Development and Validation of a Simple and Rapid HPLC Method for the Quantitative Determination of Voriconazole in Rat and Beagle Dog Plasma

Shan Cheng[1],*, Feng Qiu[2], Jia Huang[3], and **Junqi He[1]**

[1]Department of Biochemistry and Molecular Biology, School of Basic Medical Sciences, Capital Medical University, No.10 Xitoutiao, You An Men, Beijing 100069, China; [2]College of Chemistry and Molecular Engineering, Peking University, No. 202 Cheng Fu Road, Beijing 100871, China; and [3]Beijing Tiantan Hospital Affiliated to Capital Medical University, No. 6 Tiantanxili, Beijing 100050, China

Abstract

A simple and rapid high-performance liquid chromatographic method with UV detection is developed and validated to determine the concentration of voriconazole in rat and beagle dog plasma. After protein precipitation using acetonitrile, the supernatant solution is chromatographed on a Diamonsil C_{18} column (250 × 4.6-mm i.d., 5 μm). The mobile phase used is a combination of acetonitrile–water–acetic acid (55:45:0.25, v/v/v) with a pH of 4.0. Detection is achieved by a UV detector monitored at a wavelength of 256 nm. The matrix calibration curves are obtained both in the concentration range of 0.10–50.0 μg/mL in rat and beagle dog plasma, with the lower limit of quantitation of 0.10 μg/mL. The intra- and inter-assay precisions in terms of % relative standard deviation are lower than 8.6% and 6.0% in rat and beagle dog plasma, respectively. The accuracy in terms of % relative error ranged from –0.5% to 8.0% and –0.5% to 6.0% in rat and beagle dog plasma, respectively. This validated method is successfully applied to determine the concentration of voriconazole in plasma after intravenous administration of 36 mg/kg voriconazole to rats and 10 mg/kg voriconazole to beagle dogs, respectively.

Introduction

Voriconazole (VFEND) [UK-109, 496, (2*R*,3*S*)-2-(2,4-difluo-rophenyl)-3-(5-fluoro-4-pyrimidinyl)-1-(1*H*-1,2,4-triazol-1-yl)-2-butan-2-ol] is a new antifungal agent that is a derivative of fluconazole, with one triazole moiety replaced by a fluoropy-rimidine ring and a methyl group added to the propanol back-bone (1). This new form has an enhanced and a wide-spectrum activity in vitro and a fungicidal action against various mold species, including *Aspergillus* (2,3). As other azole antifungal agents, such as fluconazole and itraconazole, its primary mode of action is inhibition of fungal cytochrome P450-dependent

14α-sterol demethylase, an essential enzyme in ergosterol biosynthesis (4). Voriconazole shows a greater selectivity for the fungal enzyme than for the corresponding rat liver enzyme compared with both ketoconazole and itraconazole (5). Voriconazole is moderately lipophilic (log $D_{7.4}$ = 1.8) and has a single diastereomer with *R*- and *S*-stereochemistry by virtue of two chiral centers (2*R*, 3*S*) as shown in Figure 1.

The quantitation of voriconazole levels in plasma samples in preclinical and clinical trials is an important objective because it will permit a comprehensive characterization of its pharma-cological features and monitoring its levels in preclinical and clinical plasma samples. This was the reason our laboratory was interested in the development of an adequate analytical method. A few analytical techniques have been published for the determination of voriconazole in biological fluids, like the microbiological method (6) and chemical methods such as high-performance liquid chromatography (HPLC)–UV detection (6–9) and liquid chromatography (LC)–mass spectrometry (MS) (10). By using the reported microbiological
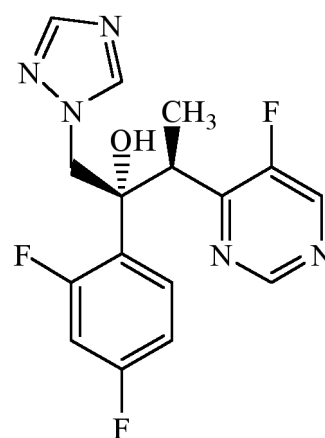

Figure 1. Chemical structure of voriconazole ($C_{16}H_{14}F_3N_5O$, MW: 349.31).

* Author to whom correspondence should be addressed: e-mail firhill@sina.com.

method, the lower limit of quantitation (LLOQ) was only 0.25 µg/L, and voriconazole couldn't be separated from its probable metabolites, which might have some antibacterial effect and would interfere with the determination of voriconazole. Several HPLC methods have been developed to determine voriconazole in human plasma, but special columns, such as size-exclusion columns or internal surface reverse-phase columns, were needed. Also, a sensitive LC–MS method has been developed to determine voriconazole in aqueous humor of rats with a LLOQ of 5 ng/mL. Because MS detection methods are not so easily used, a simple HPLC-UV method was developed for the quantitative analysis of voriconazole in biological fluids as there was a need for preclinical and clinical trials to define single and multi-dose safety, efficacy, and pharmacokinetic profiles.

In the present study, a simple and a rapid HPLC method with UV detection is developed and validated for the determination of voriconazole in rat and beagle dog plasma after an intravenous administration of 36 mg/kg voriconazole to rats and 10 mg/kg voriconazole to beagle dogs, respectively.

Experimental

Chemicals and reagents

Reference standard of voriconazole was provided by the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). HPLC-grade acetonitrile was purchased from Yuwang Chemical (Shandong, China). HPLC water used was from a Milli-Q water purification system from Millipore (Molsheim, France). All other chemicals used were of analytical grade.

Instrumentation

Chromatographic separation was performed on a Shimadzu LC-10AD pump equipped with a Shimadzu SPD-10Avp UV-Vis detector (Shimadzu Co., Kyoto, Japan). Data acquisition was performed using N-2000 data software of Zhejiang University (Hangzhou, Zhejiang, China). Injections were carried out manually by using a 20-µL loop.

Chromatographic conditions

The HPLC separation was performed on a Diamonsil C18 column (250 × 4.6 mm i.d., 5 µm) (Dikma, Beijing, China) with a Shim-pack GVP-ODS C18 guard column (10 × 4.6 mm i.d.) kept at 20°C. The mobile phase, consisting of acetonitrile–water–acetic acid (55:45:0.25, v/v/v), was delivered isocratically at a flow-rate of 1.0 mL/min. Prior to use, the mobile phase was filtered through 0.45-µm Millipore membrane filters and degassed by sonication in an ultrasonic bath. Voriconazole detection was performed using a UV–vis detector monitored at 256 nm.

Sample preparation

An aliquot of 100 µL of methanol and 200 µL of acetonitrile were added to 100 µL of rat or beagle dog plasma samples, respectively. Then the mixture was vortexed for 30 s and sonicated for 5 min. After centrifugation at 5000 × g for 5 min, an aliquot of 20 µL of the upper solution was injected onto the chromatographic column for analysis.

Before sample preparation, those samples whose concentrations were higher than the highest calibration point were diluted with blank plasma to get concentrations within the range of the standard curve.

Method validation

Rat or beagle dog plasma samples were quantitated by using the peak area of voriconazole as the assay response. Peak areas were plotted against standard concentrations and the concentrations of voriconazole in plasma were calculated by a weighted ($1/x^2$) least squares linear regression.

To evaluate linearity, rat or beagle dog matrix calibration curves were prepared and assayed in triplicate on 3 consecutive days. Accuracy and precision were also assessed by determining quality control (QC) samples at three concentration levels on the 3 different days. The accuracy was expressed by (mean observed concentration – spiked concentration)/(spiked concentration) × 100% and the precision by relative standard deviation (RSD).

The recoveries of voriconazole at three QC levels were determined by comparing the peak areas of voriconazole in a sample that had been spiked with analyte prior to preparation with samples to which the analyte had been added post-preparation.

The stability of voriconazole in rat or beagle dog plasma was investigated under a variety of storage and process conditions. The storage stability under –20°C was evaluated for at least 30 days. The freeze-and-thaw stability of voriconazole was assessed by analyzing QC samples at three concentrations undergoing three freeze-and-thaw cycles at –20°C and room temperature, respectively. The stability in the reconstituted solution was investigated by placing the QC samples (spiked at three levels) under ambient conditions for 24 h.

Application of the method to determine plasma concentration in rats and beagle dogs

Six Wistar rats (three males and three females, Laboratory Animal Center of Beijing Vitalriver, Beijing, China) weighing 200 to 250 g were used in the studies. The rats were certified and had not been dosed with any pharmaceutical before the experiment. The rats were housed under standard conditions and had *ad libitum* access to water and a standard laboratory diet. Food and water was available *ad libitum* throughout the experiments. Polyethylene cannulas were implanted in the femoral vein 2 days before the experiment while the rats were anesthetized with pentobarbital (50 mg/kg, i.v.). The cannulas were externalized at the back of the neck and filled with heparinized saline (20 units/mL). The rats were intravenously dosed with voriconazole at 36 mg/kg (9 mL/kg, 4 mg/mL) through caudal vein. Serial blood samples (0.3 mL) were post dose collected at 0, 0.083, 0.167, 0.50, 1.0, 2.0, 4.0, 6.0, 8.0, 10.0, and 12.0 h. Plasma was separated by centrifugation at 5000 × g for 5 min and stored frozen at –20°C until analysis.
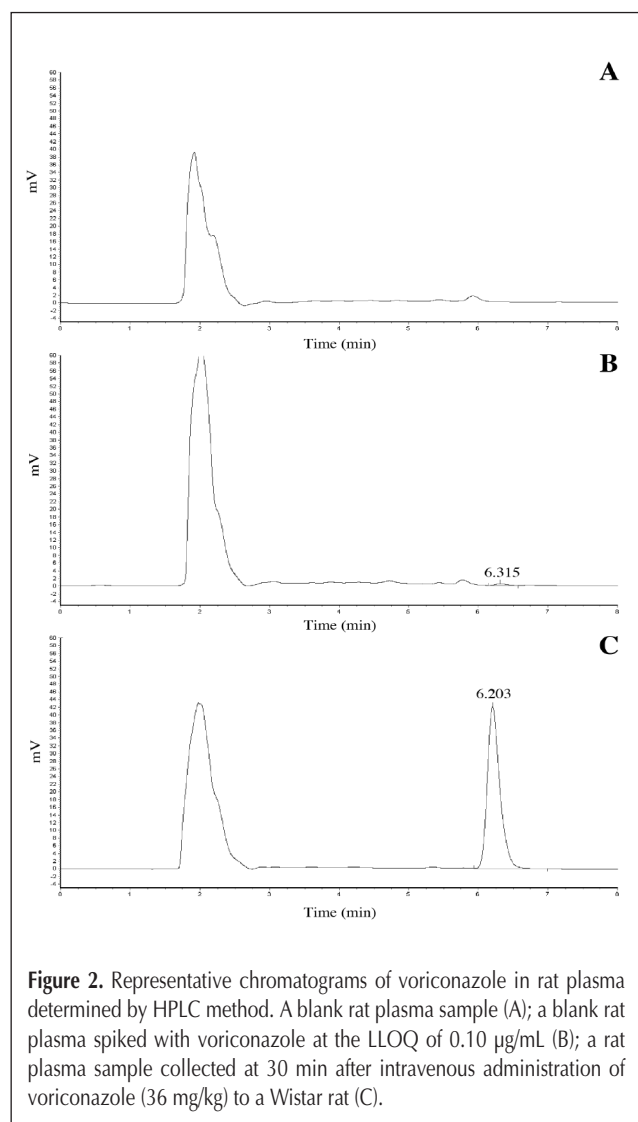
Six beagle dogs (three males and three females, Laboratory Animal Center of Beijing Vitalriver, Beijing, China) weighing 10 to 12 kg were used in the studies. The dogs were certified

and had not been dosed with any pharmaceutical before the experiment. The dogs were housed under standard conditions and had *ad libitum* access to water and a standard laboratory diet. Food and water was available *ad libitum* throughout the experiments. The beagle dogs were intravenously infused with voriconazole at 10 mg/kg (10 mL/kg, 1 mg/mL, infusion velocity about 30 drops/min) through the foreleg vein using a polyethylene cannula during a period of 1 h. Serial blood samples (0.5 mL) were collected post dose from another foreleg vein at 0, 0.17, 0.50, 1.0, 2.0, 3.0, 4.0, 6.0, 8.0, 10.0, 12, 18, 24, 30, 36, and 48 h. Plasma was separated by centrifugation at $5000 \times g$ for 5 min and stored frozen at –20°C until analysis.

Results

### Selectivity of the chromatographic conditions

During the method development, top priority was given for complete separation of voriconazole from the potential endogenous interferences of rat or beagle dog plasma.

Figure 2. Representative chromatograms of voriconazole in rat plasma determined by HPLC method. A blank rat plasma sample (A); a blank rat plasma spiked with voriconazole at the LLOQ of 0.10 µg/mL (B); a rat plasma sample collected at 30 min after intravenous administration of voriconazole (36 mg/kg) to a Wistar rat (C).

Selectivity was assessed by comparing the chromatograms of six different batches of blank rat or beagle dog plasma with the corresponding spiked plasma. Figure 2 shows the typical chromatograms of a blank rat plasma sample, a blank rat plasma sample spiked with voriconazole at the LLOQ of 0.10 µg/mL, and a plasma sample from a Wistar rat 30 min after an intravenous administration. Figure 3 shows the typical chromatograms of a blank beagle dog plasma sample, a blank beagle dog plasma sample spiked with voriconazole at the LLOQ of 0.10 µg/mL, and a plasma sample from a beagle dog 60 min after an intravenous infusion. No significant interferences from endogenous substances of rat or beagle dog plasma with voriconazole were detected. Typical retention time for voriconazole was 6.4 min.

Linearity

Calibration standards were prepared by spiking 100 µL of the appropriate standard solutions of voriconazole to 100 µL of blank rat or beagle dog plasma. Plasma concentrations were 0.10, 0.25, 0.50, 1.00, 2.00, 5.00, 20.0, and 50.0 µg/mL for

Figure 3. Representative chromatograms of voriconazole in beagle dog plasma determined by HPLC method. A blank beagle dog plasma sample (A); a blank beagle dog plasma spiked with voriconazole at the LLOQ of 0.10 µg/mL (B); a beagle dog plasma sample collected at 60 min after intravenous administration of voriconazole (10 mg/kg) to a beagle dog (C).
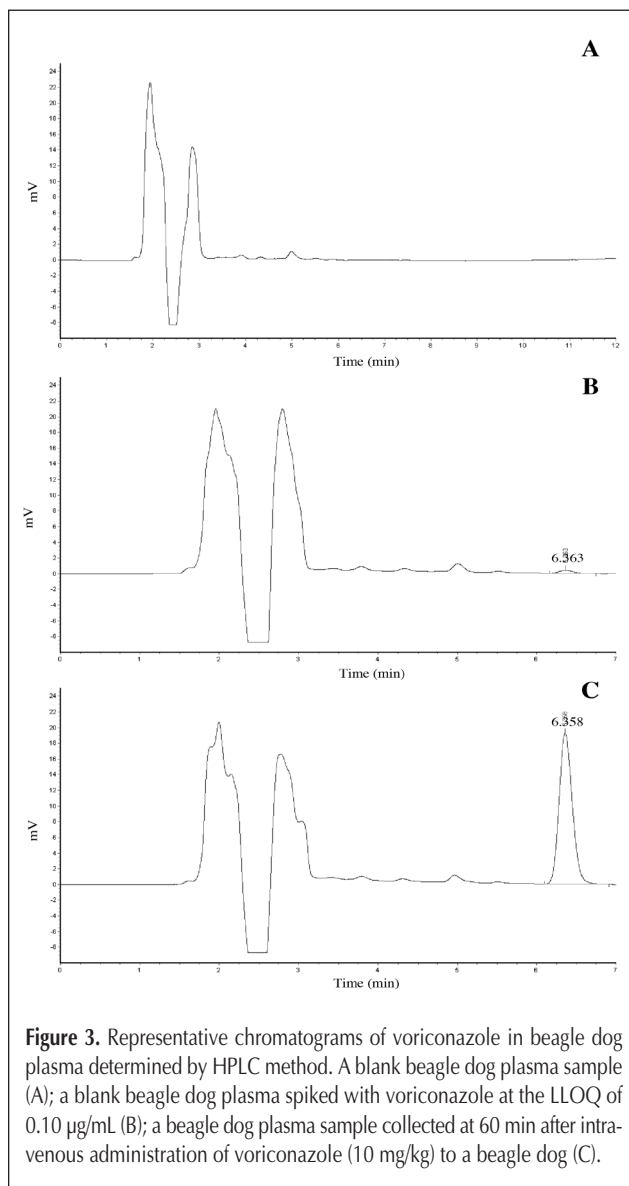
voriconazole. The peak area (Y) and concentration of voriconazole (X) were subjected to a weighted ($1/X^2$) least squares linear regression analysis to calculate calibration equation and correlation coefficients. Typical equations of the calibration curve were as follows: $Y = 69.1 + 10795X$ (R = 0.9982, $n = 5$) for rat plasma samples and $Y = 591 + 10878X$ (R = 0.9977, $n = 5$) for beagle dog plasma samples. The linear ranges of voriconazole in rat and beagle dog plasma were both from 0.10 to 50.0 µg/mL. The results show that an excellent correlation existed between the peak area and concentration of voriconazole.

LLOQ

The LLOQ was experimentally determined by diluting known concentrations of voriconazole in rat or beagle dog plasma for five replicate determinations. The present assay method offered an LLOQ of 0.10 µg/mL in rat plasma with an accuracy of 12.9% and a precision of 10.4% ($n = 5$), which was sufficient for monitoring voriconazole plasma levels over a period of 12 h after a single intravenous administration to Wistar rats. The present assay method had an LLOQ of 0.10 µg/mL in beagle dog plasma with an accuracy of 13.3% and a precision of 6.4% ($n = 5$), which was sufficient for monitoring voriconazole plasma levels over a period of 48 h after a single intravenous infusion to beagle dogs.

Accuracy and precision

Accuracy and precision were assessed by determining QC samples at three concentration levels (0.20, 2.00, and 45.0 µg/mL) on 3 different validation days. The accuracy was expressed by (mean observed concentration – spiked concentration)/(spiked concentration) × 100% and the precision by RSD. Tables I and II summarize the intra- and inter-day precision and accuracy for voriconazole from QC samples in rat and beagle dog plasma, respectively. In the assay of voriconazole in rat plasma, the intra- and inter-day precisions ranged from 3.7% to 8.6% and from 2.0% to 4.7% for each QC level, respectively. The accuracy ranged from –0.5% to 8.0%. In the assay of voriconazole in beagle dog plasma, the intra- and inter-day precisions ranged from 4.7% to 6.0% and from 3.2% to 5.8% for each QC level, respectively. The accuracy ranged from –0.5% to 6.0%. The results, calculated with a one-way ANOVA (analysis of variance), indicated that the values were within the acceptable range; thus the method is accurate and precise (11).

Recovery

The recoveries of voriconazole in rat plasma, determined at three concentrations (0.20, 2.00, 45.0 µg/mL), were 93.2%, 95.4%, and 94.2% ($n = 6$), respectively, with the RSD lower than 4.7%.

Table I. Precision and Accuracy of the HPLC Method in Determining Voriconazole in Rat Plasma ($n = 6$)

Concentration (µg/mL)		RSD (%)		
Added	Found	Intra-day	Inter-day	RE (%)
0.20	0.199	8.6	4.7	–0.5
2.00	2.14	5.9	2.4	7.0
45.0	48.6	3.7	2.0	8.0

Table II. Precision and Accuracy of the HPLC Method in Determining Voriconazole in Beagle Dog Plasma ($n = 6$)

Concentration (µg/mL)		RSD (%)		
Added	Found	Intra-day	Inter-day	RE (%)
0.20	0.199	6.0	5.8	–0.5
2.00	2.06	5.6	3.2	3.0
45.0	47.7	4.7	4.5	6.0

Table III. Stability of Voriconazole in Rat Plasma Determined by HPLC Method ($n = 6$)

	Voriconazole concentration (µg/mL)		
	0.20	2.00	45.0
Storage stability ($\leq$ –20°C)			
Mean (µg/mL)	0.204	2.00	45.9
RSD (%)	10.8	1.5	4.2
RE (%)	2.0	0.0	2.0
Freeze-thaw stability			
Mean (µg/mL)	0.199	2.04	48.7
RSD (%)	4.5	5.1	2.8
RE (%)	–0.5	2.0	8.2
Processed plasma samples at room temperature			
Mean (µg/mL)	0.195	2.09	49.6
RSD (%)	11.0	8.7	1.9
RE (%)	–2.5	4.5	10.2

Table IV. Stability of Voriconazole in Beagle Dog Plasma Determined by HPLC Method ($n = 6$)

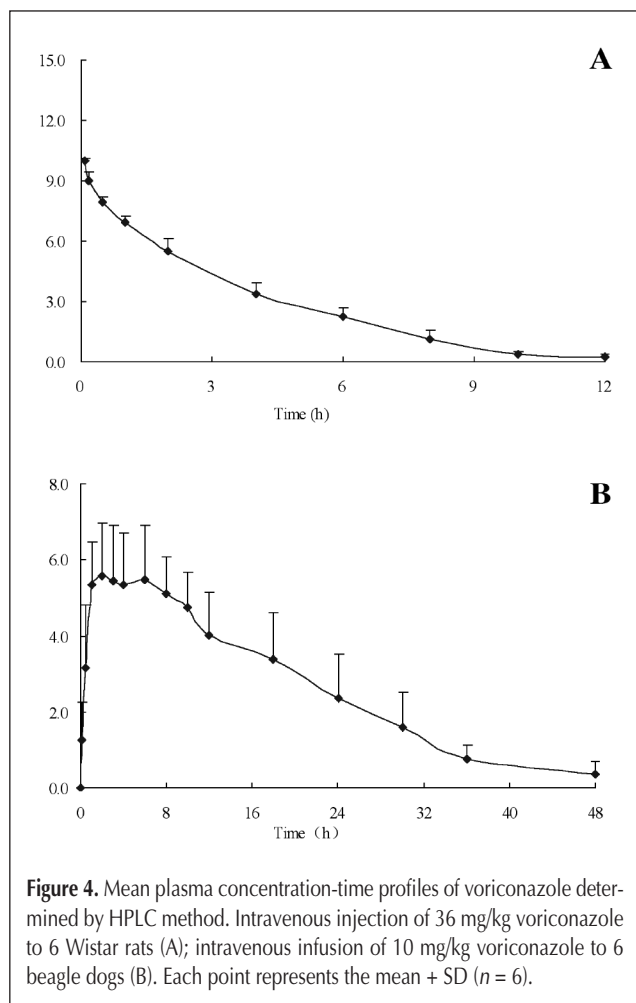|	Voriconazole concentration (µg/mL)		
	0.20	2.00	45.0
Storage stability ($\leq$ –20°C)			
Mean (µg/mL)	0.202	2.20	45.5
RSD (%)	8.5	0.9	1.9
RE (%)	1.0	10.0	1.1
Freeze-thaw stability			
Mean (µg/mL)	0.211	1.85	48.2
RSD (%)	3.2	2.1	4.8
RE (%)	5.5	–7.5	7.1
Processed plasma samples at room temperature			
Mean (µg/mL)	0.201	1.85	48.3
RSD (%)	6.0	0.4	2.6
RE (%)	0.5	–7.5	7.3

The recoveries of voriconazole in beagle dog plasma, determined at three concentrations (0.20, 2.00, 45.0 μg/mL), were 90.1%, 92.0%, and 91.2% ($n = 6$), respectively, with the RSD lower than 3.4%.

Stability

Voriconazole in rat or beagle dog plasma was shown to be stable for at least 30 days stored at –20°C ($n = 6$). The relative error (RE%) of voriconazole in rat or beagle dog plasma between the initial concentrations and the concentrations of the following three freeze-thaw cycles ranged from –7.5% to 8.2%, which indicated that voriconazole was stable during the three freeze-thaw cycles ($n = 6$). Processed samples were also found to be stable in the reconstituted solution for at least 24 h at room temperature ($n = 6$). The described stability data are summarized in Tables III and IV.

Application of the method to determine the concentration of voriconazole in rat and beagle dog plasma

The method was successfully applied to determine the plasma concentration of voriconazole in plasma after intravenous administration of 36 mg/kg voriconazole to rats and 10 mg/kg voriconazole to beagle dogs, respectively. Mean plasma concentration-time profiles of voriconazole in rats and beagle dogs are presented in Figure 4.

Figure 4. Mean plasma concentration-time profiles of voriconazole determined by HPLC method. Intravenous injection of 36 mg/kg voriconazole to 6 Wistar rats (A); intravenous infusion of 10 mg/kg voriconazole to 6 beagle dogs (B). Each point represents the mean + SD ($n = 6$).

Discussion

The mobile phase was chosen after several trials with acetonitrile, methanol, and water in various proportions and in different pH values (3.5, 4.0, and 5.0). When acetonitrile–water (55:45, v/v) was adopted as the mobile phase, it was found that good separation was achieved in rat or beagle dog plasma. But the peak shape of voriconazole was not symmetrical. Symmetrical peak shape was obtained by addition of 0.25% acetic acid in the mobile phase. Thus, a mobile phase consisting of acetonitrile–water–acetic acid (55:45:0.25, v/v/v) with a pH of 4.0 was finally selected in order to achieve optimal separation, high sensitivity, and good peak shape.

Precipitation of protein provided a simple method of sample preparation and has been widely used for the analysis of drugs in plasma. Several reagents were usually used to precipitate protein in plasma, such as methanol, acetonitrile, 6% $HClO_4$, 10% CCl_3COOH, etc. But $HClO_4$ and CCl_3COOH were both strong acids, which would cause the degradation of voriconazole. The samples prepared by methanol precipitation with the proportion of methanol–plasma (2:1, v/v) caused a rise in column pressure and distortion of peak shape. These problems were overcome by using acetonitrile as the precipitator with the proportion of acetonitrile–plasma (1:1, v/v).

By using the proposed chromatographic conditions and sample preparation method, voriconazole could be rapidly prepared and well separated in rat or beagle dog plasma. The retention times of voriconazole was 6.4 ± 0.1 min. Total analysis time of one sample was less than 8 min.

The present HPLC method offers a LLOQ of 0.10 μg/mL in rat or beagle dog plasma sample. Under the present LLOQ, the concentration of voriconazole could be determined in plasma samples up to 12 h after an intravenous injection of 36 mg/kg voriconazole to rats and 48 h following an intravenous infusion of 10 mg/kg voriconazole, which was sensitive enough to investigate the pharmacokinetic behaviors of voriconazole, to establish the relationship between dose and pharmacological effect, and identify doses that produce toxic responses.

Conclusion

The developed HPLC method for the determination of voriconazole in rat or beagle dog plasma offers sufficient selectivity, accuracy, precision and a short running time. The validated method is simple and rapid and can be used for the determination of voriconazole in biological samples.

References

1. M.M. Pearson, P.D. Rogers, J.D. Cleary, and S.W. Chapman. Voriconazole: a new triazole antifungal agent. *Ann. Pharmacother.* **37:** 420–32 (2003).
2. A.L. Barry and S.D. Brown. In vitro studies of two triazole antifungal agents (UK-109, 496 and fluconazole) against *Candida* species. *Antimicrob agents Chemother.* **40:** 1948–49 (1996).

3. M. Murphy, E.M. Bernard, T. Ishimaru, and D. Armstrong. Activity of voriconazole (UK-109, 496) against clinical isolates of *Aspergillus* species and its effectiveness in an experimental model of invasive pulmonary aspergillosis. *Antimicrob. Agents Chemother.* **41:** 696–98 (1997).
4. H. Sanati, P. Belanger, R. Fratti, and M. Ghannoum. A new triazole, voriconazole (UK-109, 496), blocks sterol biosynthesis in *Candida albicans* and *Candida krusei. Antimicrob. Agents Chemother.* **41:** 2492–96 (1997).
5. M.V. Martin, J. Yates, and C.A. Hitchcock. Comparison of voriconazole (UK-109,496) and itraconazole in prevention and treatment of Aspergillus fumigatus endocarditis in guinea pigs. *Antimicrob. Agents Chemother.* **41:** 13–16 (1997).
6. S. Perea, G.J. Pennick, A. Modak, A.W. Forthergill, D.A. Sutton, D.J. Sheehan, and M.G. Rinaldi. Comparison of high-performance liquid chromatographic and microbiological methods for determination of voriconazole levels in plasma. *Antimicrob. Agents Chemother.* **44:** 1209–13 (2000).
7. D.A. Stopher and R. Gage. Determination of a new antifungal agent, voriconazole, by multidimensional high-performance liquid chromatography with direct plasma injection onto a size-exclusion column. *J. Chromatogr. B* **691:** 441–48 (1997).
8. R. Gage and D.A. Stopher. A rapid HPLC assay for voriconazole in human plasma. *J. Pharm. Biomed. Anal.* **17:** 1449–53 (1998).
9. F. Pehourcq, C. Jarry, and B. Bannwarth. Direct injection HPLC micro method for the determination of voriconazole in plasma using an internal surface reversed-phase column. *Biomed. Chromatogr.* **18:** 719–22 (2004).
10. L. Zhou, R.D. Glickman, N. Chen, W.E. Sponsel, J.R. Graybill, and K.W. Lam. Determination of voriconazole in aqueous humor by liquid chromatography–electrospray ionization-mass spectrometry. *J. Chromatogr. B* **776:** 213–20 (2002).
11. V.P. Shah, K.K. Midha, J.W. Findlay, H.M. Hill, J.D. Hulse, I.J. McGilveray, and G. McKay. Bioanalytical method validation-A revisit with a decade of progree. *Pharm. Res.* **17:** 1551–57 (2000).