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ORIGINAL RESEARCH

Development and Validation of an HPLC Method for Voriconazole Active Substance in Bulk and its Pharmaceutical Formulation

Neslihan ÜSTÜNDAĞ OKUR, Emre Şefik ÇAĞLAR, Vildan YOZGATLI

ABSTRACT

The aim of the present study was to develop and validate a High-Performance Liquid Chromatography (HPLC) method for the determination of voriconazole in drug substances and *in situ* gel. A mixture of acetonitrile and ultrapure water (50:50) (v/v) was used as mobile phase. The column was a C18 column (150x4.6mm with 5µm particles). The eluent was monitored with UV detection at 256 nm and flow rate was set to 1 mL/min. The method was validated partially with respect to system suitability, linearity, limits of detection (LOD) and quantitation (LOQ), precision, accuracy, specificity, selectivity and stability. Obtained results showed that the analytical method had good linearity, accuracy, precision, selectivity and stability. Analytical method development results indicated that the LOD was 0.022 µg/mL; LOQ was 0.065 µg/mL and assay exhibited a linear range of 1- 30 µg/mL.

Keywords: Voriconazole, HPLC, method, validation

INTRODUCTION

Voriconazole is chemically named as [(2R,3S)-2-(2,4-difluorophenyl)-3-(5-fluoro-4-pyrimidinyl)-1-(1*H*-1,2,4-triazol-1-yl)-2-butanol] with an empirical formula of $C_{16}H_{14}F_3N_5O$ (Figure 1) (1) and its molecular weight is 349.3 Da (2). It is the Biopharmaceutics Classification System (BCS) class II antifungal drug with low aqueous solubility (3).

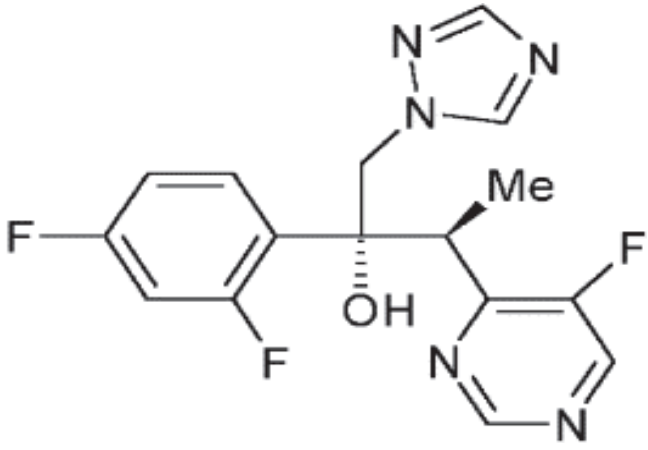

Figure 1. Chemical structure of voriconazole molecule

Neslihan Üstündağ Okur, Emre Şefik Çağlar, Vildan Yozgatlı
Istanbul Medipol University, School of Pharmacy, Department of Pharmaceutical Technology, Beykoz, 34810 Istanbul, Turkey

Corresponding Author: Neslihan Üstündağ Okur
Istanbul Medipol University, School of Pharmacy, Department of Pharmaceutical Technology, Beykoz, 34810, Istanbul, Turkey.
E-Posta: *neslihanustundag@yahoo.com*
Tel: *+90216 6815100/5190*
Fax: *+090212 5317555*

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Antifungal drugs carrying azole rings are the most commonly used agents in terms of treating superficial and systemic fungal infections. They work through a common mechanism of action; they selectively inhibit the synthesis of a major component of fungal cell; ergosterol and they alter the permeability of cell membrane by binding to the phospholipids of fungal cell membrane (4, 5). Voriconazole, a new-generation antifungal agent, possesses phenomenal characteristics like broad-spectrum activity against resistant fungal species, and acceptable tolerability. Almost 100% *in vitro* susceptibility was observed against various fungal isolates associated with keratitis and endophthalmitis. Moreover, studies suggested an excellent efficacy of voriconazole against several ocular mycoses following topical administration (6).

Fungal keratitis is a leading cause of serious ocular morbidity and blindness. It shows worldwide spread, but is more common in the tropics and subtropical regions. In fungal keratitis, early diagnosis and antifungal therapy is necessary in terms of preventing further complications such as hypopyon formation, endophthalmitis, or loss of vision (7). Whilst reports on the use of 1% voriconazole eye drops in the management of ophthalmic fungal keratitis have been promising, the voriconazole concentrations achieved in the eye were not sufficiently high to treat all types of fungal keratitis (8).

Voriconazole was studied for HPLC method and validation for bulk samples and tablet dosage forms (9, 10). Furthermore voriconazole was determined in human plasma and serum by HPLC for application to pharmacokinetic studies of voriconazole (11, 12, 13). Although several HPLC methods can be found in the literature for Voriconazole, to date, no HPLC methods for voriconazole determination in *in situ* gel formulation have been described. In the present investigation, a simple, optimized, and validated HPLC method was developed for the standardization of voriconazole. The aim of this study was to develop and validate an analytical method for voriconazole in bulk and *in situ* gel (containing poloxamer 188, poloxamer 407 and benzalkonium chloride), which is a novel drug delivery system for voriconazole active ingredient.

EXPERIMENTAL

Materials

HPLC grade Acetonitrile (Sigma, Germany) was used for HPLC. Voriconazole was purchased from Sigma, USA. Poloxamer 407 and poloxamer 188 were purchased from BASF, Turkey. All the other chemicals and solvents were analytical or HPLC reagent grade. Ultrapure water was obtained from Direct-Q® Water Purification System, Germany.

Instrumentation

The HPLC system consisted of a gradient pump, thermostable column department and a UV detector supplied by Agilent 1100. The column was a C18 column (150x4.6mm,5µm) (GL Sciences, Japan). The column compartment was temperature controlled and UV detector was employed throughout the analysis. Chromatographic data was acquired by using empower software.

Chromatographic Conditions

The voriconazole samples were analyzed using a UV spectrometer (UV-1800, Shimadzu, Japan) covering the range of 190–400 nm. The optimum wavelength selected was 256 nm (Figure 2), which represents the wavelength of maximum response for all impurities in order to permit simultaneous determination of related impurities of voriconazole.

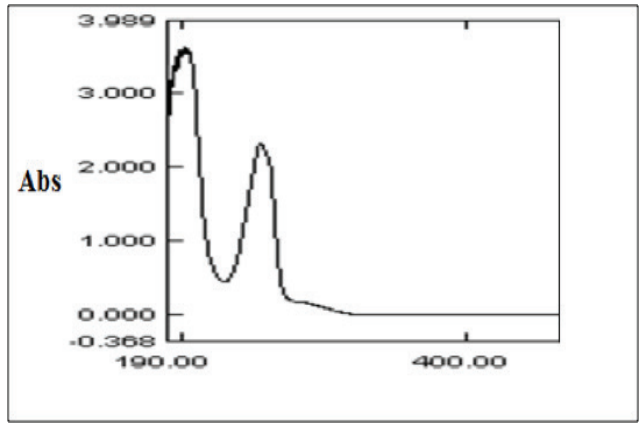

Figure 2. UV spectrum of voriconazole

The mobile phase was prepared by mixing acetonitrile and ultrapure water (50:50) (v/v) with flow rate of 1 mL/min (14). All solutions were filtered through a 0.45 µm membrane (Sartorius, Germany) prior to use. The flow rate was 1 mL/min and the column temperature was maintained at 25±1 °C. The volume of injection was adjusted 10 µl. The column was equilibrated for at least 40 min with the mobile phase flowing through the system before the injection of the drug standards. The run time was set at 10 min with the system operating at air-conditioned temperature (25±1 °C).

Preparation of stock solutions and standard working solution

Stock solutions of voriconazole (100 µg/mL) was prepared by dissolving 6.2 mg of drug in 62 ml mixture of acetonitrile and ultrapure water (50:50) (v/v). The standard solutions were stored at 4±1 °C in a clear glass volumetric flask and light-protected with aluminum foil. Voriconazole concentrations in the working solution chosen for the calibration curves were 1, 3, 5, 8, 11, 14, 17, 20, 25 and 30 µg/mL. Quality control (QC) samples (of low, medium and high concentration) at 8, 11 and 14 µg/mL were prepared in the same ways as the calibration standards. These working solutions were prepared fresh daily by making further dilutions of the stock solution in mobile phase. All samples were filtered through an aqueous 0.2 µm pore size membrane filter before injection.

HPLC Method Validation

The described method was validated partially with respect to system suitability, linearity, limit of detection (LOD) and quantitation (LOQ), precision, accuracy and specificity, selectivity and stability.

Specificity/Selectivity

The specificity of an analytical method is its ability to measure accurately and specifically the analyte in the presence of components that may be expected to be present in the sample matrix. To evaluate the specificity of the analytical method, the blank and drug loaded pharmaceutical formulation has been injected into the chromatographic system. These parameters were determined by comparing the chromatograms of the voriconazole standard, drug loaded *in situ* gel and blank *in situ* gel (15).

Linearity

The linearity between peak area and concentration was analyzed using calibration curve obtained from standard solutions of voriconazole (1 to 30 µg/mL) (16). In addition the linearity was evaluated by linear regression analysis, which was calculated by the least-square regression analysis.

Accuracy

The accuracy of an analytical method is the closeness of test results obtained by the method to the true value and is defined recovery. The prepared three standard solutions (8, 11, 14 µg/mL) were injected five times at different levels as a test sample (17).

Precision

8 µg/mL voriconazole solution and voriconazole loaded i*n situ* gel was injected ten times in order to evaluate method precision, standard deviation (SD) and coefficient of variation (CV%) (16). For intermediate precision study, three samples which different concentration were prepared and injected ten times. Intermediate precision is a measure of repeatability within laboratory variation. In order to evaluate the intermediate precision parameter, six different test solutions prepared by different analysts using the same drug substance sample have been analyzed by using different chromatographs and the difference of the experimental results obtained by two analysts has been calculated (18).

Stability of the Solution

A sample solution of voriconazole was prepared and analyzed initially and also at the end of the 48 hour by keeping the solution at room temperature (19).

Robustness

To determine the robustness of the developed method, experimental conditions were deliberately altered (18). The flow rate of the mobile phase was 1.0 mL/min. To study the effect of flow rate on the resolution, flow was ranged in between 0.5 to 1.5 mL/min.

RESULTS AND DISCUSSION

The UV spectrum of voriconazole in mobile phase was scanned in the region between 190 to 400 nm. The λ_{max} was determined at 256 nm (Figure 2). In this method the retention time of voriconazole was about 4.09 min.

The specificity and selectivity describe the capacity for each concentration of the analytical method to measure the drug in the presence of impurities, excipients, degradation products or matrix components. These parameters were determined by comparing the chromatograms of the voriconazole standard, drug loaded *in situ* gel and blank *in situ* gel.

There was no interference from the blank and also from the impurities at voriconazole peaks. The chromatogram of the voriconazole standard presented a peak in the retention time of 4.09 (Figure 3A). The chromatogram of standard solution without voriconazole is Figure 3B .The chromatogram of voriconazole loaded in situ gel sample (Figure 3C) showed a peak and retention time similar to voriconazole standard.

The chromatogram of *in situ* gel without voriconazole is Figure 3D.

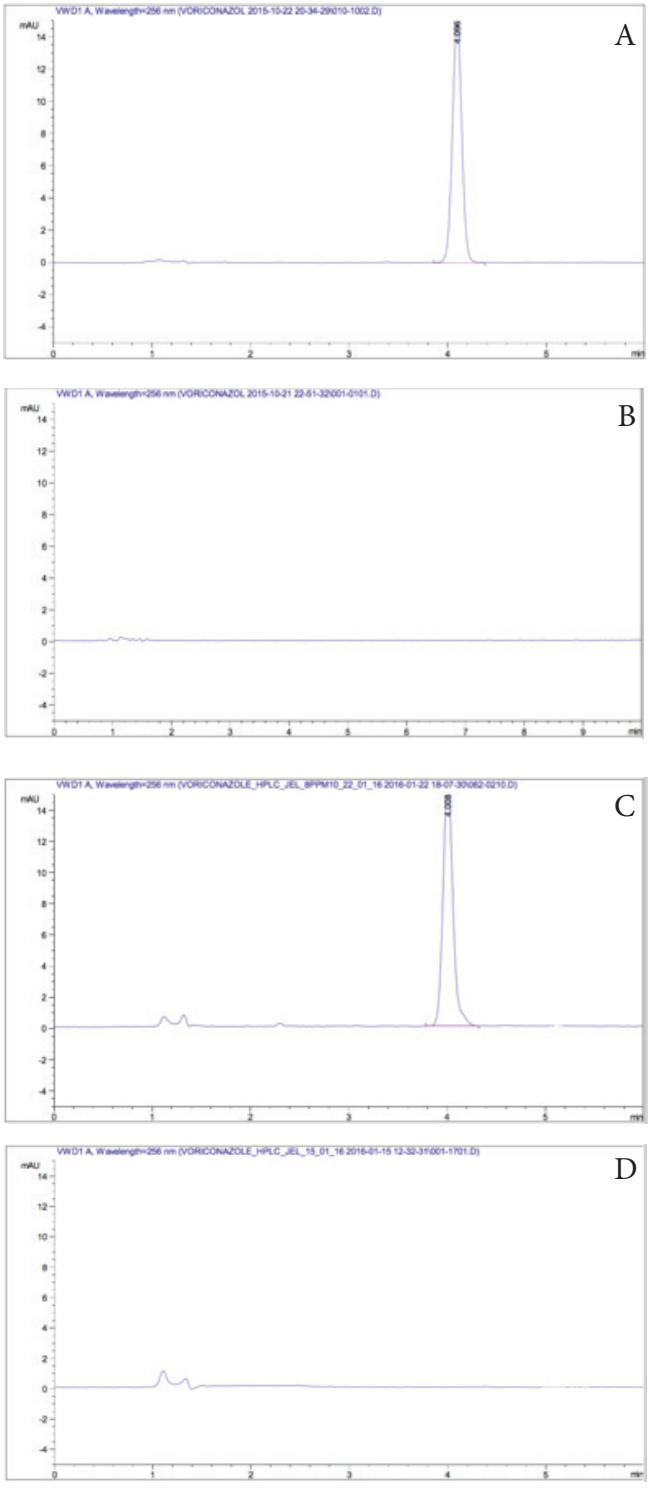

Figure 3. The chromatograms obtained in the presence (A), and absence (B) of voriconazole in standard solution, standard solution of voriconazole loaded in situ gel (C) and blank in situ gel (D). (Concentration of voriconazole (8 µg/mL))

Ten points calibration graphs was constructed covering a concentration range 1-30 µg/mL for standard solution of bulk voriconazole. Three independent determinations were performed at each concentration. Data indicate that the voriconazole peak areas are linear over concentration range of 1- 30 µg/mL. Linear relationship between the peak area and concentration of voriconazole was observed. The standard deviation of the slope and intercept were low. The determination coefficient R^2 for regression line is 0.999 with slope of 13.764x and y + intercept of - 0.656 for standard solution of voricanozole. The analyses of calibration are shown in Figure 4.

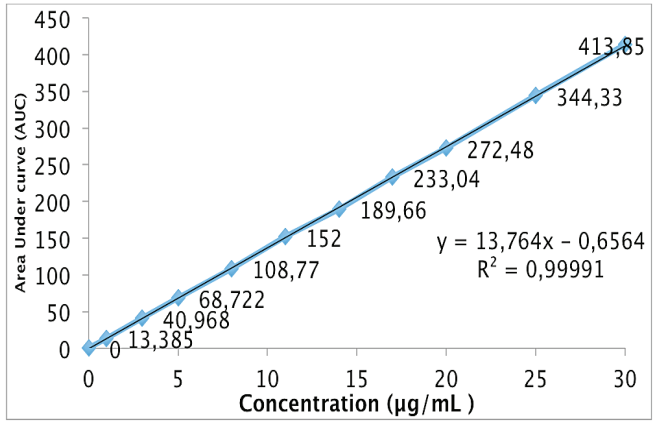

Figure 4. The regression line for voriconazole

Precision studies for an analytical procedure are being done in terms of expressing the closeness of agreement between series of measurements, which are obtained from same homogenous samples under the same conditions.

In terms of method precision study of our experiment, 8 µg/mL solutions were injected ten times into the system and the percentage of recovery was evaluated. As Table 1 shows, the percentage of mean recovery value was 99.09 with standard deviation of 0.29 for bulk voriconazole solution. Furthermore, the percentage of mean recovery value was 97.41 with standard deviation of 0.22 for voriconazole loaded *in situ* gel. Since the percentage of recovery has been found almost 100 and the standard deviation less than the acceptance criteria which is 2%, the analysis system for the determination of assay is verified. Low values of standard deviation denoted very good repeatability of the measurement. Thus it was showing that the equipment used for the study was correct and hence the developed analytical method is highly repetitive.

Table 1. The results of precision study for 8 *μg/mL* bulk voriconazole solution and voriconazole loaded *in situ* gel

	Recovery (%)	
injection times of test solution	bulk voriconazole solution	voriconazole loaded *in situ* gel
1	98.89	97.13
2	98.72	97.32
3	98.82	97.32
4	99.33	97.5
5	99.26	97.41
6	99.38	97.95
7	99.52	97.41
8	98.82	97.23
9	98.97	97.41
10	99.15	97.41
Mean	99.09	97.41
SD	0.29	0.22
RSD %	0.29	0.22

Intermediate precision study can be defined as measurement of repeatability within laboratory variations. In order to evaluate the intermediate precision parameter, two analysts prepared six different samples with the same concentration (Table 2).

Table 2. Intermediate precision study results (8 *μg/mL)*

Test Solution	Voriconazole[%] Analyst 1	Voriconazole[%] Analyst 2
1	109.72	105.45
2	107.82	106.90
3	107.89	107.96
4	111.2	109.49
5	108.47	108.99
6	107.16	108.37
Mean	108.84	107.95
SD	1.77	1.37
RSD %	1.63	1.27

The accuracy of an analytical method is the closeness of test results obtained by that method to the true value. The accuracy of an analytical method can be defined as the ratio of obtained concentration values to true values and its percentage of recovery (20).

The mean recovery data of voriconazole in sample were within the range of 99.49 and 100.7 %. The mean % R.S.D. was in between 0.09 to 0.24 % satisfying the acceptance criteria for the study. Hence the accuracy of the method was confirmed, this developed method can be used for further studies. Recovery (%) of voriconazole was given in Table 3.

Table 3. Percent recovery and coefficient of variations

		Recovery (%)	
Voriconazole concentrations	Injection times of test solution	Bulk	Voriconazole loaded *in situ* gel
8 μg/mL	1	99.66	97.13
	2	99.65	97.32
	3	99.64	97.32
	4	99.13	97.5
	5	99.38	97.41
	Mean	99.49	97.33
	SD	0.24	0.13
	RSD %	0.24	0.14
11 μg/mL	1	100.63	98.32
	2	100.43	98.38
	3	100.44	98.19
	4	100.42	97.92
	5	100.51	98.19
	Mean	100.49	98.2
	SD	0.09	0.18
	RSD %	0.09	0.18
14 μg/mL	1	100.83	98.27
	2	100.59	97.75
	3	100.48	97.64
	4	100.65	97.38
	5	100.94	97.38
	Mean	100.7	97.69
	SD	0.18	0.36
	RSD %	0.18	0.37

Samples should be tested over at least a 48 hour period (depends on intended use), and quantitation of components should be determined by comparison to freshly prepared standards. The stability of voriconazole in 11 μg/mL standard solutions was determined by storing the solutions at ambient temperature (25 ± 1 ^{0}C). After two successive days of storage, the freshly prepared solution and the previous ones were injected into the system and afterwards the data were compared to each other. In each case, it could be noticed that solutions were stable for 48 hours. Furthermore, the percentage of mean recovery of freshly prepared and 48 hour old of 11 μg/mL solution was found 100.05 and 100.10,

respectively. This denotes that voriconazole in stable in standard solutions for at least 2 days at ambient temperature. For 11µg/mL standard solutions of voriconazole loaded *in situ* gel, results of percentage recovery for freshly prepared and 48 hour old solutions are shown in Table 4.

Table 4. The percentage of mean recovery of two 11 µg/mL solutions, which are prepared in two different days

Injection times	Bulk Voriconazole solution		Voriconazole loaded *in situ* gel	
	Recovery (%) 0 h	Recovery (%) 48 h	Recovery (%) 0 h	Recovery (%) 48 h
1	99.91	99.79	97.13	100.86
2	100.05	99.87	97.32	100.86
3	100.00	100.18	97.32	100.77
4	100.3	100.14	97.5	100.95
5	100.02	100.13	97.41	100.68
6	100.11	100.4	97.95	100.77
7	100.02	100.32	97.41	101.22
8	99.89	99.92	97.23	100.95
9	100.08	99.98	97.41	100.86
10	100.15	100.27	97.41	101.13
Mean	100.05	100.10	97.41	100.9
SD	0.12	0.20	0.22	0.17
RSD %	0.12	0.20	0.22	0.17

The Limit of Detection (LOD) and Limit of Quantitation (LOQ) tests for the procedure are performed on samples containing very low concentrations of analyses. LOD is defined as the lowest amount of analyze that can be detected above baseline noise; typically, three times the noise level. LOQ is defined as the lowest amount of analyze which can be reproducibly quantitated above the baseline noise, that gives S/N = 10 (21). LOD was found 0.022 µg/mL and LOQ was found 0.065 µg/mL.

To evaluate the effect of the flow rate on assay results, 11 µg/mL voriconazole solution was analyzed at different flow rates. Changes on the flow rate showed understandable and proportional extension over the retention time of peaks (Table 5).

Table 5. Effect of the flow rate variation on assay results

Flow rate	Retention time
0.5 mL/min	8.43
0.8 mL/min	5.15
1 mL/min	4.1
1.2 mL/min	3.43
1.5 mL/min	2.75

CONCLUSION

A simple, precise, accurate, reproducible, highly sensitive and effective stability indicating HPLC method was developed and validated for simultaneous quantitative determination of voriconazole. The method was validated for accuracy, precision, specificity, and linearity. The developed method is stability indicating and it is LOD and LOQ values are in the range of 0.022 µg/mL and 0.065 µg/mL for voriconazole, respectively. In this study, the high recovery and low relative standard deviation confirm the suitability of the method for determination of voriconazole in pharmaceutical dosage forms. In conclusion, this method can be used for the routine determination of voriconazole in pure and pharmaceutical formulations.

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Declaration of Interest

The authors declare no conflict of interest.

Vorikonazol Etken Maddesi Ve Farmasötik Formülasyonları İçin HPLC Yönteminin Geliştirilmesi Ve Validasyonu

ÖZ

Mevcut çalışmanın amacı, vorikonazol etkin maddesi ve farmasötik formları için yüksek performanslı sıvı kromatografisi kullanılarak bir analitik yöntemin geliştirilmesi ve validasyonunu kapsamaktadır. Mobil faz olarak asetonitril ve ultra saf su karışımı (50:50) kullanılmıştır. Analiz işlemlerinde C18 kolonu (150x4.6mm; 5µm) kullanılmıştır. Eluent, dakikada 1ml' ye ayarlanan akış hızı ve 256 nm dalga boyunda UV detektör yardımıyla gözlenmiştir. Yöntemin validasyon işlemleri için sırasıyla sisteme uygunluk, doğrusallık, tespit ve miktar limiti, kesinlik, özgünlük, doğruluk, seçicilik ve stabilite deneyleri yapılmıştır. Elde edilen sonuçlar, geliştirilen analitik yöntemin iyi düzeyde doğrusallığa, doğruluğa, kesinliğe, seçiciliğe ve stabiliteye sahip olduğunu göstermiştir. Analitik yöntem geliştirme sonuçları, 1-30µg/mL doğrusal aralıkta, tespit sınırını 0.02176 µg/mL ve miktar sınırını ise 0.06528 µg/mL olarak göstermiştir.

Anahtar kelimeler: vorikonazol, HPLC yöntem validasyonu

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