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Measurement of voriconazole in serum and plasma

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Abstract

Objectives: Voriconazole is an antifungal agent structurally related to fluconazole. While regular drug monitoring is not indicated in most patients, it may help guide dosing in patients with reduced hepatic/renal function, on concurrent therapy with drugs that affect CYP2C9, with altered CYP2C9 genotypes, or during adverse drug reactions. Here we describe an HPLC method for determination of voriconazole.

Methods: Samples, calibrators and controls were extracted using a liquid/liquid extraction. Chromatographic separation achieved using gradient solvent delivery with detection at 254 nm with a run time of 10 min. Concentration was calculated by comparison of peak height ratio of the drug with that of internal standard (IS) against a standard curve.

Results: The assay is linear from 0.29 to 57 μmol/L (0.1–20 μg/mL) and shows good linearity ($y=0.98x+0.36$, $r^2=0.9978$). The assay has inter- and intra-day precisions of <10%. The stability of the drugs in specimens was tested for up to 7 days at room temperature, for 30 days frozen at −20 °C, and through 3 freeze–thaw cycles and was found to be stable under those conditions.

Conclusions: This describes a robust method for the determination of voriconazole in serum and plasma.

Keywords: Voriconazole; Therapeutic drug monitoring; Analysis

Introduction

Voriconazole (VFEND™) is a triazole antifungal derived from the structure of fluconazole by replacement of one triazole moiety by a fluoropyrimidine group and alpha methylation [1]. Voriconazole is designated chemically as (2*R*,3*S*)-2-(2,4-difluorophenyl)-3-(5-fluoro-4-pyrimidinyl)-1-(1*H*-1,2,4-triazol-1-yl)-2-butanol (Fig. 1) and a molecular weight of 349.31 [2]. Compared to fluconazole, voriconazole has an enhanced antifungal spectrum that includes filamentous fungi. Voriconazole was designed to enhance the potency and spectrum of activity of fluconazole [3] used against a broad spectrum of significant clinical isolates like *Aspergillus*, *Candida*, *Scedosporium* and *Fusarium* [4–7].

Like other azole antifungals, voriconazole exerts its effect by altering the fungal cell membrane. Voriconazole inhibits ergosterol synthesis by interacting with 14-alpha demethylase, a cytochrome *P*-450 (CYP) enzyme that is needed to convert lanosterol to ergosterol, an essential component of the membrane [2]. Other antifungal effects have been proposed [8] and include: inhibition of endogenous respiration, interaction with membrane phospholipids and inhibition of the transformation of yeasts to mycelial forms. Other mechanisms may involve inhibition of purine uptake and impairment of triglyceride and/or phospholipid biosynthesis [9].

Early clinical studies suggested that treatment with voriconazole carried some risk of toxicity [10,11]. Plasma voriconazole concentrations of >6 μg/mL have been associated with serious clinical events. The most frequent adverse effects in voriconazole clinical trials have been visual impairment or photopsia, elevated hepatic enzymes (Alanine aminotransferase (ALT), aspartate transaminase (AST) and alkaline phosphatase) and elevated bilirubin [12]. Hepatitis, hepatic failure and death

Abbreviations: CYP, cytochrome *P*-450; C_{max}, maximum plasma concentration; AUC, area under the curve; LOD, limit of detection; LOQ, limit of quantitation; SD, standard deviation; CV, coefficient of variation; RT, ambient (room) temperature; EDTA, potassium ethylenediaminetetraacetic acid; HPLC, high performance liquid chromatography; HPLC-UV, HPLC-ultraviolet detection; LC-MS, liquid chromatography mass spectrometry; LC-MS/MS, liquid chromatography-tandem mass spectrometry.

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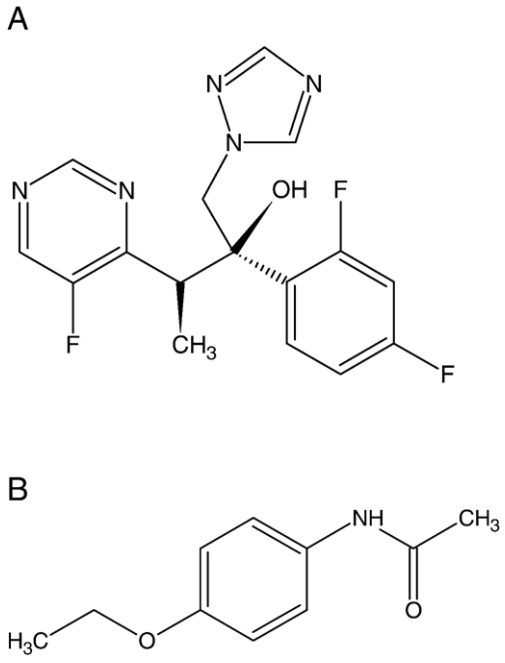

Fig. 1. Structure of voriconazole (A) and phenacetin (B).

have also been rarely documented, and monitoring of hepatic enzymes should be considered for all patients [2,11,13,14].

Voriconazole is rapidly and almost completely absorbed following oral administration. The oral bioavailability is up to 96%, with a maximum plasma concentration (C_{max}) occurring 1–2 h after dosing [2,11,15,16]. The apparent volume of distribution is 4.6 L/kg indicating widespread distribution in the body. Oral steady state plasma concentrations have ranged from 2.1 to 4.8 mg/L (peak) and 1.4 to 1.8 mg/L (trough) [2]. Voriconazole is extensively metabolized by the liver, and patients with pre-existing hepatic disease have been shown to have altered pharmacokinetics and displays nonlinear pharmacokinetics due to saturation of its metabolism. While regular blood level monitoring is not indicated in most patients, the test is useful to guide dosing in patients with reduced hepatic function, undergoing renal dialysis, or while on concurrent therapy with drugs that affect its metabolism, or when adverse drug reactions occur. Additionally, because the half-life is dose dependent, it is not useful in predicting the accumulation or elimination of voriconazole. A test used for monitoring voriconazole therapy is therefore necessary. Here we describe a high performance liquid chromatography (HPLC) assay for measurement of voriconazole in serum and plasma.

Materials and methods

Voriconazole ($C_{16}H_{14}F_3N_5O$; CAS# 137234-62-9) standard was obtained from Toronto Research Chemicals North York, ON, Canada, and phenacetin ($C_{10}H_{13}NO_2$; CAS# 62-44-2) was obtained from Sigma, St. Louis, MO. All other solvents and chemicals were of the best available commercial grade.

The assay for voriconazole in biological specimens was developed in our laboratory; 500 μL of standards, controls, and samples, 100 μL of internal standard (10 μg/mL phenacetin in methanol, IS) and 0.5 mL 0.5 N sodium hydroxide (NaOH) were added and extracted into 7.0 mL of ether. The extract was dried under nitrogen at room temperature and reconstituted in 200 μL of reconstitution solution which is made up of 50% of mobile phase A and 50% of mobile phase B (v/v). Using a Shimadzu CLASS-VP LC (Shimadzu Scientific Instruments, Columbia, MD, U.S.A.), 60 μL of reconstituted sample was injected onto a Supelcosil LC-18-DB column (25 cm × 4.6 mm, 5 um, Supelco # 58355-U, Sigma-Aldrich, Bellefonte, PA) held at 50 °C. Chromatographic separation was achieved using gradient solvent delivery using a mobile phase A made up of 10% acetonitrile (ACN) and 90% of 0.01 mol/L potassium phosphate pH 3.0 (v/v) and mobile phase B which is 100% ACN with a flow rate of 1.5 mL/min. The mobile phase goes from initial conditions of 20% B to 35% B at 4.0 min, 40% B at 4.5 min, 50% B at 6.1 min, 60% B at 7.3 min and 70% B at 8.0 and equilibrates to 20%B. The total run time was 10 min. Detection was achieved by monitoring the absorbance at 254 nm. Voriconazole eluted at 8.0 min and IS at 6.1 min (Fig. 2). The concentrations were calculated by comparison of peak height ratio of the drug with that of IS against a 4-point standard curve. Samples with concentrations exceeding the linearity were diluted with drug free matrix.

Results

Limit of detection

Limit of detection (LOD) is defined as the concentration at which the analyte can be distinguished from background. This was determined by measuring the peak height that is greater than or equal to the average of the blanks +3 standard deviation (SD) of the blanks [17]. Five replicates of the blank and incrementally increasing amounts of voriconazole were used in the determination. The LOD of voriconazole for serum and plasma in this assay was found to be 0.09 μmol/L (0.03 μg/mL).

Linearity and limit of quantitation (LOQ)

Samples ranging from 0.00 to 573 μmol/L (0.0–200 μg/mL) were assayed on 5 separate occasions to determine the linearity and LOQ of the assay. The concentration range resulting in the best fit line ($y = 0.98x + 0.36$, $r^2 = 0.9978$) was determined to be from 0.29 to 57 μmol/L (0.1–20 μg/mL). The graph of the mean and SD of those runs are shown in Fig. 3. The (LOQ) was defined in our laboratory as the lowest concentration where the coefficient of variation (CV) is less than or equal to <10%. The LOQ of voriconazole for serum and plasma in this assay was determined to be 0.29 μmol/L (0.10 μg/mL). No carryover was seen in a blank sample following the 573 μmol/L (200 μg/mL) standard.

Precision

Intra-assay (within run) precision was assessed by extracting 20 replicates of three different concentrations; the mean standard deviation (SD) and percent CV are shown in Table 1. The

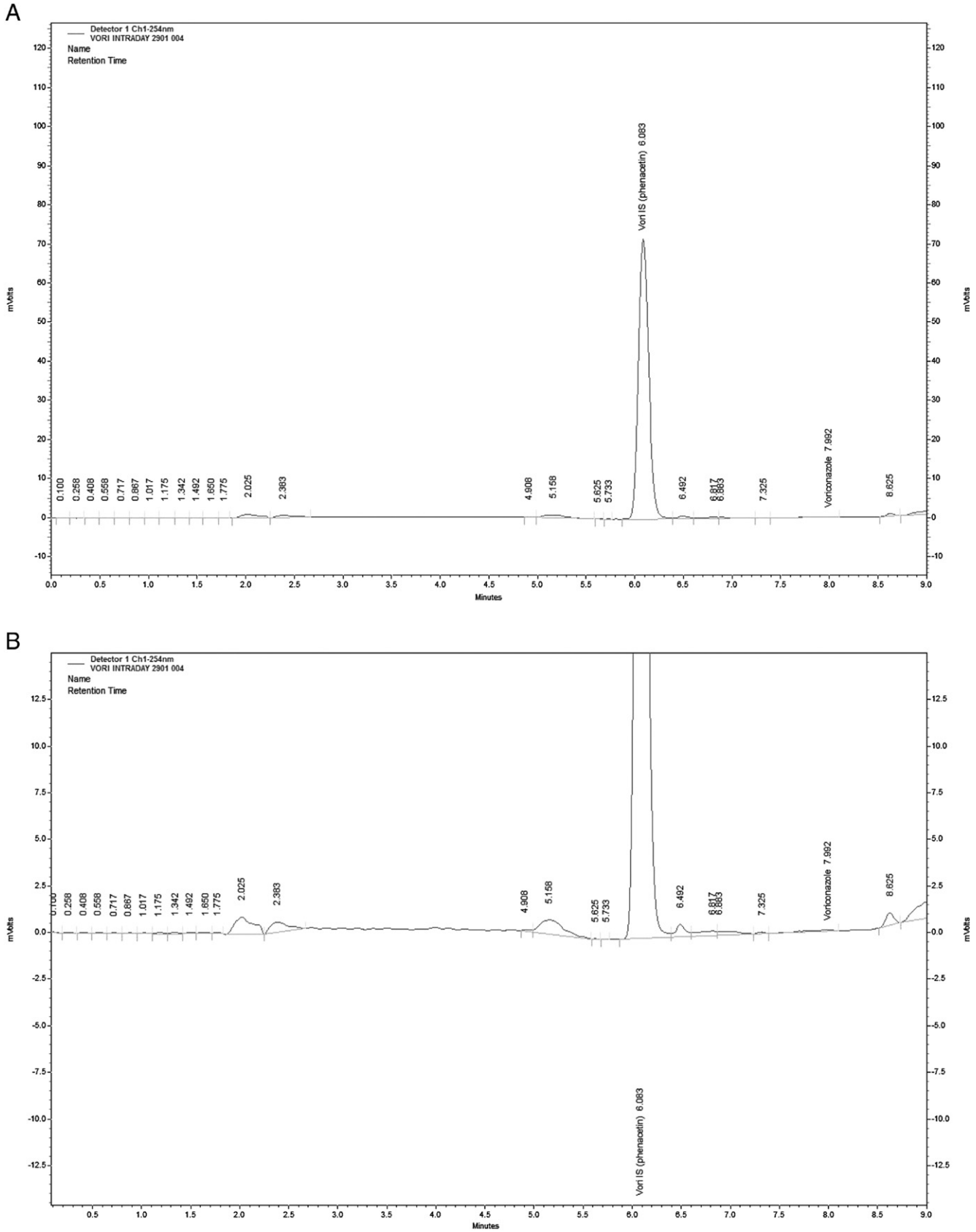

Fig. 2. Chromatograms of phenacetin (IS), (A) sample blank, (B) sample blank expanded baseline, (C) 0.29 μmol/L (0.10 μg/mL, LOQ) voriconazole, (D) 0.29 μmol/L (0.10 μg/mL) expanded baseline and (E) 29 μmol/L (10 μg/mL) voriconazole.

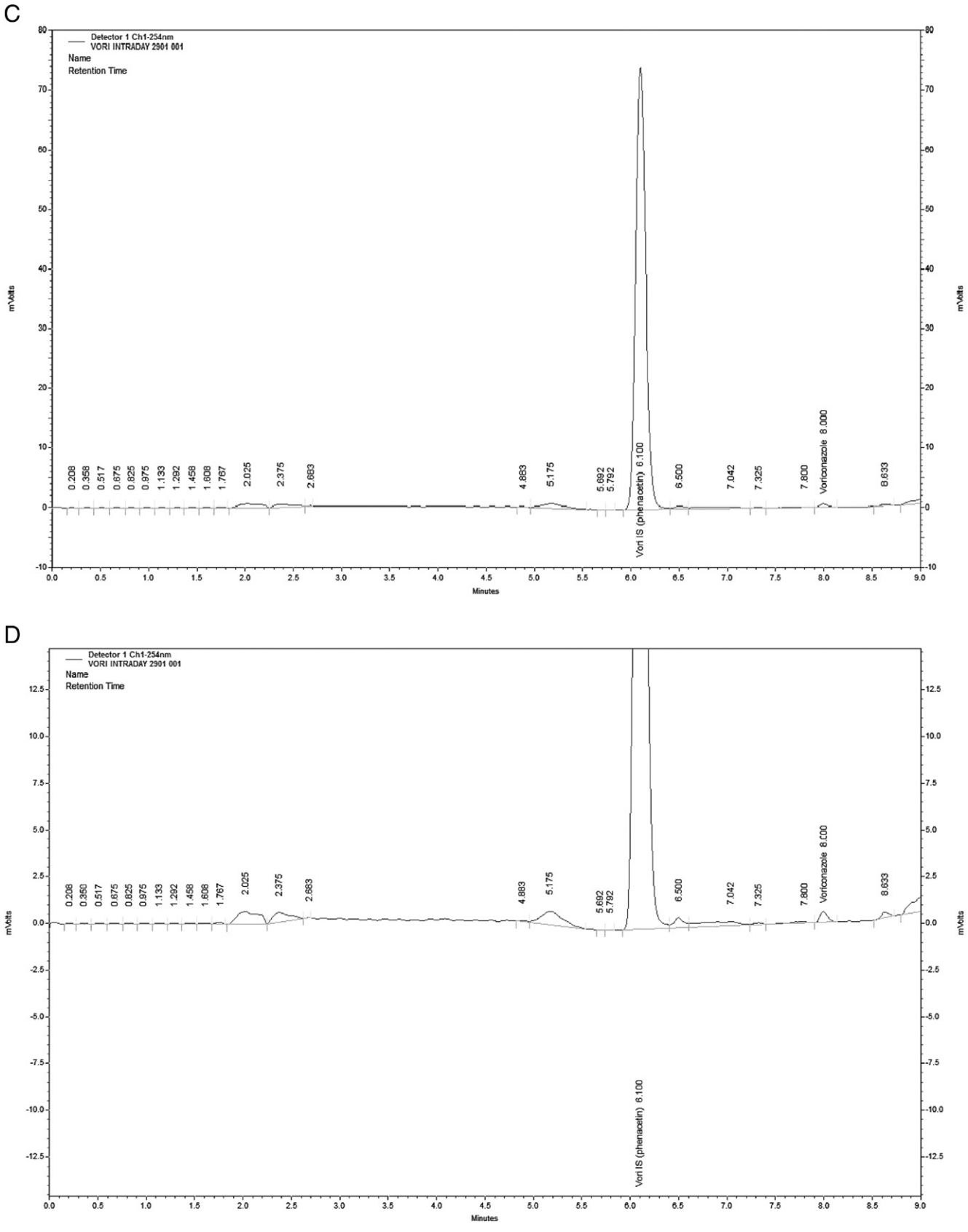

Fig. 2 (*continued*).

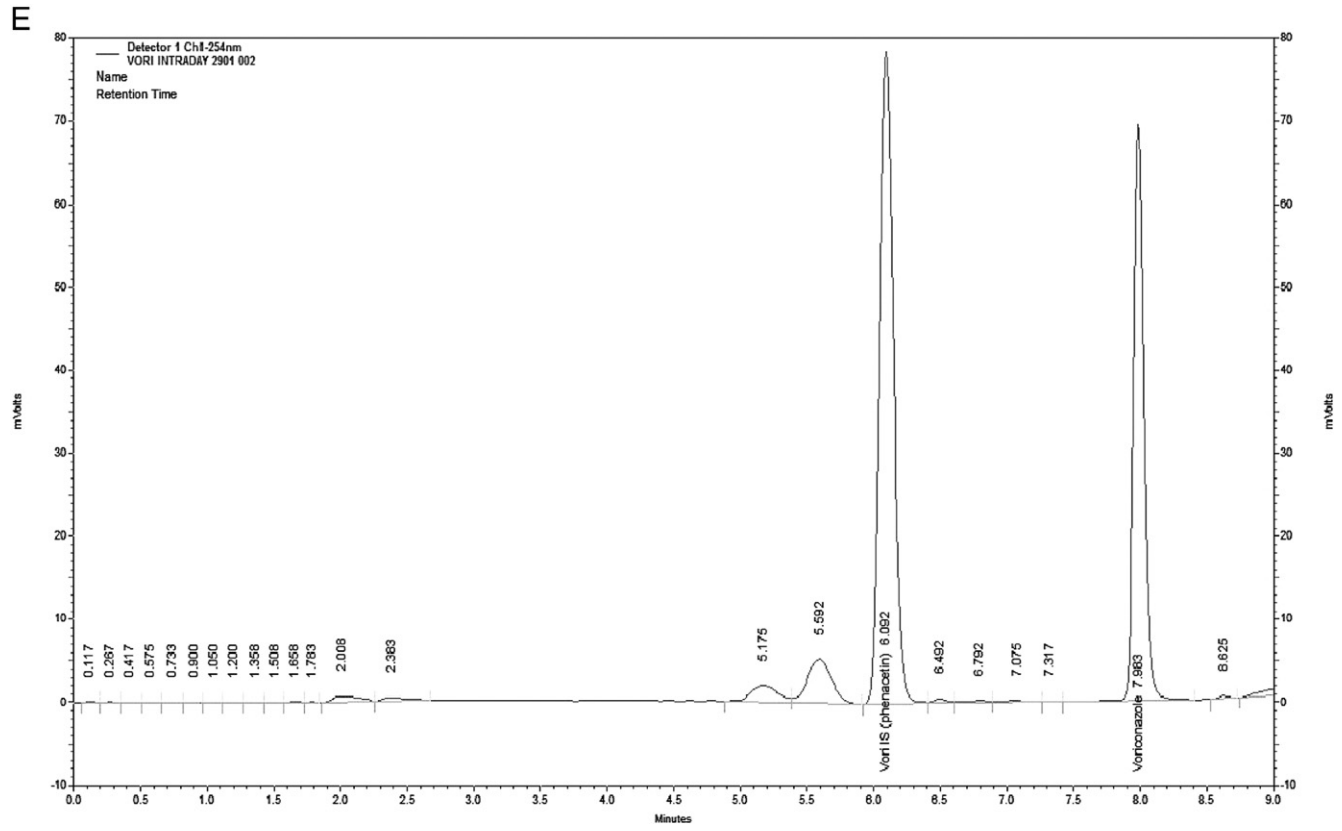

Fig. 2 (*continued*).

precision was <10% at all the concentrations tested. The inter-assay (between run) precision was assessed by running three different concentrations in five separate runs. The mean, SD and % CV are shown in Table 2. The inter-assay precision was <10% at all the concentrations tested.

Stability

Samples spanning the linear range were evaluated for analyte stability at ambient (room) temperature (RT), frozen at −20 °C and after freeze–thaw cycles. The mean and SD of the difference of the samples are shown in Table 3. Individual samples showed less than 20% deviation from the initial concentration indicating stability at RT for up to 7 days and samples were able to undergo three freeze–thaw cycles and were stable at −20 °C for 30 days. Stability of voriconazole beyond this was not evaluated.

Sample extract stability was evaluated by assaying samples run on day 0 and re-injecting the samples the following day. The concentration was determined using the previous day's calibration curve. The samples showed less than a 10% difference from the day 0 values and gave a correlation equation as follows: $y=0.95x+0.098$, $r^2=0.9993$.

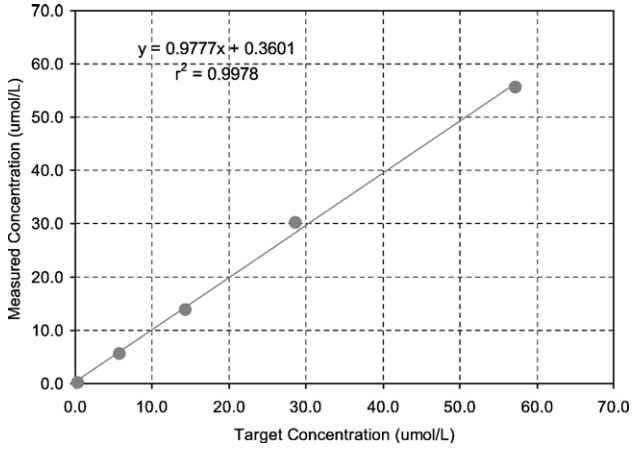

Fig. 3. Average±SD measured concentration of voriconazole vs. target concentration.

Anticoagulant suitability

The suitability of common anticoagulants and collection tubes was assessed. Bovine serum was spiked at three different concentrations of voriconazole and pipetted into the evacuated blood collection tubes containing the following additives: no additive (red top), lithium heparin (green top), sodium fluoride/

Table 1
Intra-assay (within run) precision

Mean (μmol/L)	7.4	18.4	43.7
SD (μmol/L)	0.5	1.6	1.9
% CV	6.8	8.9	4.4
N	20	20	20

Table 2
Inter-day (between run) precision

Mean (uμmol/L)	5.7	14.3	55
SD (μmol/L)	0.4	1.0	7.2
% CV	7.7	7.0	2.5
N	5	5	5

potassium oxalate (grey top) and potassium ethylenediaminetetraacetic acid (EDTA) (lavender top). Three milliliters of spiked sample were pipetted into one of each of the tube types. Tubes were recapped, mixed well and allowed to stand for at least 30 min at room temperature. The contents of each tube were analyzed in triplicate and the results are shown in Table 4. None of the anticoagulants tested showed an analytical interference as defined by a greater that 10% variation from the non-anticoagulant sample. Any of the anticoagulants tested would be acceptable for collection of samples from patients.

Interference

No interferences were found after twenty of the most commonly prescribed drugs (http://www.rxlist.com/top200.htm) and other anti-fungal drugs (Table 5) at 10 μg/mL were spiked into serum matrix and extracted. Other patient samples randomly extracted from other assays from that for other assays in the laboratory showed no interferences with either voriconazole or phenacetin.

Discussion

Voriconazole is rapidly and almost completely absorbed following oral administration. The oral bioavailability is up to 96%, with a maximum plasma concentration (C_{max}) occurring 1–2 h after dosing [2,11,15,16]. The apparent volume of distribution is 4.6 L/kg indicating widespread distribution in the body. Oral steady state plasma concentrations have ranged from 2.1 to 4.8 mg/L (peak) and 1.4 to 1.8 mg/L (trough) [2].

Voriconazole is extensively metabolized by human hepatic CYP enzymes, CYP2C19, CYP3A4 and CYP2C9 [2,15,18]. The affinity of voriconazole appears to be highest for 2C19, followed by 2C9, and is appreciably lower for 3A4 [19]. Enzyme CYP2C19 exhibits genetic polymorphism resulting in an approximately four-fold higher voriconazole exposure in poor metabolizers vs. extensive metabolizers [2]. Inducers or inhibitors of the CYPs may decrease or increase voriconazole plasma concentrations, respectively. Additionally, voriconazole is a known inhibitor of CYP2C19, CYP2C9 and CYP3A4 [2].

Table 4
Mean and SD of the percent difference of different anticoagulants from no additive tube

	Mean (%)	SD (%)	*N*
Green top	6.0	12.1	8
Grey top	3.7	9.3	7
Lavender tube	1.7	4.7	9

Apparently, voriconazole inhibits CYP2C9 and 3A4 to a greater extent than 2C19 [19]. The *N*-oxide metabolite ((a*R*,b*S*)-a-(2,4-Difluorophenyl)-5-fluoro-b-methyl-a-(1*H*-1,2,4-triazol-1-ylmethyl)-1-oxide-4-pyrimidineethanol) has minimal antifungal activity, it does not contribute to the overall efficacy of voriconazole [2,15]. However, the *N*-oxide metabolite also inhibits the metabolic activity of CYP2C9, CYP3A4 and, to a lesser extent, CYP2C19 [19]. Patients with pre-existing hepatic disease have been shown to have altered pharmacokinetics. Dosage adjustments are recommended for mild or moderate hepatic impairment; however, voriconazole use should be avoided in severe hepatic impairment [2].

Voriconazole displays nonlinear pharmacokinetics due to saturation of its metabolism. Increasing the IV dose from 3 mg/kg to 4 mg/kg twice daily and the oral dose from 200 mg to 300 mg twice daily results in roughly a 2.5-fold increase in the AUC [2,3]. As a result of nonlinear pharmacokinetics, the terminal half life ($T_{1/2}$) depends on the dose and is approximately 6 h at 3 mg/kg IV or 200 mg oral [2,3,15]. Drugs that display nonlinear pharmacokinetics often require TDM.

While regular blood level monitoring is not indicated in most patients, the test is useful to guide dosing in patients with reduced hepatic function, undergoing renal dialysis or while on concurrent therapy with drugs that affect CYP2C9 or with altered CYP2C9 genotypes or when adverse drug reactions occur. Additionally, because the half-life is dose dependent, it is not useful in predicting the accumulation or elimination of voriconazole.

The optimal voriconazole concentrations for efficacy and minimization of adverse events have not been fully determined. The substantial interpatient variability in peak and time to peak concentration, as well as $T_{1/2}$, make simple algorithms for therapeutic monitoring of voriconazole (as with the aminoglycosides) difficult. Patients with undetectable concentrations of drug (usually because of non-compliance, drug interactions or in young children) are unlikely to respond to therapy [14].

Early clinical studies suggested that treatment with voriconazole carried some risk of toxicity [10,11]. Plasma voriconazole concentration of >6 μg/mL has been associated with

Table 3
Mean and SD of the percent decrease measured from day 0

	Day 1			Day 3			Day 7			Day 30		
	Mean	SD	*N*	Mean	SD	*N*	Mean	SD	*N*	Mean	SD	*N*
RT light	5.8%	2.6%	10	2.1%	7.8%	10	−5.2%	5.1%	8			
RT dark				0.9%	6.8%	10	−4.8%	6.1%	10			
−20 °C	9.0%	4.0%	10							11.3%	8.7%	5
Three freeze–thaw cycles				4.7%	3.1%	10						

Table 5
Drugs evaluated At 10 μg/mL for analytical interferences

Drugs evaluated for interferences at 10 μg/mL
Acetaminophen
Atorvastatin
Amoxicillin
Lisinopril
Hydrochlorothiazide
Furosemide
Alprazolam
Amlodipine
Metformin
Sertraline
Ibuprofen
Cephalexin
Zolpidem
Prednisone
Hydrocodone
Esomeprazole
Lorazepam
Propoxyphene
Warfarin
Flucanozole
Itraconazole

serious clinical events and dose reduction is probably appropriate. The most frequent adverse effect in voriconazole clinical trials has been visual impairment or photopsia, elevated hepatic enzymes (Alanine aminotransferase (ALT), aspartate transaminase (AST) and alkaline phosphatase) and elevated bilirubin [12]. Hepatitis, hepatic failure and death have also been rarely documented and monitoring of hepatic enzymes should be considered for all patients [2,11,13,14]. Voriconazole should be used extremely cautiously in patients with liver disease and has CYP2C19 genetic polymorphisms resulting in a poor metabolizer phenotype [2]. Drug interactions are numerous for voriconazole and will pose an additional therapeutic challenge for the clinician [2,11]. A suggested plasma therapeutic concentration below 0.5 μg/mL may not be therapeutic while those above 5.0 μg/mL may be associated with increased adverse drug reactions [1,14,20,21].

Previously described assays for voriconazole included bioassays, HPLC with ultraviolet detection (HPLC-UV), liquid chromatography mass spectrometry (LC-MS) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) [1,22–24]. Discrepancies have been previously reported with direct comparison of HPLC and bioassay results for itraconazole, primarily because of bioactive metabolites [22]. This is not the case for voriconazole since the drug has no active metabolites. Therefore, comparison between the assays will not be affected by this mechanism. HPLC is therefore a useful alternative method. The bioassay method is inexpensive in terms of equipment and consumable costs; however, it lacks required sensitivity. The bioassay has several other deficiencies including poor turnaround time compared with the chromatographic methods because of the long 18-hour incubation time, and combination antimicrobial therapy may also be a problem with the bioassay but not with chromatographic techniques [1,23,24]. Finally, the large dynamic range required for voriconazole measurement cannot be achieved with any accuracy using bioassay. The clinical benefit of the more rapid and specific HPLC procedure is therefore clear [24].

The first published HPLC procedure [23] was based on size exclusion chromatography coupled with on-line reverse phase chromatography with column switching and was therefore complex. Other HPLC assays utilizing an acetonitrile precipitation as the sample preparation followed by reverse phase chromatography have been described [1,25]. However, this type of sample preparation has the possibility of drug entrapment in the precipitant, thereby decreasing drug recovery. Another method used a direct injection micro-HPLC method and small sample volume [26]. Additionally a method of Zhou et al. used direct injection of untreated aqueous humor [24]. None of these methods used an internal standard.

There are HPLC methods previously described for the determination of voriconazole that utilize an internal standard. One method utilizes solid phase extraction for sample preparation. However, the internal standard used in this assay was a gift to the researchers, and not a commercially available compound [27]. The ideal internal standard would behave with the similar extraction characteristics as the compound of interest. We believe that phenacetin is a good internal standard because the extractions recovery for voriconazole and phenacetin were comparable (89.6% and 80.7 respectively). Additionally, and from a practical perspective, phenacetin is unavailable as a prescription drug in the USA, and it has the distinct advantage of being commercially available. Another HPLC UV method for voriconazole utilized ketoconazole as an internal standard [28]. In this method, the sample volume was 1 mL, the limit of quantitation was 0.29 μmol/L (0.1 μg/mL) and the highest calibrator was 23 μmol/L (8.0 μg/mL) [28]. Although our method has a comparable limit of quantitation of 0.29 μmol/L (0.10 μg/mL) our method has a higher reportable value of 57 μmol/L (20 μg/mL) and uses much less sample volume (100 μL).

There are also several LC-MS or LC-MS/MS methods described [24,29,30]. While these methods are highly sensitive compared with the other techniques [29] and generally thought to be superior to HPLC-UV and bioassays, they are expensive and the equipment is not available to all laboratories [28].

We describe here a robust assay for the detection and quantization of voriconazole in serum and plasma with no identified interferences. This method shows good linearity ($y=0.98x+0.36$, $r^2=0.9978$) from 0.29 to 57 μmol/L (0.1–20 μg/mL). The assay has inter- and intra-day precisions of <10%. The stability of the drug in specimens was tested for up to 7 days at room temperature, for 30 days frozen at −20 °C, and through three freeze–thaw cycles and was found to be stable under those conditions. The extracted samples are stable for 24 h at 4 °C. Both serum and plasma samples are suitable for analysis. In conclusion, we have developed and validated a sensitive assay for voriconazole with the advantage of an internal standard, rapid analysis and small sample size that should prove useful for therapeutic drug management and pharmacokinetic studies.

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