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Short communication

Validation of rapid and simple LC–MS/MS method for determination of voriconazole in rat plasma

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Abstract

A rapid, simple and sensitive LC–MS/MS analytical method was developed and validated for the determination of voriconazole (VRC) in rat plasma, using ketoconazole as internal standard (IS). Analysis was performed on a Shimadzu® HPLC system using a Shimadzu® C18 column and isocratic elution with acetonitrile–water–formic acid (60:40:0.05, v/v/v), at a flow of 1.0 mL/min (split ratio 1:5), and a mass spectrometer Micromass®, equiped with a double quadrupole and an electrospray ionization interface, operated in a positive mode. Plasma samples were deproteinized with methanol (1:2) and 30 μL of the supernatant was injected into the system. The retention times of VRC and IS were approximately 3.3 and 2.7 min, respectively. Calibration curves in spiked plasma were linear over the concentration range of 50–2500 ng/mL with determination coefficient >0.98. The lower limit of quantification was 50 ng/mL. The accuracy of the method was within 5%. Intra- and inter-day relative standard deviations were less or equal to 12.5 and 7.7%, respectively. The applicability of the LC–MS–MS method for pharmacokinetic studies was tested using plasma samples obtained after intravenous administration of VRC to male Wistar rats. The reported method provided the necessary sensitivity, linearity, precision, accuracy, and specificity to allow the determination of VRC in pre-clinical pharmacokinetic studies.
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Keywords: Voriconazole; LC–MS/MS; Pharmacokinetics; Biologic fluid; Validation

1. Introduction

Voriconazole (VRC) (2*R*,3*S*)-2-(2,4-difluorophenyl)-3-(5-fluoro-4-pyrimidinyl)-1-(1H-1,2,4-triazol-1-yl)-2-butanol (Fig. 1) is a new antifungal agent that belongs to the triazole family and was the first drug in its pharmacological group, to be used in human clinical practice [1].

The compound exhibits a high activity against a broad spectrum of medically important pathogens including *Aspergillus* [2] and *Candida* species [3], as well as emerging fungal pathogens such as *Scedosporium* and *Fusarium* species [4].

The pharmacokinetics of VRC has been investigated in different animal species such as mice [5], Sprague–Dawley rats and guinea pigs [6]. Furthermore, there are several clinical pharmacokinetic evaluation of VRC in humans, including healthy volunteers, patients and children [7–10].

Many researchers have been using free plasma levels of triazolic antifungal drugs to evaluate their pharmacokinetic/pharmacodynamic indices and predict efficacy in pre-clinical studies, assuming that these levels are a good surrogate for the concentrations in the interstitial space [5,11,12]. However, the relationship between plasma and free tissue levels of voriconazole has not been established in animals or humans. To allow this investigation in animals, a voriconazole quantification method that deals with small sample volumes and presents a low limit of quantification is needed.

Voriconazole quantification in all studies is performed using HPLC with drug detection by ultraviolet [6–8,13,14,15] or mass spectrometry (MS) [16–19]. The first report using LC/MS for voriconazole quantification in biological fluids, aqueous humor, was made by Zhou et al. [16] who used solid-phase extraction for sample cleaning. The majority of the reported analytical methods

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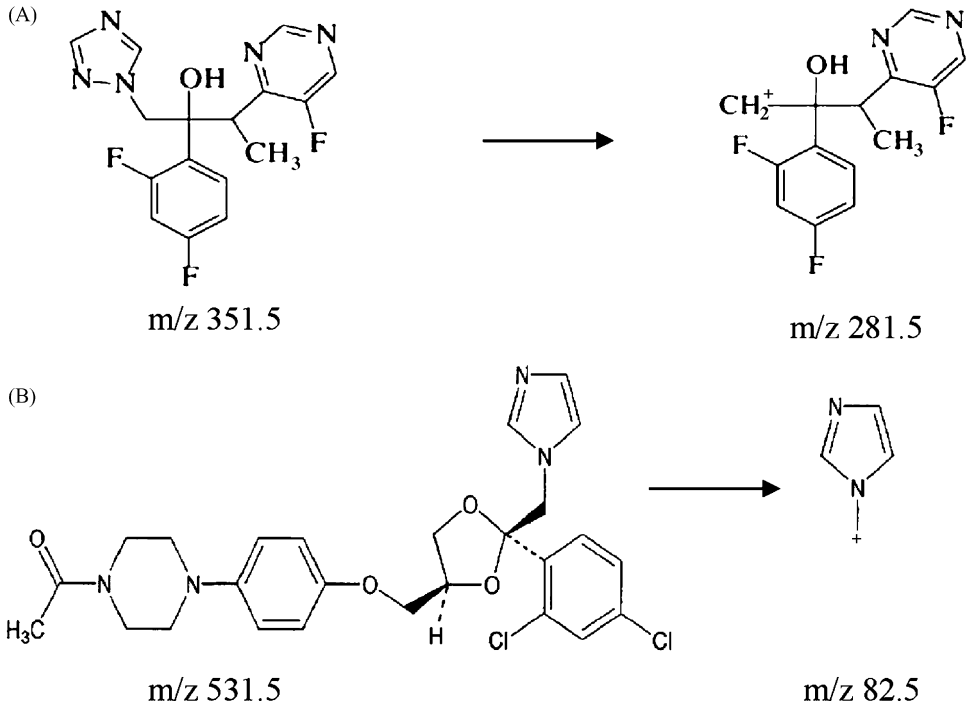

Fig. 1. Chemical structure of voriconazole (A) and the internal standard ketoconazole (B), and fragment ions formed from protonated molecules.

quantifying voriconazole in plasma by UV or LC/MS require big sample volumes, around 1–2 mL [6–8,13–15] or introduce a step to improve the limit of quantification, such as solid-phase extraction [17–19], which raises the cost of analysis. Using some these methods it is not possible to determined voriconazole pharmacokinetic profile following sequential sampling in the same animal [6,13,14] or to investigate drug disposition after the administration of small doses to animals, which are expected to produce drug levels in the low ng/mL range [6,17,18].

In this work, a liquid chromatography-tandem mass spectrometry (LC–MS/MS) method was developed and validated for the determination of voriconazole in rat plasma using small sample volumes and ketoconazole (Fig. 1) as internal standard (IS). The bioanalytical methodology was validated considering the specificity, linearity, precision, accuracy and lower limit of quantification. The applicability of the LC–MS/MS method for pharmacokinetic studies was tested using plasma samples obtained after intravenous (i.v.) administrations of voriconazole in a small dose (2.5 mg/kg) to male Wistar rats.

2. Experimental

2.1. Solvents and chemicals

Voriconazole (Mikatub, India) was kindly donated by Mikatub Laboratory. Ketoconazole was obtained from Delaware (Porto Alegre, Brazil). Analytical grade formic acid and HPLC grade acetonitrile and methanol were purchased from Merck (Darmstat, Germany). HPLC water from Millipore's Milli-Q System was used throughout the analysis.

2.2. Preparation of standard solutions and quality control samples

Two separate weighted amounts of voriconazole were used to prepare standard stock solutions at 500 μg/mL dissolved in methanol. One stock solution was used to prepare working solutions for the calibration curve standards. The other stock solution was used to prepare standard working solutions for the quality control (QC) samples. The calibration curve samples were prepared by spiking 100 μL of Wistar rat plasma with 10 μL of the appropriate standard working solution to obtain VRC final concentrations of 50, 100, 250, 500, 1000 and 2500 ng/mL. Quality control samples were prepared at 100, 800 and 2000 ng/mL. New standard stock solutions, calibration curve and QC were prepared on each analysis day.

Standard stock solution of ketoconazole (IS) was prepared at 500 μg/mL in methanol. This solution was successively diluted to result in a final concentration of 4 μg/mL of ketoconazole. The IS solutions were prepared on the day of the samples analysis, due to the low stability of IS in methanol.

Chromatographic analysis was carried out on a Shimadzu® liquid chromatograph and Micromass® model Quattro LC mass spectrometer, using LC-10ADVP solvent delivery system with low-pressure gradient flow control valve FCV-10ALVP, SCL-10AVP system controller and DGU-14A degasser. The injection was made with a Shimadzu® SIL-10ADVP automatic injector and analyses were performed using MassLynx® software (Version 3.5).

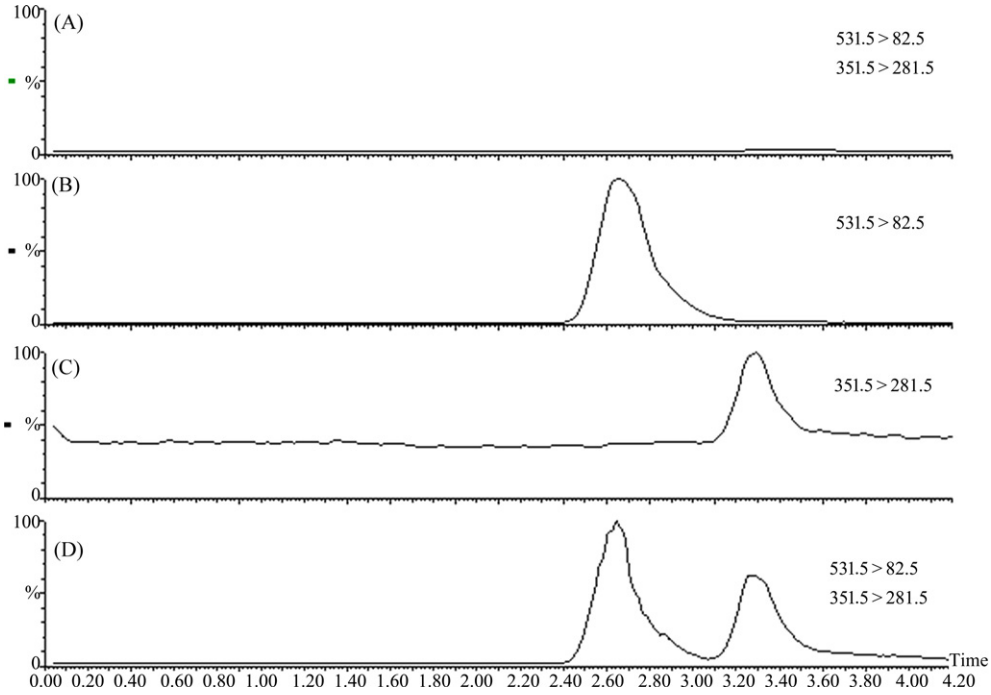

Fig. 2. Representative total ion chromatograms in MRM-ESI$^+$ mode in rat plasma: (A) blank plasma, (B) plasma spiked with VRC and IS (50 ng/mL and 133 μg/mL, respectively), (C) plasma spiked with VRC and IS (2500 and 133 μg/mL, respectively), (D) plasma sample 6 h post-administration of 2.5 mg/kg i.v. VRC (plasma concentration 74 ng/mL) with IS (133 ng/mL). The following daughter ion *m/z* ratios were monitored 350.5 > 281.5 for VRC and 531.5 > 82.5 for IS. The retention times observed were: I.S.: 2.7 min and VRC: 3.3 min.

2.3. Sample preparation

Prior to the chromatographic analysis, 100 μL samples were deproteinized by addition of 190 μL of ice-cold methanol and 10 μL of IS (4 μg/mL), vortexed for 30 s and centrifuged at 12,000 rpm, 4 °C for 15 min. The final concentration of ketoconazole in the samples was 133 ng/mL. All plasma samples, including calibration curve, QC and samples from pharmacokinetic experiments were processed in the same manner.

2.4. Instrumentation

2.4.1. Chromatographic and mass spectrometer conditions

The mobile phase used for the chromatographic separation was composed of acetonitrile–water–formic acid (60:40:0.05, v/v/v). The mobile phase was filtered before using and it was delivered isocratically at a flow rate of 1.0 mL/min (split ratio 1:5). The analysis was carried out at 40 °C using Shimadzu® Shim-pack HPLC column (150 mm × 4.6 mm i.d.) packed with 5 μm ODS stationary phase, protected by Waters μbondapack guard column packed with 50 μm RP18 material. The autosampler was set to inject 30 μL sample aliquots.

The analyte was monitored using mass spectrometer equiped with a double quadrupole and an electrospray ionization interface, operated in a positive mode (ESI$^+$). Samples were introduced into the interface through a heated nebulizer probe set at 250 °C. Nitrogen was the nebulizer and desolvation gas, and argon was used as collision gas. The other operating conditions were: nebulizer gas flow = 35 L/h; desolvation gas flow = 365 L/h; ion spray voltage = 3.2 kV; cone voltage = 36 V; ion source temperature = 130 °C; entrance potential = −13 V; collision energy = −16 V; collision cell exit potential = −17 V; collision gas pressure = 18 psi. The spectrometer was programmed in multiple reaction monitoring (MRM) mode to allow the specific transition of precursor ion to fragment for each compound. The detection of ion species was performed by monitoring the decay of the mass-to-charge (*m/z*) ratio 350.5, corresponding to the parent molecular ion of voriconazole, to the *m/z* 281.5 product ion, which correspond to the loss of triazole group from the parent molecule. IS was detected by decay of the 531.5 *m/z* precursor ion to the 82.5 *m/z* daughter ion (Fig. 2). This decomposition of the molecular ion of voriconazole was previously documented by Zhou et al. [16] and Egle et al. [19].

2.5. Validation procedures

Standard calibration curve and QC samples were analyzed in triplicate in two consecutive days. Linearity of calibration curves based on peak area ratio (area of analyte/area of IS) as function of the nominal concentration was assessed by weighted (1/concentration) least square regression. Slopes, intercepts and determination coefficients were calculated. The selectivity of the method was investigated for potential interferences of endogenous substances by using six independent batches of rat plasma samples. Moreover, the chromatograms of the experimental samples obtained after i.v. administration of VRC to Wistar rats were compared to the calibration curve standards chromatograms in order to detect interfering peaks. Intra- and inter-day precision and accuracy of the analytical method were shown by triplicate processing and following anal-

ysis of QC samples (100, 800 and 2000 ng/mL). Precision was calculated as relative standard deviation (R.S.D.) of the experimental concentrations and accuracy as the comparison between the experimental and nominal samples concentration. The criteria for acceptability of the data included accuracy within ±15% deviation from the nominal values and precision within ±15% R.S.D., except for the lower limit of quantification (LLOQ), where it should not exceed 20% of R.S.D. [20]. Recovery experiments (extraction efficiency) was performed by comparing the analytical results for extracted samples ($n = 3$) with unextracted standards that represent 100% recovery.

2.6. Stability preliminary studies

Preliminary studies were carried out to evaluate VRC stability under the conditions used in this work. Analytes stability was determined during blood sample collection and handling at room temperature for 2 h and after freezing plasma for 15 days at −36 °C. Furthermore, the stability of the processed samples was determined at 4 °C for 12 h in the autosampler.

2.7. Pharmacokinetic study

The animal experiments were approved by UFRGS Ethics in Research committe (# 2004300).

The applicability of the LC–MS/MS method for pharmacokinetic studies was tested using plasma samples obtained after intravenous administration of VRC to male Wistar rats ($n = 6$). The animals were kept under controlled 12 h light–dark cycle during the acclimation period with access to water and food *ad libitum*. For the experiments the animals were anesthetized with urethane (1.25 g/kg i.p.) and the carotid artery was used for blood collection by an indwelling catheter. The exposed artery and catheter were irrigated with heparinized (125 IU/mL) saline after inserting the catheter. For intravenous administration, the solution was prepared by dissolving VRC in saline containing 20% (v/v) of dimethyl sulfoxide (DMSO). About 0.5 mL of the VRC solution was administered by the femoral vein, corresponding to a 2.5 mg/kg dose. Blood samples (200 μL) were harvested immediately before drug administration (time zero) and at 0.08, 0.16, 0.25, 0.5, 1, 2, 4, 6, 8 and 10 h after administration. Blood were harvested into heparinized tubes and immediately centrifuged at 12,000 rpm at 4 °C for 15 min to obtain plasma. The plasma samples were frozen and stored at −36 °C until assayed. Plasma (100 μL) samples were processed as described in Section 2.4 for VRC quantification.

3. Results and discussions

3.1. Selectivity and recovery

The selectivity of the analytical methodology can be observed in Fig. 2 by comparing representative chromatograms of independent blank plasma (A), blank plasma spiked with ketoconazole (400 ng/mL) (B), plasma spiked with VRC (50 ng/mL) (C) and a sample rat containing VRC and IS (D). No additional peaks due to endogenous substances were observed that would interfere with the detection of the target compounds. Furthermore, the metabolites described for voriconazole in rats [6] cannot generate the same daughter fragment used (*m/z* 281.5), confirming that only the drug is quantified in this method.

Table 1
Calibration curve parameters and statistics for VRC in rat plasma[a]

Curve	Slope	*y*-intercept	Determination coefficient
Day 1			
1	0.00024	0.0013	0.998
2	0.00024	0.0011	0.994
3	0.00026	0.0016	0.999
Day 2			
1	0.00027	0.0012	0.999
2	0.00028	−0.0004	0.999
3	0.00028	−0.0004	0.999
Mean ($n = 6$)	0.00026		
S.D.	0.000019		
R.S.D. (%)	7.5		

[a] S.D., standard deviation; R.S.D., relative standard deviation reference.

The retention times of VRC and IS were approximately 3.3 and 2.7 min, respectively. The extraction recovery, 72.1 ± 2.1%, was concentration independent of VRC.

3.2. Linearity

The linearity of method was observed in the investigated concentration range (50–2500 ng/mL). The media calibration curve was determined in two consecutive validation days for VRC. Calibration curves of peak area ratio (VRC/IS) as function of nominal concentration were linear using weighted (1/concentration) linear regression, with a determination coefficient greater than or equal to 0.994 for all curves (Table 1).

3.3. Lower limit of quantification

The lower limit of quantification (LLOQ) was 50 ng/mL, which was accepted as the lowest level on the calibration curve that could be determined with appropriate precision and accuracy under the experimental conditions of this analytical method (Tables 2 and 3) [18]. Previously Keevil et al. [17] described a LC–MS/MS method for voriconazole quantification in serum also using small volume sample but a different daughter fragment (*m/z* 224.1), less abundant than the one used in the present study, was employed. For that method the reported lower limit of quantification was 100 ng/mL. Using the present method the limit of quantification could be reduced in 50%. Furthermore, only protein precipitation was needed to clean the sample in opposition to the solid-phase extraction described by those authors, showing the advantages of the present method for pharmacokinetic investigation is rodents.

3.4. Precision and accuracy

The intra- and inter-day relative standard deviation values for VRC are shown in Table 2. The intra-assay and inter-assay precision (R.S.D.) values for QC samples were less than or equal

Table 2
Intra- and inter-day variation of voriconazole in rat plasma

Nominal concentration	Day	Experimental concentrations		
		Mean (ng/mL)	S.D.	R.S.D. (%)
Intra-day variation				
50 ng/mL (LLOQ)	1	56	0.6	0.99
	2	49	8.1	6.8
100 ng/mL	1	105	8.0	7.7
	2	94	3.8	4.1
800 ng/mL	1	828	41.8	5.0
	2	748	48.9	6.5
2000 ng/mL	1	2069	67.9	3.2
	2	1829	232.1	5.6
Inter-day variation				
50 ng/mL (LLOQ)		53	6.6	12.5
100 ng/mL		100	8.2	8.1
800 ng/mL		788	59	7.5
2000 ng/mL		1950	151	7.7

Values (mean and S.D.) represent $n = 3$ observations/day for intra-day and $n = 6$ observations for inter-day variation.

to 7.7 and 12.5%, respectively. The method showed an accuracy within 15%, which can be observed in Table 3. The data obtained for VRC was within acceptable limits stated for bioanalytical methods validation [20].

3.5. Stability

Preliminary studies showed that VRC was stable under the conditions used in this work. The analyte was stable in blood samples at room temperature for 2 h (99.4%) and in plasma samples after freezing at −36 °C for 30 days (99.4%). The stability of processed samples was determined at 4 °C in the autosampler for 12 h. VRC and internal standard did not degrade at these condition, showing 99.2 and 97.6%, recovery, respectively.

3.6. Applicability of the analytical method

To investigate the suitability of this analytical method for pharmacokinetic studies, it was applied to determine VRC plasma concentration after i.v. administration of a single dose (2.5 mg/kg) to Wistar rats ($n = 6$). The mean plasma concentration–time profile of VRC is shown in Fig. 3. The results indicated that the analytical method is suitable to measure plasma concentrations of the compound in rat plasma. The peak plasma concentration was 455.5 ± 300.2 ng/mL. The estimated half-life was found to be 2.4 ± 0.6 h and the $AUC_{0-\infty}$ was 1.54 ± 0.35 μg h/mL. The elimination phase of VRC was appropriately characterized considering that the pharmacokinetic study was carried out for 8 h which, which corresponds to three elimination half-lives. The result prove the method developed here has adequate sensitivity for the intended application.

Table 3
Accuracy for the analysis of voriconazole in rat plasma

Concentration (ng/mL)	Range	Accuracy
50 (LLOQ)	45–59	88.4–118.4
100	93–113	96.8–113.0
800	739–848	92.4–106.9
2000	1837–2147	91.6–107.3

$n = 6$ observations.

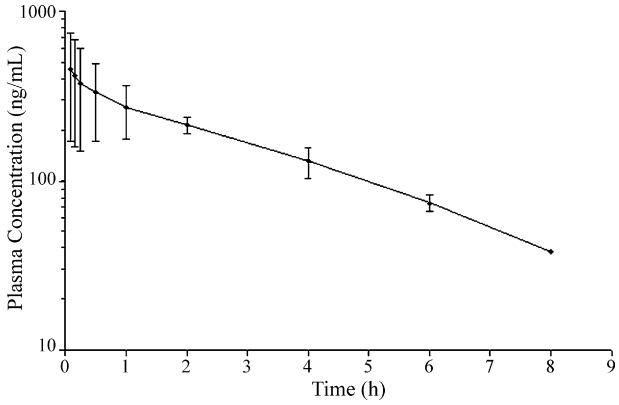

Fig. 3. Mean plasma concentration–time of VRC after single intravenous dose of 2.5 mg/kg to male Wistar rats. The data points are means and positive error bars are S.D. of six animals.

4. Conclusions

A LC–MS/MS bioanalytical method for the determination of VRC in rat plasma using ketoconazole as IS was established. This method showed adequate sensitivity, linearity, precision and accuracy and it has been successfully applied to determine the concentration–time profiles in pharmacokinetic studies employing rodents.

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