, we developed a method for the determination of VRCZ and VNO plasma concentrations by column-switching LC-MS/MS, which was shown to be rapid, simple, precise, and accurate. This method allows physicians to be rapidly informed of a patient's VRCZ concentration, and thus can determine the appropriate VRCZ dosage for outpatients immediately. Moreover, in cases where VRCZ has been administered to an outpatient for a long period, the plasma concentrations can be properly maintained in the therapeutic range, which is beneficial for the treatment of fungal infections and maintenance of quality of life. We believe that by monitoring the VRCZ and VNO plasma concentrations in individual patients, we may further our understanding of pharmacokinetic changes and adherence, providing useful information for VRCZ treatment.

Acknowledgments: We would like to thank Editage (www.editage.com) for English language editing.

The authors declare no conflict of interest.

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