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Short communication

Fast and reliable determination of the antifungal drug voriconazole in plasma using monolithic silica rod liquid chromatography

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Abstract

In the present study, we developed a fast and reliable HPLC assay for the determination of the new triazole antifungal agent voriconazole in plasma, using a Chromolith® RP 18e (100 mm × 4.6 mm) monolithic silica rod HPLC column. After liquid–liquid extraction, plasma samples were separated with a mobile phase consisting of ammoniumdihydrogencarbonate buffer (pH 5.8)–acetonitrile–tetrahydrofuran (72:25:3) at a flow-rate of 3.5 mL/min and UV detection at 255 nm. The retention times for voriconazole and internal standard (UK-115794) were 2.3 and 2.7 min, respectively, and total run time was 4 min. The calibration curves were linear between 0.05 and 10 μg/mL, and within-assay and between-assay coefficients of variation were <4%. The proposed assay for voriconazole in plasma is fast, sensitive and reliable, and, thus, well suited for routine therapeutic monitoring of patients and for pharmacokinetic studies. It can be predicted that the use of monolithic silica rod chromatography will substantially shorten the turn-around time in the therapeutic drug monitoring laboratory.

Keywords: Voriconazole; Monolithic columns; HPLC; Therapeutic drug monitoring

1. Introduction

Voriconazole ((*2R*,*3S*)-2-(2,4-difluorophenyl)-3-(5-fluoro-4-pyrimidinyl)-1-(1*H*-1,2,4-triazol-1-yl)-2-butan-2-ol) (Fig. 1) is a novel wide-spectrum triazole antifungal agent against clinically significant and emerging pathogens, including *Aspergillus* and *Candida* species [1–3]. In humans about 80% of voriconazole is hepatically eliminated, primarily via the cytochrome P450 (CYP) 2C9, CYP2C19, and to a lesser extent by CYP3A4 [4]. Patients, who are at risk for acquiring fungal infections, e.g. patients after bone marrow or solid organ transplantation, are typically receiving multiple medications, and are prone to drug–drug interactions [5]. In addition, a non-linear pharmacokinetic behaviour has been observed in healthy volunteers [6] and in patients [7] for this drug. Therefore, monitoring of voriconazole in plasma might be warranted in some patients at higher risk, especially in patients under polymedication [8]. Another patient population, for whom voriconazole plasma level monitoring might be of advantage, are patients with impaired oral bioavailability of voriconazole due to inflammation of the intestinal tract, e.g. patients exhibiting a graft versus host (GvH) reaction after organ transplantation. Optimal voriconazole plasma concentrations in terms of efficacy and toxicity are not yet defined, and, thus, measured concentrations of voriconazole in patients are compared with results from pharmacokinetic studies.

Several CE, HPLC and HPLC/MS methods have been developed for the determination of voriconazole in biological fluids [9–15]. Because voriconazole determinations to monitor therapeutic regimens are not yet routinely established, often only a few samples have to be measured in a single series. Thus, taking into account the inclusion of calibrators and quality control samples, the determination of only a few plasma samples can be rather time consuming, considering the typical chromatographic run times of up to 12 min for HPLC methods. On the other hand, clinicians often require the results on the same day, which makes the turn-around time for drug analysis critical, especially if also other drugs have to be measured with the same equipment. Recently, highly porous monolithic silica rod columns have been introduced, which have a bimodal pore structure with a large surface area [16–18]. Due to this fact, higher flow-rates

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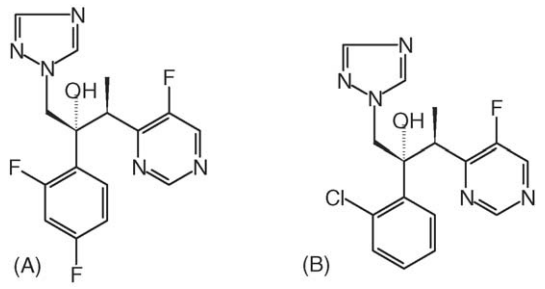

Fig. 1. Structure (A) of voriconazole and (B) internal standard (UK-115794).

can be applied while the column back-pressure is still low. These new columns not only enable flow-rates up to 10 mL/min with good column performance, resulting in very short run times, but also a very rapid column equilibration allowing a fast method change-over.

On this basis, the aim of the present study was to develop and optimise a fast and reliable HPLC method using monolithic silica rod column technology for the routine determination of voriconazole in plasma samples from patients.

2. Experimental

2.1. Chemicals

Voriconazole (UK-109496) and internal standard (UK-115794) were generous gifts from Pfizer Global R&D, Sandwich, UK.

Analytical grade ammoniumacetate, ammoniumdihydrogen phosphate, ethylacetate, diethylether, HPLC grade tetrahydrofurane, methanol and acetonitrile were purchased from Merck, Darmstadt, Germany. Double distilled water was used for all solutions.

2.2. Instrumentation and liquid chromatography

HPLC analyses have been performed on a LaChrome Elite® system (VWR International, Darmstadt, Germany) which consisted of a model 2130 quarternary pump with on-line degassers, model 2200 autosampler, model 2300 UV detector set at 255 nm and a model 2400 column oven. The LaChrom Elite software was used for controlling the HPLC system and for data acquisition and processing. Voriconazole and internal standard were separated on a RP 18e (100 mm × 4.6 mm) Chromolith™ Performance monolithic silica rod column (VWR International, Darmstadt, Germany), including the corresponding 5 mm × 4.6 mm guard column, maintained at 32 °C. The mobile phase consisted of 0.025 M ammoniumdihydrogenphosphate buffer (pH 5.8)–acetonitrile–tetrahydrofurane (74: 25: 1, v/v/v). The flow-rate was 3.5 mL/min, leading to a back-pressure of 87 bar, and the run time was set at 4 min.

2.3. Sample preparation and standards

To 0.25 mL of plasma, 50 μL of internal standard solution (20 μg/mL in water) and 0.5 mL of 0.2 M ammoniumacetate buffer, pH 9.0, were added and extracted with 7 mL ethylacetate–diethylether (1:1, v/v) by vortexing at maximal speed for 90 s. After centrifugation at 1500 × *g* for 3 min, the two phases were separated by freezing out of the water phase, and the organic layer was transferred in a second tube and evaporated to dryness in a turbovap® evaporator (Zymark Corporation, Hopkinton, MA, USA) at 40 °C under a stream of nitrogen. The residues were dissolved in 0.2 mL of mobile phase, centrifuged at 10,000 × *g* for 6 min, and 30 μL of the supernatant were injected onto the column for HPLC analysis. Standards containing 0.055, 0.111, 0.333, 1.0, 3.0 and 9.0 μg/mL voriconazole were prepared by dilution of a stock solution of voriconazole (1 mg/mL) in 50% methanol (v/v) by appropriate volumes of drug free plasma and stored frozen at −20 °C.

2.4. Assay validation

Calibration curves were prepared in concentration ranges of 0.055–9 mg/L. Precision and accuracy were determined by running quality controls at three different concentrations covering the calibration range on the same (intra-day) and on different days (inter-day variability). Extraction recovery was calculated by comparing the concentration of extracted spiked plasma or liquor samples with the determination of the spiking solution without extraction. The limit of detection (LOD) was calculated at signal-to-noise level of 3 and the approximate limit of quantitation (LOQ) was defined for the lowest concentration measured with a relative standard deviation of <20%.

2.5. Routine determination of patient's samples

To examine for potential drug interferences between 1 and 4 voriconazole trough levels of 12 patients under polymedication receiving 200–400 mg voriconazole (p.o. or i.v.) were measured during the period of assay introduction into clinical routine. The suitability of the proposed assay for voriconazole determinations in liquor was tested by spiking liquor samples from patients with known amounts of the drug.

3. Results

3.1. Chromatography

With a flow-rate of 3.5 mL/min and a back-pressure of 87 bar, the internal standard and voriconazole could be separated with retention times of 2.4 and 2.8 min, respectively, and a total run time of 4 min. Examples of chromatograms of a blank plasma and two plasma samples from patients at low (0.12 mg/L) and high (3.5 mg/L) voriconazole concentrations are shown in Fig. 2. No carryover has been observed between patient's samples with high voriconazole concentrations and blank plasma samples.

3.2. Precision, accuracy, recovery and limit of quantitation

Standard curves showed an excellent linearity between 0.1 and 10 μg/mL. The correlation coefficients (R^2) of the

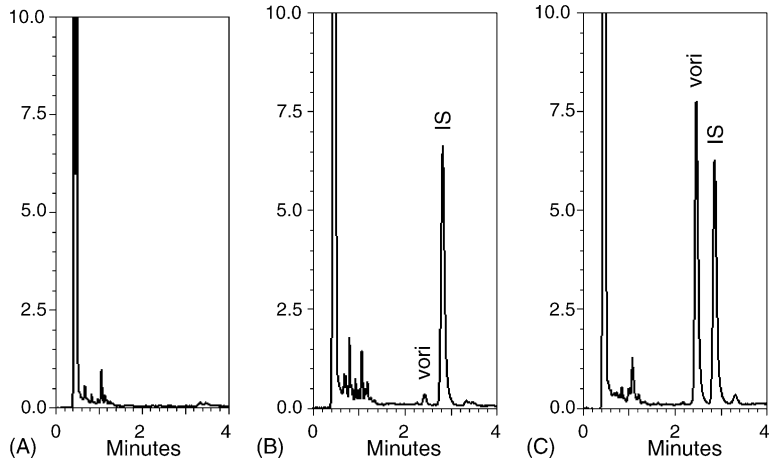

Fig. 2. Chromatograms of blank plasma (A), and plasmas from patients containing 0.1 μg/mL of voriconazole (B), and 3.5 μg/mL voriconazole (C). Abrreviations: Vori, voriconazole; IS, internal standard.

Table 1
Intra-day and inter-day precision and accuracy for voriconazole

Theoretical concentration (mg/L)	Intra-day				Inter-day			
	N	Mean concentration found (mg/L)	R.S.D. (%)	Accuracy (%)	*N*	Mean concentration found (mg/L)	R.S.D. (%)	Accuracy (%)
0.225	6	0.224	3.8	99.5	10	0.240	6.06	106
0.900	6	0.898	0.34	99.8	10	0.910	1.58	101
4.50	6	4.61	1.2	102	10	4.48	2.47	99.5

linear calibration curves were between 0.9998 and 1.0000. The inter-day variability of the slopes was less than 4%. Extraction recoveries for voriconazole and internal standard were 94.6 ± 2.9 and 95.8 ± 2.3%, respectively ($n = 5$). Intra- and inter-day precision and accuracy at low, medium and high voriconazole concentrations are summarized in Table 1. The lower limit of quantitation (LLOQ) was 50 ng/mL and the lover limit of detection (LLOD) was 15 ng/mL.

3.3. Assay application

At present more than 30 patients receiving voriconazole (200–400 mg i.v. or p.o., b.i.d.) were monitored on several occasions to ensure effective voriconazole concentrations. Pre-dose concentrations of voriconazole were between 0.2 and 1.5 mg/L, and post dose concentrations (1 h after the end of infusion) were between 1.5 and 4.0 mg/L. In one patient also the concentrations in cerebrospinal fluid (CSF) were determined during three different dosing intervals. The mean concentration ratio between plasma and CSF was 2.05.

4. Discussion

The use of the silica rod technology for the fast and effective separation of drugs and other compounds is a very promising concept. Unfortunately, the available selection of stationary phases is quite limited so far. Thus, the first step in the present assay development using the available RP-18 stationary phase was the optimisation of the mobile phase. Because reproducible gradient separation is difficult to achieve with short run times, we aimed for a good separation of voriconazole from the structurally related internal standard (Fig. 1) with isocratic separation within the shortest time possible to take advantage from the time saving using high flow-rates. A good separation could thus be achieved by optimising the concentration of tetrahydrofuran as organic modifier, resulting in a total run time of 4 min. If the proposed internal standard compound (UK-115794) is not available, naproxen can be used without changing the current method. Naproxen elutes with a retention time of about 1 min. The within-day and between-day coefficients of variation observed in the proposed assay were better than in most studies in the literature [9–15] and, thus, this method is also suitable for pharmacokinetic investigations. Although all the patients we have monitored for voriconazole levels so far were under polymedication (patients after bone marrow transplantation), we observed no interferences in the chromatograms caused by other drugs or endogenous substances. Appling a flow of 3.5 mL/min resulted in a back-pressure of 83 bar. In our experience even higher flow-rates (up to 6 mL/min) times can be used without loosing significant peak resolution. However, an additional speeding up of the assay was found unnecessary and would have interferred with the duration of wash cycles of our autoinjector.

As a result of the short retention times, high and narrow peaks were achieved, leading to a good assay sensitivity with a LOQ of about 50 ng/mL which is far below the currently proposed

therapeutic range [10], but useful for pharmacokinetic studies. The monolithic column proved to be very stable. At present time about 700 injections have been applied onto the same column without any loss of separation performance. The present HPLC method was also applied to the determination of voriconazole in cerebrospinal fluid (CSF) in patients with cerebral aspergillosis. Although we could not yet fully perform a complete quality control study for this matrix, the hitherto achieved results for the assay performance are at least as good as for plasma samples. Between plasma and CSF, we found a concentration ratio of about 2, which is in accordance to published observations [19,20].

The use of very fast HPLC columns with a good performance is of great advantage in the field of therapeutic drug monitoring (TDM), especially for drugs for which so far no alternative automatic immunoassays exist. The TDM laboratory is often faced with the situation that different HPLC methods have to be applied for only a few patient samples, and the results have to be forwarded to the clinicians on the same day. Thus, the application of high flow-rates allows not only short run times but also the short equilibration times after column exchange.

However, at present, the number of published applications for the use of silica rod HPLC for TDM drug monitoring is rather scarce. This might change, if additional stationary phases are introduced in the near future.

5. Conclusion

A rapid, reliable and robust HPLC assay has been developed for the determination of voriconazole in plasma using a monolithic silica rod column at a high flow-rate. The application of fast monolithic columns proved to be a very useful approach in the field of therapeutic drug monitoring because short assay turnover times are achieved.

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