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Journal of Chromatography B, 852 (2007) 223–228
JOURNAL OF CHROMATOGRAPHY B
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Simultaneous quantification of voriconazole and posaconazole in human plasma by high-performance liquid chromatography with ultra-violet detection

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Received 3 August 2006; accepted 12 January 2007
Available online 1 February 2007

Abstract

A sensitive and selective high-performance liquid chromatographic (HPLC) method with ultra-violet detection has been developed and validated for the simultaneous determination of posaconazole and voriconazole, two systemic anti-fungal agents. An internal standard diazepam was added to 100 μL of human plasma followed by 3 mL of hexane–methylene chloride (70:30, v/v). The organic layer was evaporated to dryness and the residue was reconstituted with 100 μL of mobile phase before being injected in the chromatographic system. The compounds were separated on a C8 column using sodium potassium phosphate buffer (0.04 M, pH 6.0): acetonitrile:ultrapure water (45:52.5:2.5, v/v/v) as mobile phase. All compounds were detected at a wavelength of 255 nm. The assay was linear and validated over the range 0.2–10.0 mg/L for voriconazole and 0.05–10.0 mg/L for posaconazole. The biases were comprised between −3 and 5% for voriconazole and −2 and 8% for posaconazole. The intra- and inter-day precisions of the method were lower than 8% for the routine quality control (QC). The mean recovery was 98% for voriconazole and 108% for posaconazole. This method provides a useful tool for therapeutic drug monitoring.
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Keywords: Posaconazole; Voriconazole; Liquid chromatography; UV detection

1. Introduction

Fungal infections are life-threatening conditions, whose incidences are steadily rising with the increased frequency of various immunosuppressive situations (cancer chemotherapy, organ transplant, HIV infection). To improve therapeutic possibilities, the advent of several new triazole anti-fungal agents provides increased efficacy and safety. They were developed to expand the spectrum of activity of existing azoles by inhibiting the synthesis of ergosterol, the major sterol component in the fungal cell membrane [1].

Voriconazole and posaconazole are two of these new compounds; voriconazole became in France the recommended first line treatment for invasive aspergillosis [2]. Few clinical pharmacokinetic–pharmacodynamic studies are available for posaconazole and voriconazole but an *in vivo* study based on a murine model of disseminated candidiasis identified the ratio between the 24 h-area under the concentration-time curve divided by the minimum inhibitory concentrations of the drug (AUC/MIC) to be predictive of the treatment efficacy [3,4]. In humans, Smith et al. showed a relationship between disease (aspergillosis, blastomycosis infections ...) progression

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doi:10.1016/j.jchromb.2007.01.021

which was defined as an increase in size or number of lesions on follow-imaging and voriconazole serum concentrations with a favourable response in patients with concentrations above 2.05 μg/mL [5].

Furthermore, voriconazole is characterized by a saturable metabolism, a high interindividual variability of its pharmacokinetic parameters [2] and is involved in many pharmacokinetic interactions involving immunosuppressive agents, antiretroviral protease inhibitors and anti-tuberculosis drugs such as rifampin and rifabutin [7,8]. Due to the high interindividual variability expected, therapeutic drug monitoring should be suggested. Monitoring voriconazole levels can also be useful for selected patients, especially those with abnormal liver function [6].

Up to date drug interactions with posaconazole were not characterized but are nevertheless expected in immunossuppressed patients, because of its ability to inhibit CYP3A4 [9], the main metabolic pathway of calcineurin inhibitors [10]. Besides, if posaconazole target concentrations in patients are still to be determined, its interindividual pharmacokinetic variability [11] could support an individual dose adjustement.

Taken together, these aspects suggest the potential interest of performing a therapeutic drug monitoring (TDM) of these compounds in order to manage drug interactions, to balance interindividual pharmacokinetic variability and to ensure an effective exposure to the drug [12].

The quantification of voriconazole in plasma by high-performance liquid chromatoghraphy assays [13–16] and by liquid chromatography–tandem mass spectrometry methods [17–19] were reported. In addition, a method for the quantification of posaconazole by high-performance liquid chromatoghraphy in human serum was also described [20]. To our knowledge, no simultaneous assay for the measurement of posaconazole and voriconazole concentrations has been reported. Although both drugs are not supposed to be combined in the treatment of fungal infections, an analytical method as simple and rapid as possible for the simultaneous determination of voriconazole and posaconazole is very useful in a clinical laboratory as it saves both time and solvents.

2. Experimental

2.1. Chemicals

Voriconazole pure drug substance was kindly supplied by Pfizer (Paris, France) and posaconazole powder by Schering Plough (Hérouville-Saint-Clair, France). The internal standard (IS) diazepam was purchased from Sigma chemical company (St Louis, MO, USA). The structures of voriconazole, posaconazole and the IS diazepam are described in Fig. 1.

Chromanorm® methanol for HPLC was purchased from Prolabo (Fontenay-sous-Bois, France). LiChrosolv® acetonitrile gradient grade for liquid chromatography, *n*-hexane and methylene chloride were obtained from Merck (Darmstadt, Germany).

The potassium dihydrogen phosphate and sodium monohydrogen phosphate were purchased from Prolabo (Fontenay-sous-Bois, France). Ultrapure water was obtained from Milli-Q water purification system.

2.2. Chromatographic and detection conditions

The HPLC system consisted of a SpectraSYSTEM SCM1000® degassing system, a SpectraSYSTEM P1000XR® pump, a SpectraSYSTEM AS3000® autosampler, a SpectraSYSTEM UV6000LP® UV detector and a SpectraPHORESIS SN4000® interface (ThermoFinnigan, Les Ulis, France).

The chromatographic separation was accomplished on a C8 plus Satisfaction column (250 mm × 3 mm, 5 μm; Cluzeau, Sainte-Foy la Grande, France) protected by a guard column (15 mm × 3 mm) of the same phase and a A-103X filter (Cluzeau).

The mobile phase consisted of sodium potassium phosphate buffer (0.04 M, pH 6.0):acetonitrile:ultrapure water (45:52.5:2.5, v/v/v) and was filtered on a 0.45 μm hydrophilic

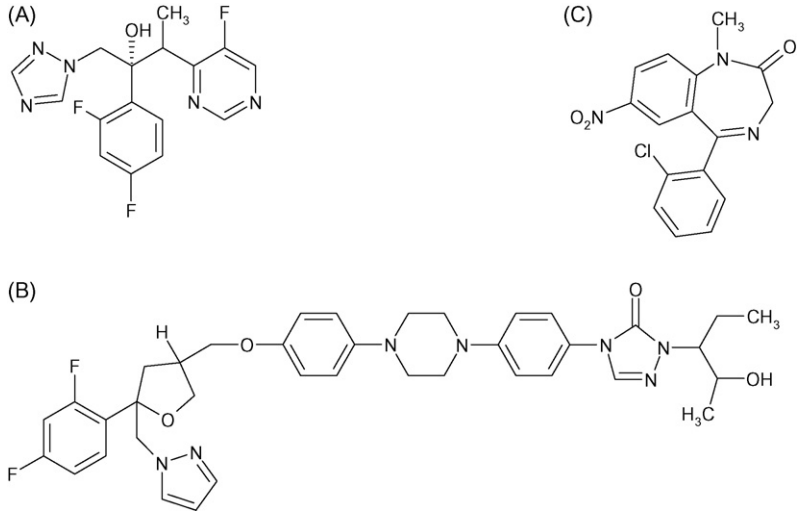

Fig. 1. Structure of voriconazole (A) posaconazole (B) and IS diazepam (C).

polypropylene filter. Chromatography was performed at room temperature using a flow rate of 0.5 mL/min isocratically.

2.3. Preparation of stock solutions, working solutions and quality control samples

Two stock solutions of each compound were prepared by dissolving voriconazole and posaconazole in methanol, both at a concentration of 1 mg/mL. One solution was used to spike the calibration standards and the other one to prepare the quality control (QC) samples. The stock solutions were stored at −20 °C.

Working solutions of combined voriconazole and posaconazole were prepared from stock solutions by diluting the appropriate aliquot in methanol: water (1:1, v/v).

A stock solution of IS diazepam was prepared in methanol at a concentration of 1 mg/mL, and was diluted in ultrapure water to obtain a working solution of 1 mg/L.

QC samples were prepared with voriconazole and posaconazole at concentrations level of 0.2, 0.4, 1.5 and 7.5 mg/L of voriconazole, and 0.05, 0.1, 2.0, and 7.5 mg/L of posaconazole by evaporating to dryness at 30 °C under a stream of nitrogen of appropriate amounts of the 1 mg/mL stock solution of voriconazole and posaconazole. The dry residue was then reconstituted with drug free human plasma. After 3 h of mixing, the QC samples were stored in polypropylene tubes at −30 °C. The last three concentration levels of the QC samples were used for routine analysis and in the validation of the method, whereas the 0.2 mg/L for voriconazole and 0.05 mg/L for posaconazole were used only for the validation of the lower limits of quantification (LOQ).

2.4. Sample preparation

The calibration standards were obtained from appropriate dilutions of the stock solution in methanol:water (1:1, v/v) in the same flasks.

One hundred microlitres of each dilution were then evaporated to dryness at 30 °C under a stream of nitrogen. The dry residue was reconstituted with 100 μl of human blank plasma in order to obtain the final simultaneous calibration standards. The calibration curves were obtained using seven calibration standard levels (from 0.2 to 10.0 mg/L for voriconazole and from 0.05 to 10.0 mg/L for posaconazole). One hundred microlitres of the IS working solution was added to plasma samples, QC and calibration standards. After 30 s of mixing, 3 mL of hexane–methylene chloride (70:30, v/v) were added for the extraction of voriconazole, posaconazole and diazepam from plasma. After an agitation of 20 min with a mechanical shaker, the tubes were centrifuged 5 min at $2200 \times g$. The supernatant was transferred in a glass tube and evaporated to dryness at 30 °C under a stream of nitrogen, the dry residue was reconstituted with 100 μL of the mobile phase. After mixing, the extracts were transferred into the autosampler vials and 50 μL of each sample was injected in the chromatographic system.

2.5. Validation procedures

2.5.1. Linearity

Complete calibration curves were analysed in triplicate on 3 separate days. QCs were assayed twice with each standard curve. A linear regression with a weighting factor of 1/(peak height ratio)^2 was used to plot the peak height ratio (each compound to IS) versus the corresponding analyte concentration in the range of 0.2–10.0 mg/L for voriconazole and 0.05–10.0 mg/L for posaconazole. Slope, *y*-intercept and correlation coefficient were calculated for each standard curve.

2.5.2. Accuracy and precision

The accuracy and the precision of the method were evaluated on the QC by multiple analysis. In order to study the intra day accuracy and precision, the QC samples were consecutively measured in one single day. This procedure was repeated on three different days to test the inter-day accuracy and precision. Accuracy was determined as the difference between back calculated concentration (Cobs) of voriconazole and posaconazole in spiked plasma with theoretical concentration (Ctheo) expressed in percent. Precision at each concentration level was expressed as the relative standard deviation (R.S.D.) of the calculated concentrations. The limit of quantification was set at the lowest calibration standard value (0.20 mg/L for voriconazole and 0.05 mg/L for posaconazole).

2.5.3. Recovery

Recovery of voriconazole and posaconazole was evaluated at concentration levels corresponding to the three routine QC values (0.4, 1.5 and 7.5 mg/L for voriconazole and 0.1, 2.0 and 7.5 mg/L for posaconazole) analysed in triplicate. The peak height ratio of extracted samples containing one of the anti-fungal compounds (voriconazole or posaconazole) including IS were compared to the ratio of samples containing the same amount of IS but not the anti-fungal compound which was added after the extraction step (on the dry residue). The same method was employed to study the recovery of the IS with the analytes used as reference compounds.

2.5.4. Selectivity and specificity

Selectivity was indicated by the absence of any endogenous interference at retention times of peaks of interest evaluated by chromatograms of five blank plasmas. The specificity has been evaluated with respect to all registered anti-infectious drugs commonly used by immunocompromised patients and was tested by comparing chromatograms with the chromatogram of plasma spiked with voriconazole, posaconazole and IS.

2.5.5. Stability

Freeze-thaw stability of both analytes was determined by assaying the three routine QC samples in triplicate over three freeze-thawing cycles. The stability of voriconazole and posaconazole in plasma was investigated by assaying each week the three concentration levels of routine QC samples stored at −30 °C for a 2-months period. Peak heights were compared

with the one obtained from QC samples freshly made and concomitantly analysed. The stock solution of voriconazole and posaconazole stored at −30 °C was compared monthly to a stock solution freshly made during a 3-months period. The stability of voriconazole, posaconazole and IS in the extract was assayed by two ways. First, the dry residues of the extracts obtained from the three levels of routine QC samples analysed in duplicate were stored at −30 °C for a 2-weeks period and were reconstituted with 100 μL of the mobile phase before injection in the chromatographic system. Second, reconstituted extracts of the three levels of the routine QC samples were left at room temperature in the autosampler for 3 days (i.e. approximately twice the time necessary for a routine analysis). For each experiment, peak heights were compared with the one obtained from QC samples freshly analysed.

3. Results

3.1. Validation procedures

3.1.1. Linearity

The nine standard curves were linear over a concentration range of 0.2–10.0 mg/L for voriconazole and of 0.05–10.0 mg/L for posaconazole. The average correlation coefficient was higher than 0.998 in both cases, with a mean slope (±S.D.) of 0.526 ± 0.029 and a mean *y*-intercept (±S.D.) of −0.006 ± 0.011 for voriconazole and a mean slope (±S.D.) of 0.312 ± 0.017 and a mean *y*-intercept (±S.D.) of −0.002 ± 0.004 for posaconazole.

3.1.2. Accuracy and precision

The intra- and inter-day accuracy and precision data of both compounds are summarized in Table 1.

The within-day precision, expressed as the coefficient of variation of observed concentrations of the routine QC was lower than 6.6% for both analytes, while the within-day accuracy, expressed by the calculated bias between observed and theoretical concentrations of the routine QC, ranged from −2.48 to 1.33% for voriconazole and from −0.87 to 4.93% for posaconazole. The between-day precision and bias were respectively, less than 7.3 and 4.2% for both analytes. The within-day precision for the LOQ of voriconazole and posaconazole were 10.4 and 12.2%

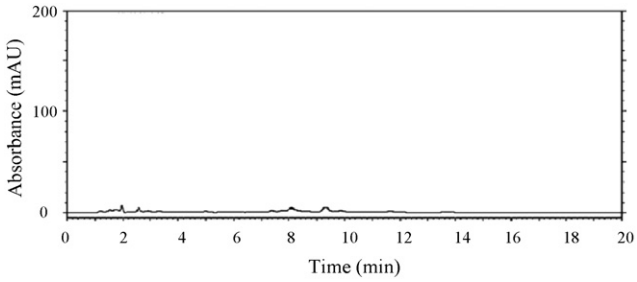

Fig. 2. Chromatogram of blank plasma.

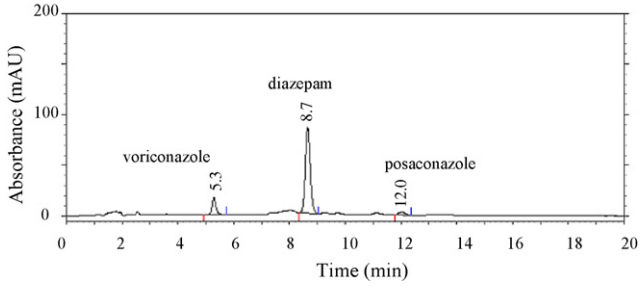

Fig. 3. Chromatogram of LOQ.

respectively, whereas the within-day accuracy for each LOQ was −5.2% for voriconazole and 0.7% for posaconazole. The inter-day precision and bias for each LOQ were below 11 and 8.2%, respectively. Representative chromatograms of a blank human plasma and human plasma spiked with 0.2 mg/L of voriconazole and 0.05 mg/L of posaconazole corresponding to the respective LOQ are shown in Figs. 2 and 3 respectively. A good separation between posaconazole, voriconazole and IS diazepam peaks and plasma components peaks was achieved.

3.1.3. Recovery

The mean recovery from human plasma was in the range 90.5–101.5% for voriconazole and in the range 96.9–105.1% for posaconazole. The mean recovery of the internal standard was 95.2%.

3.1.4. Specificity–selectivity

No interference has been found between voriconazole and posaconazole or the IS and endogenous substances or antiretro-

Table 1
Precision and accuracy of posaconazole and voriconazole determination in human plasma

	Voriconazole				Posaconazole			
	Theoretical (mg/L)	Observed (mg/L)	Bias (%)	R.S.D. (%)	Theoretical (mg/L)	Observed (mg/L)	Bias (%)	RS.D. (%)
Within-day ($n = 6$)	0.2	0.190	−5.17	10.4	0.05	0.050	0.66	12.2
	0.4	0.405	1.33	3.48	0.1	0.099	−0.87	6.11
	1.5	1.46	−2.48	4.17	2.0	2.10	4.93	5.85
	7.5	7.52	0.32	3.95	7.5	7.61	1.43	6.60
Between-day ($n = 18$)	0.2	0.211	5.09	10.1	0.05	0.054	8.16	11.0
	0.4	0.410	2.56	4.24	0.1	0.100	0.22	7.25
	1.5	1.47	−2.18	3.75	2.0	2.09	4.20	5.36
	7.5	7.43	−0.96	3.73	7.5	7.30	−2.76	6.14

Table 2
Retention times of other anti-infective drugs

Therapeutic class	Compounds	Retention Time (min)
Analytes	Voriconazole	5.3
	Posaconazole	12.1
	Diazepam	8.7
Antiretroviral drugs	Abacavir	NR
	Amprenavir	8.1
	Atazanavir	13.2
	Didanosine	3.2
	Efavirenz	21.9
	Emtricitabine	2.2
	Indinavir	6.0
	Lamivudine	9.3
	Lopinavir	19.8
	Nelfinavir	NR
	Nelfinavir metabolite M8	14.6
	Nevirapine	3.3
	Ritonavir	16.3
	Saquinavir	19.9
	Tenofovir	2.6
	T20	NR
	Tipranavir	NR
	Zidovudine	2.2
Antifungal drugs	Amphotericine B	NR
	Fluconazole	3.1
	Itraconazole	NR
Antituberculosis drugs	Isoniazide	NR
	Pyrazinamide	13.2
	Rifabutine	25
	Rifampicine	4.9
Antibacterial drugs	Imipenem	NR
	Ofloxacin	4.2
	Ciprofloxacin	3.7
Antiviral drugs	Cidofovir	NR
	Ganciclovir	NR
	Ribavirine	NR

NR: not retained by the column.

viral, anti-tuberculosis, other anti-fungal or antiviral drugs, retention times of which are listed in Table 2. Some of these drugs are not retained by the column and are therefore detected at the void volume time. A representative chromatogram of a patient treated with posaconazole or voriconazole is displayed on Figs. 4 and 5.

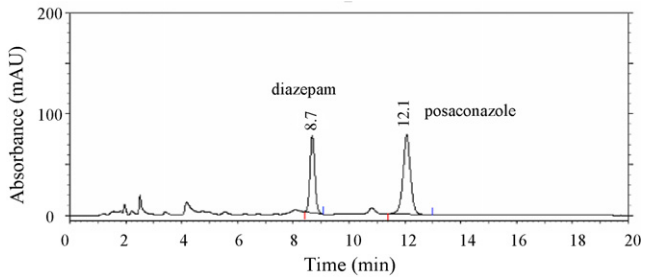

Fig. 4. Chromatogram of a plasma from patient treated with posaconazole (concentration of posaconazole: 2.6 mg/L).

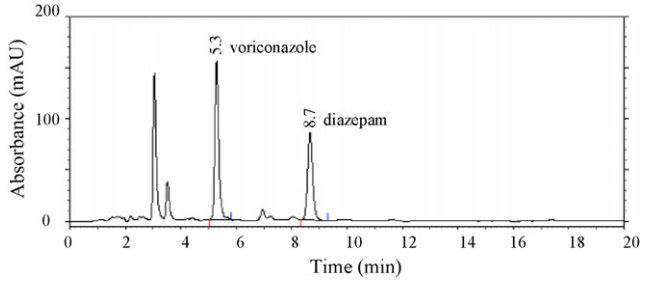

Fig. 5. Chromatogram of a plasma from patient treated with voriconazole (concentration of voriconazole: 6.4 mg/L).

3.1.5. Stability

Freeze-thaw stability of voriconazole and posaconazole was evaluated by the calculated bias between observed and theoretical concentrations (Table 3). These biases were lower than 10.3% for voriconazole and than 11.8% for posaconazole. The assay of the stability of voriconazole and posaconazole in plasma for 2 months at −30 °C showed the absence of degradation products. The stock solution of voriconazole and posaconazole stored for 3 months at −30 °C was comparable to the stock solution freshly made. The assay of stability of the extracts showed a difference less than 5% for peak heights of all analytes (voriconazole, posaconazole and IS) between the QC extracted the day of the injection and the extracts stored at −30 °C for 15 days as well as the extracts left at room temperature for 3 days.

Table 3
Freeze-thaw stability cycles of voriconazole and posaconazole in human plasma ($n = 9$)

	Voriconazole				Posaconazole			
	Theoretical (mg/L)	Observed (mg/L)	Bias (%)	C.V. (%)	Theoretical (mg/L)	Observed (mg/L)	Bias (%)	C.V. (%)
Freeze-thaw cycle 1	0.4	0.36	−10.3	3.64	0.1	0.10	−0.89	2.30
	1.5	1.38	−8.70	2.02	2.0	1.85	−8.28	5.21
	7.5	7.17	−4.63	1.56	7.5	7.02	−6.78	9.42
Freeze-thaw cycle 2	0.4	0.38	−5.19	3.86	0.1	0.10	−1.56	2.01
	1.5	1.41	−6.25	3.41	2.0	1.83	−9.08	6.67
	7.5	7.22	−3.94	4.46	7.5	6.73	−11.4	1.66
Freeze-thaw cycle 3	0.4	0.37	−7.56	3.12	0.1	0.10	−3.51	5.79
	1.5	1.39	−7.13	3.92	2.0	1.77	−11.8	6.33
	7.5	7.06	−5.90	1.51	7.5	6.65	−11.4	3.94

4. Discussion

A simple analytical method was developed to quantify simultaneously posaconazole and voriconazole using a small volume of human plasma. This method has been found accurate enough to perform TDM in immunocompromised adults and children.

An analytical interference was observed between posaconazole and endogenous compounds when sample extractions were performed with terbutylmethylether. The use of the extraction solution described in the method avoided this interfering peak.

Because of its poor water solubility, posaconazole and therefore stock solutions had to be diluted in a methanol:water (1:1, v/v) solvent in order to have linear calibration curves. Therefore due to methanol, these dilutions had to be evaporated before adding the blank plasma in order to obtain limpid extracts.

Diazepam is a marketed drug that can be administered to patients and could provide an analytical interference. However the therapeutic plasma concentration of diazepam achieved is about one hundred times lower than the concentration of diazepam used as internal standard in our method. Therefore it is likely that the presence of diazepam in plasma samples due to drug intake would not compromise posaconazole and voriconazole quantification [21].

As analytical interferences could be expected with endogenous compounds during renal or hepatic failure, several plasma samples from cirrhotic and hemodialyzed patients were analysed. No analytical interference was noticed (not shown).

This method can be applied by clinical researchers to monitor plasma concentrations of posaconazole or voriconazole in pharmacokinetic studies. It can also be used for therapeutic drug monitoring in order to optimize drug dosage on an individual basis.

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