Hacettepe University Journal of the Faculty of Pharmacy
Volume 33 / Number 2 / July 2013 / pp. 149-162

A Fully Validated Chromatographic Determination of Voriconazole from Infusion Solution and Tablet

Received : 20.12.2013
Revised : 03.03.2014
Accepted : 14.04.2014

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Introduction

The azole derivatives constitute the largest group of commercially available antifungals. They have a broad spectrum against fungi and some gram-negative bacteria[1]. These compounds inhibit the development of fungi cells by blocking ergosterol biosynthesis[2]. The second generation triazoles including voriconazole (VOR) were introduced into the treatment with stronger activity and lower toxicity compared to other antifungals[3] in different pharmaceutical formulation as tablet, intravenous infusion, suspension and ophthalmic solution. Voriconazole (2R, 3S)-2-(2, 4-difluorophenyl)-3-(5-fluoropyrimidin-4-yl)-1-(1H-1, 2, 4-triazol-1-yl) butan-2-ol) is a triazole antifungal derived from fluconazole and used in the treatment of the fungal pathogens of *Candida Aspergillus*, *Cryptococcus* and *Fusarium* (Figure 1).

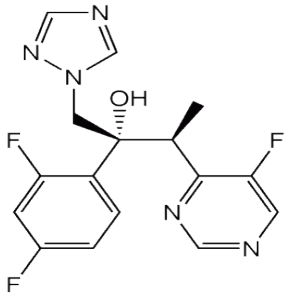

Figure 1
Chemical structure of VOR

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The analysis of VOR by high performance liquid chromatography (HPLC) from various matrices such as from bulk drug, tablet, suspension and ophthalmic solution[4-11] has been reported in the literature. The methods for pharmaceutical analysis of VOR are utilized C_{18} and C_8 functional group bonded stationary phases containing long columns (250 x 4.6 mm) packed with particles in 5 μm size. VOR is a moderately lipophilic drug (log $D_{7.4}$ = 1.8)[12] thus C_{18} functional group containing stationary phases are highly compatible for its elution. The progressive effect of reduced particle size of packing material and use of small columns on chromatographic separation is very well known, and then 50 mm x 3 mm i.d. column packed with 2 μm sized packing material was used for elution of VOR in ultra flow liquid chromatography (UFLC) system. Moreover, no liquid chromatographic method has been published in the literature for the analysis of VOR from its intravenous infusion preparation.

In presented study, UFLC method for analysis of VOR from tablet and intravenous infusion preparations was developed, and fully validated according to ICH Guidelines[13], in terms of specificity, selectivity, accuracy, precision, sensitivity, linearity, range, robustness, ruggedness and recovery. Moreover, applicability the method to the analysis of VOR from tablet and intravenous infusion formulations and long term performance in routine analysis via quality control chart for developed and validated method was also tested.

Material and Methods

Reagents

VOR is kindly donated by Pfizer Drug Inc (Turkey). Phenazoprydine (IS) was purchased from Merck (Darmstadt, Germany) while methanol was purchased from Sigma. Milli-Q water was used in the mobile phase and all reagents were in analytical grade.

Instrumentation

The UFLC equipment was comprised of a solvent delivery system (Shimadzu LC - 20AB) and a DAD detector (Shimadzu SPD - M20A). The auto sampler (Shimadzu SIL - 20AC) was used for sample injection. UV

detection was employed at 255 nm. A LC Solution software (Shimadzu Technologies) was used for data analysis. Bandalin Snorex marked RK 514 BH ultrasonicator and Titan marked filter papers were used for filtering the mobile phase.

Chromatographic conditions

Chromatographic separations were performed using an ACE Excel 2 C_{18} (50 mm x 3 mm i.d., 2 μm) column made in Aberdeen, Scotland. The mobile phase consisted of methanol and water (50: 50, v/v) was mixed during the analysis by a binary gradient pump. The flow rate was 0.40 mL/min and injection volume was 5 μL. Diode array detector was set to 255 nm and the analysis were performed in room temperature.

Standards and stock solutions

Stock solution of the VOR (1000 μg/mL) was prepared in methanol and stored in deep-freezer at -23 °C. Working solutions of VOR were daily prepared from stock solutions by diluting with mobile phase. IS were prepared at 1000 μg/mL in water, stored in deep-freezer at -23 °C and dissolved before use.

Sample Preparations

In infusion preparation analysis, content of one preparation (200 mg) was dissolved in 250 mL methanol and 12.5 μL of this solution spiked with 5 μL of IS (1000 μg/mL) and then diluted to 1 mL with mobile phase in a vial. The ingredients for the intravenous infusion preparation were not clearly explained in all sources thus to clarify the specificity of developed method in the analysis of intravenous infusion preparations was tested by standard addition technique.

In tablet analysis, five tablets were accurately weighed and fine-powdered and mixed in a mortar. A portion of the powder equivalent to the average weight of one tablet was transferred into a 250 mL volumetric flask and methanol was added. The content of the flask was treated in an ultrasonic bath for 15 min. After shaking, the solution was taken into centrifuge tubes of 20 mL's and then centrifuged for 10 min at 5000 rpm to decant insoluble excipient. VOR tablet solution (12.5 μL of supernatant) was transferred into a vial, 5 μL IS (1000 μg/mL) added and then diluted to 1 mL with mobile phase. The quantitative values in the drugs

were calculated using the regression equations of the corresponding calibration curves plotted for the method.

Synthetic tablet preparation was consisted of 200 mg VOR added 310 mg lactose, 80 mg starch, 13 mg carboximeloz Na, 13 mg magnesium stearate and povidone mixture. Tablet placebo solution was containing all above mentioned excipient but not VOR.

Assay Validation

The method was validated according to current International Conference on Harmonization Guideline for Validation of Analytical Methods (ICH) over the parameters of stability, specificity, linearity, sensitivity as limit of detection (LOD) and limit of quantification (LOQ), accuracy, precision, robustness, and ruggedness[13-15].

Results and Discussion

Method optimization

Elution of VOR in UFLC was performed through ACE Excel 2 C_{18} (50 mm x 3 mm i.d., 2 μm) column using an optimized mobile phase. Organic solvent content (type and amount) and flow rate of the mobile phase was optimized for the best resolution and peak shape. Methanol or acetonitrile in combination with water in different ratios (30% - 55%) at different flow rates (0.3 - 0.45 ml/min) were tested as optimization parameters for mobile phase. The mobile phase consisted of methanol and water (50:50, v/v) at a flow rate of 0.40 mL/min resulted in best separation for VOR, at which k' =7.08, N = 2952.54, R_s = 2.74 and A_S = 1.16, and all chromatographic parameters were acceptable on the basis of these scientific and pharmacopea criteria. Because, chromatographic parameters were satisfactory enough buffer, or any ion-pairing agent e.g. triethylamine (TEA) was not used in the mobile phase. Under selected chromatographic conditions, the back pressure was 275 bar, and retention times of VOR and IS were 6.09 and 7.35 min, respectively.

Specificity

The selectivity of the method in tablet analysis was evaluated by comparison results obtained from standard, synthetic, tablet and placebo

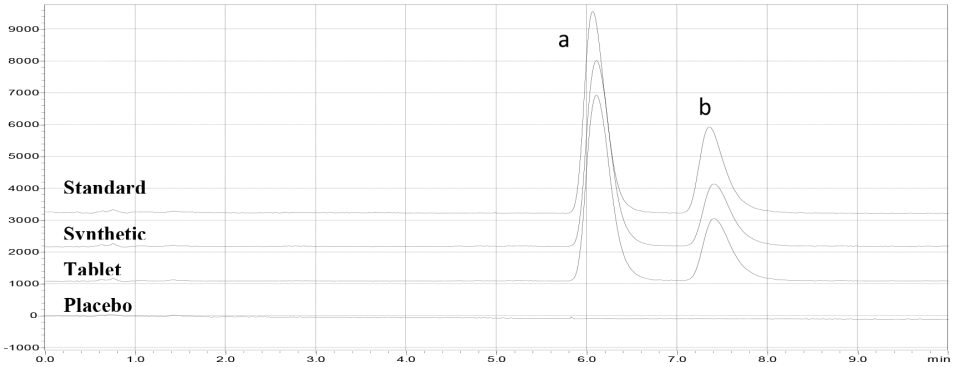

Figure 2
Representative chromatograms of standard, synthetic tablet, tablet and placebo solution for (a) VOR (10 μg/mL) spiked with (b) IS (5 μg/mL)

solutions for VOR analyzed using developed method. VOR and IS were well resolved from baseline and from each other and no interfering peak was observe from tablet matrices (Figure 2).

Selectivity

The method selectivity for infusion preparation analysis was controlled by comparing the slopes of the regression equations of VOR obtained using internal standard calibration curves and standard addition technique at 10, 20, 30 and 40 mg/ml concentration levels for VOR. The slopes for internal calibration and standard addition curves were found to be 0.1820 and 0.1818, respectively. Moreover, the concentrations detected for intravenous infusion preparation sample using both standard addition and internal calibration curves were 199.21 ± 0.28 and 199.53 ± 0.40, respectively ($n=6$, $p > 0.05$). Thus the differences in the slopes and VOR concentrations in infusion preparation found by two calibration techniques were not statistically significant. The results demonstrated that matrix effect and interference does not happen in the analysis intravenous infusion preparations by developed method.

In addition, VOR and IS peaks spectra recorded at DAD detector from samples and standard were not different, thus peak purity indexes were about unit.

Stability

The VOR standard solutions (10 μg/mL) were waited at day light, in dark and at + 4 °C for 8, 24 and 48 hours, and then analyzed by the developed method. Their responses were compared with the results in fresh VOR standard solution at the same concentration. The lowest remaining VOR values were calculated in 48 hours at all tested conditions and found to be 99.60%, 99.79% and 99.87%, respectively. For long term stability testing, VOR standard solutions (10 μg/mL) were stored in deep-freezer (-23 °C) for 1 month, and the remaining VOR ratio was 98.72%. Since the differences were lower than 2%, it was suggested that VOR was stable at day light, in dark and at +4 °C for 48 hours and in deep-freezer for 1 month.

Sensitivity

Limit of detection (LOD) and quantification (LOQ) values for VOR were decided according to signal to noise ratio values (S/N) at about 3 and 10, respectively. LOD and LOQ for VOR were found to be 50 ng/mL and 100 ng/mL, respectively, and were satisfactory sensitivity for the analysis of VOR in pharmaceutical preparations.

Linearity

Calibration curve was constructed at 11 different concentrations of VOR (0.5, 1, 5, 10, 20, 30, 40, 50, 60, 70, 100 μg/mL). The ratio of the peak area (VOR/IS) values were plotted against to the concentration of VOR for calibration curve establishment. The regression equation of the calibration curve was $y = 0.182 (\pm 0.249)x + 0.1022(\pm 0.032)$ ($R^2 = 0.9991$, n=6), where y is the ratio of peak area (VOR/IS) and x is the concentration of VOR. The validity of linear regression line was verified by means of ANOVA. There was linear regression and no deviation from linearity at 95% confidence of interval.

Recovery

In recovery studies synthetic preparations were spiked with 10 μg/mL VOR standards then analyzed via developed UFLC method. The mean of the recovery was calculated as 100.64 ± 0.48% (RSD: 1.18%, n=6), indicating that the method has a high recovery.

TABLE I
Precision and accuracy results for VOR

Standard solution	Added (mg/mL)	Inter-day			Intra-day		
		Found $\overline{X}$ (mg/mL)	Precision RSD %	Accuracy Bias %	Found $\overline{X}$ (mg/mL)	Precision RSD %	Accuracy Bias %
VOR	5.00	5.03 ± 0.04	1.81	0.64	5.03 ± 0.01	0.65	0.65
	40.00	40.00 ± 0.14	0.85	0.00	40.17 ± 0.08	0.49	0.42
	80.00	80.32 ± 0.36	1.08	0.40	80.17 ± 0.13	0.41	0.21

$\overline{X}$: Mean±Standard error (n=6); RSD%: Relative standard deviation %;
Bias%: [(Found–Added) / Added] x100

Accuracy and Precision

Precision and accuracy of the method were investigated by intra-day and inter-day determinations of VOR at three concentration levels in the linearity range. The highest RSD value was calculated as 1.81% in inter-day and intra-day studies that were lower that acceptable 2% of RSD value for developed UFLC method (Table I).

The repeatability studies for the instrument and developed method were performed by analyzing 40 μg/mL standard solution and demonstrated high repeatability with the RSD values of 0.56% and 0.71%, respectively.

Ruggedness

Ruggedness studies were performed by the comparison of two different analysts' results at 10 μg/mL of VOR which was in the linearity range, and the differences were statistically insignificant ($p > 0.05$, n=6).

Robustness

The robustness study was performed by testing the response of the method to small deliberate changes in mobile phase organic solvent content of methanol (48 and 52 %) and flow rate (0.38 and 0.42 mL/min) for 10 mg/mL VOR standard. The results were compared with the ones at optimum conditions and no statistically significant difference was found ($p > 0.05$, n=3). Therefore the method was considered robust to the small changes in experimental conditions.

TABLE II
System suitability of the method (n=6).

Tested Parameters	**VOR**	**IS**
Injection repeatability (RSD% ≤ 1) *	0.28%	0.33%
Capacity factor (k' > 2)	7.08	8.82
Resolution (Rs > 1.5) **	2.74	
Asymmetry factor (≤ 1.5)	1.16	1.36
Theoretical plate number (N >2000)	2952.54	3351.30

* : The values in the brackets are pharmacopeias limits for related parameters.
** : Resolution was calculated between VOR and IS.

System suitability

The system suitability of the method was assessed by injection repeatability, capacity factor, resolution, theoretical plate number and peak asymmetry of VOR (10.0 μg/mL) and IS (5.0 μg/mL). Criteria values were within acceptable levels stated in pharmacopeia[16] therefore developed and fully validated method was suitable in order to determine VOR in pharmaceutical preparations (Table II).

Pharmaceutical preparation applications

The developed and fully validated UFLC method was successfully applied to the analysis of tablets and intravenous infusion preparations commercially available in Turkish Pharmaceutical Market (Table III).

Quality Control Charts

Quality control charts were used to monitor long term quality of any method throughout its use in routine analysis, and in this study, single variable Shewhard's quality control chart was used. Quality control (QC) sample was containing 20 μg/mL of VOR and 5 μg/mL of IS and was analyzed 20 independent days to mimic independent runs in real life. The chart was progressed by the calculation of standard deviation (SD) values of mean values of the solutions analyzed. The values in the range mean value ± 2 SD limits were in warning zone. The values in the region between warning zone and mean value ± 3 SD limits were considered to be in the control zone. The values out of mean value ± 3 SD limits were in out of control region (Figure 3).

TABLE III
Results of pharmaceuticals analysed via developed and validated UFLC method

	VFEND® Tablet	**VFEND® Intravenous infusion**
$\overline{X}$	200.50 ± 0.47	199.53 ± 0.40
SD	1.15	0.97
RSD (%)	0.57	0.49

$\overline{X}$: Mean ± Standard error (n=6), SD: Standard deviation, RSD: Relative standard deviation

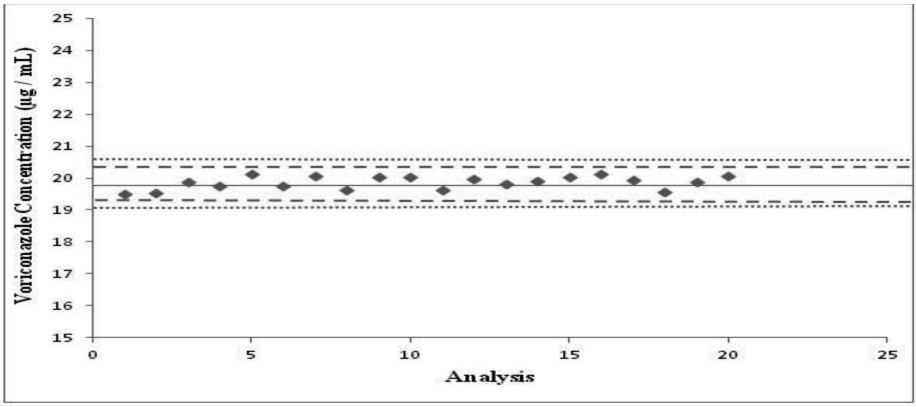

Figure 3
Shewhard's quality control chart for VOR analysis by developed and validated UFLC method

The mean value for QC sample over 20 independent analysis was 19.86 ± 0.05 µg/mL, having a variation in the range of day-to-day precision which were having RSD and bias values less than 2%. None of the QC results were at the out of control region, and also in between control zone and warning limits. All QC results were randomly dispersed in reliable reagon and at two sides of the mean value. Thus developed and validated UFLC also provides a long term quality and reliability for VOR analysis results in tablet and intravenous infusion preparation for routine testing.

Conclusion

Developed ultra flow liquid chromatographic (UFLC) method for determination of Voriconazole (VOR) in intravenous infusion and tablet preparations has been evaluated over specificity, sensitivity, linearity, precision and accuracy, ruggedness and robustness, and proven to be convenient for the analysis of VOR in its intravenous infusion and tablet preparation[14-15]. Because VOR is a moderately liphophlic, C_{18} functional group containing stationary phase resulted in a high performance for VOR elution. Additional reduced column dilution and band sharpening effect of small column (50 mm x 3 mm i.d.) packed with 2 μm sized packing material, prompt high symmetry, theoretical plate number, resolution for VOR peak in UFLC system at 0.4 ml/min flow rate. Moreover, no liquid chromatographic method has been published in the literature for the analysis of VOR from its intravenous infusion preparation. Thus, developed UFLC method was simple, sensitive, precise and accurate and cost reduced with low solvent consumption because of its low flow rate for routine quality controls.

Özet

Vorikonazol' ün İnfüzyon Çözeltisi ve Tabletten Valide Edilmiş Kromatografik Tayini

Vorikonazol (VOR) immün sistemi baskılanmış hastalarda oluşabilecek invazif aspergilloz tedavisinde kullanılan önemli bir ilaçtır. Bu çalışmada, intravenöz infüzyon ve tablet formülasyonlarından VOR tayini için bir ultra akış sıvı kromatografisi (UFLC) yöntemi geliştirildi ve ICH rehberlerine uygun olarak valide edilmiştir. İç standart Fenazopiridin (IS) ile birlikte VOR analizi ACE Excel 2 C18 (50 mm x 3 mm id, 2 um) analitik kolonu kullanılarak gerçekleştirilmiştir. Bileşenler metanol ve su (50: 50, v/v) içeren hareketli faz ile 0.40 mL/dk akış hızında elüe edildi. Dizi diyot dedektör dalga boyu 255 nm olarak ayarlanmıştır. Bu kromatografik şartlar altında VOR ve IS alikonma zamanları sırasıyla 6.09 and 7.35 dk 'dır. VOR için LOD ve LOQ değerleri 50 ng/mL and 100 ng/mL olarak bulunmuştur. Geliştirilen metot VOR için 0.5 - 100 μg/mL aralığında doğrusaldır. Gün içi- günler arası çalışmalarda en yüksek

BSS değeri %1.81 olarak bulunmuştur. VOR için ortalama geri kazanım 100.64 ± 0.48% (RSD: 1.18%, n=6) dır. Intravenöz infüzyon ve tablet preparasyonlardan VOR analizi için geliştirilmiş yöntemin; farklı analizci ve kromatografik koşullarında küçük değişiklikler açısından, sağlam ve dayanıklı olduğu bulunmuştur. Geliştirilen yöntemin farmakopelerde belirtilen kriterlere dayanan sistem uygunluk değerleri, VOR'un intravenöz infüzyon ve tablet preparasyonlardan tayini için uygun olduğunu ortaya koymaktadır. VOR analizi için geliştirilen ve valide edilen UFLC yönteminin uzun dönem güvenirlik çalışmaları Shewhard kalite kontrol kartları ile gösterilmiştir. Yöntem rutin kalite kontrol çalışmaları için basit, duyarlı, kesin, doğru olup düşük akış hızından dolayı az solvent kullanımı ile ucuzdur.

Anahtar Kelimeler: Vorikonazol, sıvı kromatografisi (LC), fenazopiridin, sistem uygunluk, kalite kontrol kartları

Summary

VOR is a major drug used in the treatment of invasive aspergillosis, which may occur in immunocompromised patients. In this study, an ultra flow liquid chromatographic (UFLC) method for determination of VOR in intravenous infusion and tablet preparations was developed and fully validated according to the ICH guidelines. The analysis of VOR together with internal standard Phenazoprydine (IS) was performed using an analytical column of ACE Excel 2 C_{18} (50 mm x 3 mm i.d., 2 μm). Substances were eluted by a mobile phase consisted of methanol and water (50: 50, v/v) at a flow rate of 0.40 mL/min. Diode array detector was set to 255 nm. Under these chromatographic conditions retention times of VOR and IS were 6.09 and 7.35 min, respectively. LOD and LOQ values for VOR were found to be 50 ng/mL and 100 ng/mL. Developed method was linear over the range from 0.5 to 100 μg/mL VOR. The highest RSD value was calculated as 1.81% in inter-day and intra-day studies. The mean recovery for VOR was 100.64 ± 0.48% (RSD: 1.18%, n = 6). Developed method for the analysis of VOR in intravenous infusion and tablet preparations was found to be rugged and robust in terms of different anaylst and minor changes in chromatographic conditions. System suitability values of developed method on the basis of criteria stated in phar-

macopeias were over the limit values suggesting that was suitable in order to determine VOR in its intravenous infusion and tablet preparations. The long term reliability of the results from developed and fully validated UFLC method presented here was demonstrated via Shewhard's quality control chart of VOR. Thus, developed UFLC method was simple, sensitive, precise, accurate and cost reduced with low solvent consumption because of its low flow rate for routine quality controls.

Key words: voriconazol, liquid chromatography (LC), phenazoprydine, system suitability, quality control chart

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