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ORIGINAL RESEARCH

Direct injection HPLC micro method for the determination of voriconazole in plasma using an internal surface reversed-phase column

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ABSTRACT: A direct plasma injection liquid chromatographic method has been developed for the determination of a new triazole antifungal agent, voriconazole, using an internal surface reversed phase column. Therapeutic drug monitoring of voriconazole is relevant for patient management, especially in the case of drug–drug interaction. The method is easy to perform and requires 10 μL of a plasma sample. The chromatographic run time is less than 9 min using a mobile phase of 17:83 v/v acetonitrile–potassium dihydrogen phosphate buffer, 100 mM, pH 6.0 and UV detection at 255 nm. The flow rate was 1 mL/min. A linear response was observed over the concentration range 0.5–10 μg/mL (r^2 = 0.977). A good accuracy (bias ≤ 7.5%) was achieved for all quality controls, with intra-day and inter-day variation coefficients inferior to 6.7%. The lower limit of quantitation was 0.2 μg/mL, without interference of endogenous components. The stability of voriconazole in plasma stored at different temperatures was checked. Finally, the possibility of direct injection of plasma samples into the column permits a reduction in reagent consumption and in analytical steps, and hence in analytical error. Copyright © 2004 John Wiley & Sons, Ltd.

KEYWORDS: internal surface reversed-phase column; HPLC; micro method; voriconazole

INTRODUCTION

Voriconazole, (2*R*,3*S*)-2-(2,4-difluorophenyl)-3-(5-fluoro-4-pyrimidinyl)-1-(1H-1,2,4-triazol-1-yl)-2-butan-2ol (chemical structure in Fig. 1), is a new antifungal agent structurally derived from fluconazole. Compared with fluconazole, voriconazole shows a broader spectrum of activity against such common fungal pathogens as *Candida* and *Aspergillus* (Jeu *et al.*, 2003). Despite its potential clinical advantages, voriconazole also has several disadvantages. It appears not to be better tolerated than other antifungal agents. In particular, transient visual abnormalities have been seen up to 30% of patients receiving treatment with voriconazole (Lazarus *et al.*, 2002). Moreover, voriconazole is both a substrate for and an inhibitor of the CYP isoenzymes CYP2C19, CYP3A4 and CYP2C9, and a number of drugs should not be used concomitantly with this antifungal agent or require dose adjustment or additional monitoring when so used (Jeu *et al.*, 2003). This extensively metabolized drug appears to exhibit nonlinear pharmacokinetics, possibly due to saturation of metabolism (Purkins *et al.*, 2002). The primary route of metabolism is N-oxidation

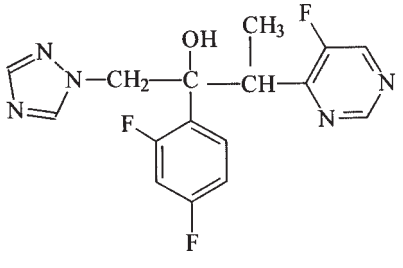

Figure 1. Chemical structure of voriconazole.

of the fluoropyrimidine ring to form a metabolite, which possesses minimal antifungal activity (Jeu *et al.*, 2003). In such a drug that displays nonlinear pharmacokinetics, small changes in the metabolic rate can have a disproportionate effect on plasma concentrations. Consequently, therapeutic monitoring of voriconazole levels may be necessary.

For this reason, there has been interest in the development of an adequate analytical method for detection of voriconazole in biological fluids. Few high-performance liquid chromatographic (HPLC) methods with UV detection have been reported for the quantitation of this drug in plasma (Stopher and Gage, 1997; Gage and Stopher, 1998; Perea *et al.*, 2000; Pennick *et al.*, 2003). The first published procedure (Stopher and Gage, 1997) was based on size exclusion chromatography coupled on-line with a reversed-phase HPLC system with column switching, and was therefore complex. Two assays utilizing a protein precipitation with

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Abbreviations used: GFF, glycine–phenylalanine–phenylalanine; IRSP, internal surface reversed-phase.

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acetonitrile as the sample preparation prior to reversed-phase HPLC were described (Gage and Stopher, 1998; Perea *et al.*, 2000). By these 'non-extraction' methods, there was always the possibility of drug entrapment in the precipitant. It was also possible that endogenous plasma components, solubilized in the organic solvent, interfere with analyte peaks. Pennick *et al.* (2003) described a solid-phase extraction method using 500 µL of plasma samples with a low limit of quantitation of 0.2 µg/mL.

In the current paper, we describe a direct plasma injection method, which requires no sample clean-up steps and involves only 10 µL of plasma sample. Its greater speed and simplicity appear to be of particular interest for therapeutic drug monitoring of voriconazole.

MATERIALS AND METHODS

Reagents and standards. All reagents used for the assay were of HPLC or analytical grade. Water was deionized and glass-distilled prior to use and human heparinized plasma of healthy volunteers was purchased from Aquitaine Establishment of Blood Transfusion (E.T.S.A, Bordeaux, France). Voriconazole was kindly supplied by Pfizer (Sandwich Kent, UK). A stock standard solution of voriconazole was prepared at concentrations of 1 mg/mL in methanol. This solution was stable at –20°C for at least 3 months.

Calibration standards and plasma controls. Daily, a standard solution of voriconazole was prepared from the stock solution by suitable dilution with distilled water and was used for the preparation of plasma standards. These calibration standards were made into drug-free human plasma to yield concentrations of 0.5, 1, 2, 5 and 10 µg/mL of voriconazole. In the same manner, plasma quality controls (QC) spiked with 0.8, 2.5 and 7 µg/mL of voriconazole were prepared to perform accuracy and precision of the method.

Apparatus. The chromatographic apparatus (ThermoQuest™, San Jose, CA, USA) was equipped with a constant flow pump M 100, a model 150 ultraviolet detector and a Datajet® integrator. Separations were carried out using a 15 cm × 4.6 mm (inner diameter) column packed with 5 µm particle size internal surface reversed-phase (IRSP) silica particles, bonded with a glycine–phenylalanine–phenylalanine (GFF) peptide (Pinkerton ISRP GFF II® from Regis; Morton Grove, IL, USA). The ISRP particle had two surfaces: a hydrophilic outer surface and a hydrophobic inner surface. When plasma is injected onto the IRSP column, plasma proteins cannot reach the inner surface and are neither retained nor denatured by this surface. Small molecules, in contrast, are able to penetrate through to the inner surface where they are retained by the hydrophilic support in a manner that is common to conventional chromatography.

Analytical procedure and chromatographic conditions. An aliquot of each sample (calibration standards, controls and patient specimens; 100 µL) was transferred to 400 µL polypropylene tubes. The samples were centrifuged at 10,300 ***g*** for 10 min; then a 10 µL aliquot was injected into the chromatograph. The mobile phase consisted of a mixture of acetonitrile–potassium dihydrogenphosphate buffer 100 mM pH 6.0 (17:83; v/v). This mobile phase was filtered through a 0.45 µm filter and degassed prior to use. The flow rate was maintained at 1 mL/min. Voriconazole was detected at 255 nm within 9 min.

Validation of the method. Standard curves were constructed by plotting the peak height values against the concentration of the standards. Peak height values were used instead of peak area values to improve precision and accuracy of low concentrations (Perea *et al.*, 2000)

The accuracy and intra-day and inter-day precision of the method were evaluated by assaying replicates of the three QC samples prepared as described above. The intra-day precision was defined by calculating the coefficients of variation (CV) for QC samples with six replicates. The inter-day precision was determined from QC samples obtained on three different days. Accuracy was expressed as the percentage of bias [(found concentration – spiked concentration)/spiked concentration] × 100 (%).

Extraction recoveries from plasma were determined by comparison of HPLC responses from extracted samples, containing 0.5, 1, 2, 5 and 10 µg/mL concentrations of voriconazole with those obtained by directly injected solutions of the analyte made in the same injection solvents.

RESULTS AND DISCUSSION

The objective of this work was the development of a specific and rapid micro method which required no sample pre-treatment step and which possessed sensitivity and reproducibility for use in voriconazole monitoring.

Under the described RP-HPLC procedure, voriconazole was adequately resolved from endogenous plasma compounds and its retention time was approximately 7 min. Typical chromatograms for spiked plasma samples with voriconazole are shown in Fig. 2.

Precision, accuracy and linearity

The results obtained for precision and accuracy are listed in Table 1 and expressed as CV (%) and percentage bias, respectively. These results indicate that the method is precise: intra-day precision less than 6.7% and inter-day precision less than 5.9%. This method is accurate (bias ranged from –1.4 to –7.5%).

From eight calibration curves, constructed with calibration points ranging from 0.5 to 10 µg/mL, a high correlation coefficient (r^2 = 0.977) was found (p < 0.0001). This linear correlation had a slope of 18.05 ± 0.43 and an intercept of –1.9 ± 1.8.

Limit of quantitation (LOQ)

The LOQ was defined as the lowest voriconazole concentration that could be determined with a precision

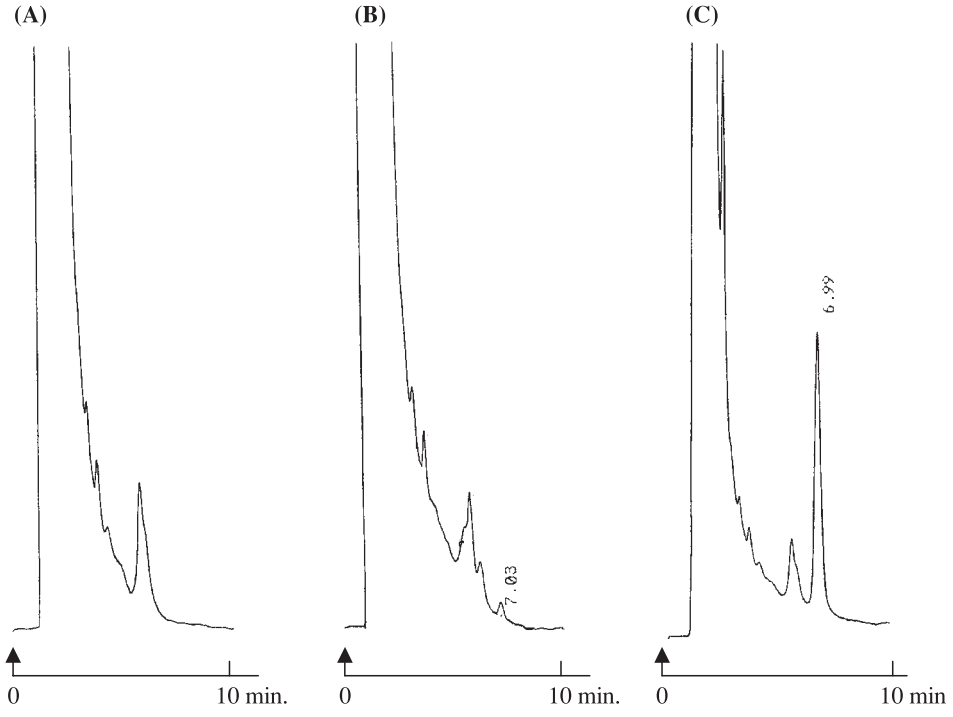

Figure 2. Chromatograms of (A) blank human plasma, (B) spiked plasma containing 0.2 μg/mL of voriconazole (LOQ) and (C) spiked plasma with 7 μg/mL. Voriconazole: *tr* = 7 min.

Table 1. Precision and accuracy of results for plasma spiked with voriconazole

	Concentrations added (ng/mL)	Concentrations found ± SD (ng/mL)	CV (%)	Bias (%)
Intra-day (n = 6)	0.8	0.75 ± 0.03	4.0	−6.2
	2.5	2.4 ± 0.16	6.6	−4.0
	7.0	7.2 ± 0.37	5.1	−4.0
Inter-day (n = 18)	0.8	0.74 ± 0.03	4.0	−7.5
	2.5	2.4 ± 0.14	5.8	−4.0
	7.0	7.1 ± 0.34	4.8	−1.4

less than 20% (CV) and with accuracy between 80 and 120%, as determined in the inter-day analytical runs. The LOQ was found to be 0.2 μg/mL. This concentration is about 2.5-fold lower than the minimum trough concentration observed after a low dose of 200 mg *per os* (Purkins *et al.*, 2002).

Lower detection limit (LOD)

The LOD of this method was 0.08 μg/mL for a 10 μL plasma sample at a signal-to-noise level of 3. However, a large injection volume might be used for detection of lower plasma concentrations, but this might result in a more frequent change of guard column. In our conditions, approximately 150 total plasma injections were made before the system pressure reached 3000 psi.

Extraction efficiency

The recovery was calculated by comparing the peak height after injection of drugs dissolved in mobile phase with the peak height after extraction of the same amount of voriconazole from plasma (0.5, 1, 2, 5 and 10 μg/mL). Over the concentrations studied, the mean recovery (mean ± SD, n = 10) was 99.7 ± 5.9%. The nearly complete recovery eliminates the need of using an internal standard.

Stability

To determine the influence of temperature on the stability of the drug, three QC samples spiked with voriconazole were stored under different conditions:

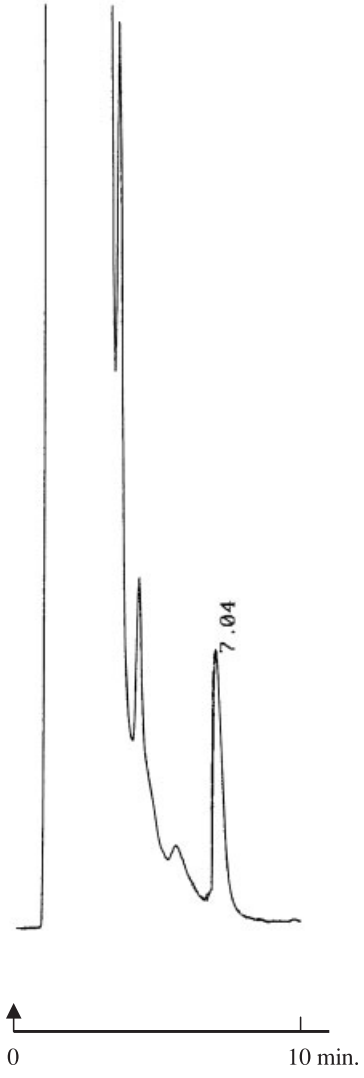

Figure 3. Chromatogram obtained from a patient who had received voriconazole (200 mg, *per os*). Voriconazole: *tr* = 7 min.

at +4°C for 48 h, at +20°C for 24 h in daylight, and in the dark.

The storage during 48 h at +4°C produced no significant decrease of voriconazole concentration (CV and percentage bias values less than 3.7%). Moreover, the storage at room temperature during 24 h in the daylight or in the dark indicated a good stability in the two cases with CV lower than 6.6% and percentage bias values less than 8.8%.

Specificity

The specificity was evaluated by analysing plasma samples from patients receiving itraconazole, fluconazole, ketoconazole or amphotericin B. This study did not test all of the other possible multiple-drug cocktails that patients may receive; however, no interference was observed by the aforementioned antifungal agents.

Application

This method was applied to the determination of concentration in plasma of one patient undergoing chronic treatment with voriconazole. This patient (female, 49 years old) took 200 mg of voriconazole twice daily. A blood sample was collected at 8.00 h in the morning, just before the first daily drug administration. The chromatogram corresponding to this plasma sample is reported in Fig. 3. No interference from endogenous compounds or co-administered drugs was found. The plasma level of voriconazole found in this sample was 2.4 μg/mL. This value was in the therapeutic range of voriconazole, as reported in clinical studies. The minimum plasma concentrations (C_{trough}) ranged from 0.5 to 2.5 μg/mL for patients treated with voriconazole at 200 mg twice daily (Jeu *et al.*, 2003).

CONCLUSION

This direct injection HPLC method for the analysis of voriconazole in human plasma is easy to perform, uses small sample volumes and requires no sample pretreatment steps. This micro method fulfils the validation requirements for procedures used for determination of drugs in biological fluids. The inter- and intra-day accuracy and precision data were less than 10%. The LOQ reached (0.2 μg/mL) was below the concentration expected in patients undergoing therapy with voriconazole. Consequently, the proposed method is suitable for a reliable therapeutic drug monitoring of this antifungal agent, especially where the sample size is limited.

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