Development and Validation of a Stability-Indicating HPLC Method for Determination of Voriconazole and Its Related Substances

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Abstract

An isocratic reversed-phase high performance liquid chromatographic (RP-HPLC) method has been developed and validated for the determination of voriconazole and its related substances. The drug substance was subjected to stress conditions of UV light, water hydrolysis, acid, base, oxidation, and deoxidization to observe the degradation products. The successful separation of voriconazole from its synthetic impurities and degradation products formed under stress conditions was achieved using an Agilent Zorbax SB-C18 (250mm × 4.6 mm i.d., 5 µm) column maintained at 25°C with a mobile phase of a mixture of ammonium phosphate dibasic buffer (pH adjusted to 6.0 using diluted orthophosphoric acid; 50 mM)–acetonitrile (52:48, v/v). The mobile phase flow rate was 1.0 mL/min, and the detection wavelength was 250 nm. The stress sample solutions were assayed against the qualified reference standard of voriconazole and the mass balance in each case was close to 99.7%, confirming its stability-indication capacity. The developed HPLC method was validated with respect to linearity, accuracy, precision, specificity, and robustness. The developed HPLC method to determine the related substances and assay determination of voriconazole can be used to evaluate the quality of regular production samples. It can be also used to test the stability samples of voriconazole.

Introduction

Voriconazole ((2R,3S)-2-(2,4-difluorophenyl)-3-(5-fluoro-4-pyrimidinyl)-1-(1H-1,2,4-triazol-1-yl)-2-butan-2-ol) (Figure 1) is a synthetic second-generation, broad-spectrum triazole derivative of fluconazole. It inhibits the cytochrome P450 (CYP)-dependent enzyme 14-alpha-sterol demethylase, thereby disrupting the cell membrane and halting fungal growth (1). Voriconazole has shown in vitro activity against many yeasts and a variety of mold and dermatophyte isolates. It can be administered either orally or parenterally, exhibiting good oral bioavailability, wide tissue distribution including distribution into the central nervous system, and hepatic metabolism (2). There are several process impurities/related substances associated with the synthesis of voriconazole. Different process related impurities are observed with various synthetic routes. Four of the known voriconazole-related substances have been studied here; chemical structures for voriconazole and its related substances, namely imp-A, imp-B, imp-C, and imp-D are provided in Figure 1.

High-performance liquid chromatographic (HPLC) and microbiological methods have been reported in the literature for the determination of voriconazole in plasma (3,4). LC–electrospray ionization-mass spectrometric (ESI-MS) determination of voriconazole in aqueous humor has also been reported in the literature (5). LC–LC–ESI-MS–MS with parallel column-switching technique was found in the literature for the analysis of voriconazole from serum (6). Reversed-phase (RP)-HPLC has been mentioned for the determination of voriconazole in pharmaceutical formulation (7). The aforementioned methods have all been used to monitor the concentration of voriconazole in biological fluids or in pharmaceutical formulation, not for dealing with the quantitative determination of related substances in bulk drug. So far, to our present knowl-

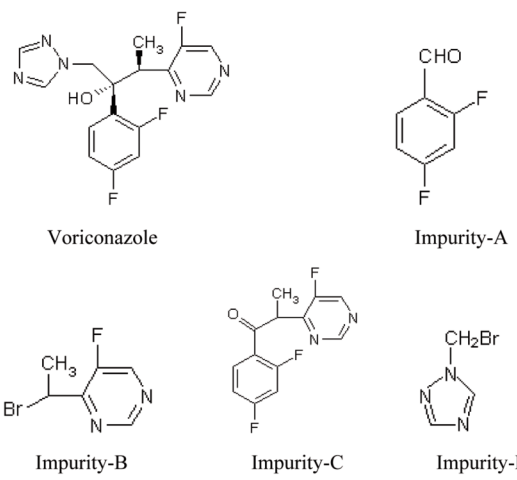

Figure 1. Chemical structures for voriconazole and its related substances.

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edge, only one method has been reported for determining voriconazole and its potential impurities (8), which was more complex and expensive; on account of a special achiral amino-based column coupled with a chiral amylose-based column being used, it might not be suitable for voriconazole routine monitoring. However, developing a simple stability-indicating LC method that could be used to determine the related substances and also the assay of bulk samples of voriconazole was highly desired. This paper describes the assay method which was developed and validated for accurate quantification of voriconazole and all four impurities in bulk samples, respectively. This paper also included the forced degradation of voriconazole under stress conditions like acid hydrolysis, base hydrolysis, oxidation, deoxidization, heat, and UV light. The developed method was validated to ensure the compliance in accordance with International Conference on Harmonization guidelines.

Experimental

Chemicals

Samples of voriconazole and its four impurities were received from Jiangsu Hengrui Medicine Co., Ltd (Lianyungang, China). Analytical-grade phosphoric acid, ammonium phosphate dibasic, and orthophosphoric acid were purchased from Shanghai Reagent Co. (Shanghai, China). HPLC-grade acetonitrile was purchased from Merck (Darmstadt, Germany). High-purity water was obtained from the purification system (Sartorius Arium 611).

Instrumentation

The HPLC system, used for the method development, forced degradation studies, and method validation was Agilent 1100 series (manufactured by Agilent Technologies, Wilmington, DE) equipped with a quaternary pump, online degasser, column heater, autosampler, and diode array-detector (DAD). The output signal was monitored and processed using Chemstation software (designed by Agilent Technologies) on a Pentium IV computer (HP Equipment Co).

Chromatographic conditions

The chromatographic column used was an Agilent Zorbax SB-C18 250 mm × 4.6 mm column with 5 µm particles. The mobile phase consisted of a mixture of ammonium phosphate dibasic buffer (pH adjusted to 6.0 using diluted orthophosphoric acid; 50 mM)–acetonitrile (52:48, v/v). The mobile phase was filtered through a nylon membrane (pore size 0.45 µm). The flow rate of the mobile phase was kept at 1.0 mL/min. The column temperature was maintained at 25ºC and the wavelength was monitored at 250 nm. The injection volume was 20 µL. The test concentration for the related substance analysis was 250 µg/mL, and for the assay, 50 µg/mL. The standard and the test dilutions were prepared in mobile phase.

Preparation of standard solutions

A stock solution of voriconazole (500 µg/mL) was prepared by dissolving appropriate amount of substance in the mobile phase. Working solutions of 250 and 50 µg/mL were prepared from this stock solution for the related substance determination and assay determination, respectively. A stock solution of impurities (mixture of imp-A, imp-B, imp-C, and imp-D) at 250 µg/mL was also prepared in the mobile phase.

Validation of the method

Specificity. Specificity is the ability of the method to measure the analyte response in the presence of its potential impurities (9). The specificity of the developed LC method for voriconazole was carried out in the presence of its impurities; namely, imp-A, imp-B, imp-C, and imp-D. Stress studies were performed for voriconazole bulk drug to provide an indication of the stability-indicating property and specificity of the proposed method. Intentional degradation was attempted under stress conditions of UV light (254 nm), water hydrolysis (heat at 60ºC), acid (0.5N HCl), base (0.5N NaOH), oxidation (3.0% H_2O_2), and deoxidization (3.0% $NaHSO_3$) to evaluate the ability of the proposed method to separate voriconazole from its degradation product. For deoxidization and light studies, the study period was 10 days, whereas for acid, base, oxidation, and heat, it was 48 h. A peak purity test was carried out for the voriconazole peak by using DAD in stress samples. Assay studies were carried out for stress samples against qualified voriconazole reference standard. The assay was also calculated for the voriconazole sample by spiking all four impurities at the specification level (i.e., 0.15%).

Precision. The precision of the assay method was evaluated by carrying out six independent assays of voriconazole test sample against a qualified reference standard and calculating the % relative standard deviation (RSD) of the assay.

The precision of the related substance method was checked by injecting six individual preparations of voriconazole (250 µg/mL) spiked with 0.15% of imp-A, imp-B, imp-C, and imp-D with respect to voriconazole analyte concentration. % RSD of area for each imp-A, imp-B, imp-C, and imp-D was calculated.

The intermediate precision of the method was also evaluated using different analyst and different instrument in the same laboratory.

Limits of detection and quantification. The limit of detection (LOD) and limit of quantification (LOQ) for imp-A, imp-B, imp-C, and imp-D were determined at a signal-to-noise ratio of 3:1 and 10:1, respectively, by injecting a series of dilute solutions with known concentrations (10). Precision study was also carried out at the LOQ level by injecting four individual preparations of imp-A, imp-B, imp-C, and imp-D and calculating the % RSD of the area.

Linearity. Linearity test solutions for the assay method were prepared from voriconazole stock solutions at six concentration levels from 20% to 200% of assay analyte concentration (10, 20, 30, 50, 75, and 100 µg/mL). The peak area versus concentration data was treated by least-squares linear regression analysis.

Linearity test solutions for the related substance method were prepared by diluting stock solutions to the required concentrations. The solutions were prepared at six concentration

levels from LOQ to 200% with respect to the impurity specification level of 0.15% of the specification level (LOQ, 0.075%, 0.15%, 0.1875%, 0.225%, and 0.3%).

These tests were carried out for three consecutive days in the same concentration range for both the assay and related substance method. The % RSD value for the slope and *Y*-intercept of the calibration curve were calculated.

Accuracy. The accuracy of the assay method was evaluated in triplicate at three concentration levels (i.e., 25, 50, and 75 µg/mL). The percentage of recoveries was calculated from the slope, and the *Y*-intercept of the calibration curve was obtained in the "Linearity" section.

The accuracy study of impurities was carried out in triplicate at 0.075, 0.15, and 0.225% of the voriconazole analyte concentration (250 µg/mL). The percentages of recoveries for impurities were calculated from the slope and *Y*-intercept of the calibration curve obtained in the "Linearity" section.

Robustness. To determine the robustness of the developed method, experimental conditions were deliberately altered, and the resolution between voriconazole, imp-A, imp-B, imp-C, and imp-D was recorded. The flow rate of the mobile phase was 1.0 mL/min. To study the effect of flow rate on the resolution, flow was changed by 0.2 units from 0.8 to 1.2 mL/min. The effect of pH on resolution of impurities was studied by varying ± 0.2 pH units (at 5.60, 5.80, 6.20, and 6.40 buffer pH). The effect of the column temperature on resolution was studied at 20ºC and 30ºC instead of 25ºC. The effect of the percent organic strength on resolution was studied by varying acetonitrile by –3% to +3% while other mobile phase components were held constant as stated in the "Chromatographic conditions" section.

Solution stability and mobile phase stability. The solution stability of voriconazole in the assay method was carried out by leaving both the test solutions of sample and reference standard in tightly capped volumetric flasks at room temperature for 48 h. The same sample solutions were assayed for 6 h interval up to the study period. The mobile phase stability was also carried out by assaying the freshly prepared sample solutions against freshly prepared reference standard solution for 6 h interval up to 48 h. Mobile phase prepared was kept constant during the study period. The % RSD for the assay of voriconazole was calculated during mobile phase and solution stability experiments.

The solution stability of voriconazole and its impurities in the related substance method was carried out by leaving spiked sample solutions in tightly capped volumetric flasks at room temperature for 48 h. Contents of imp-A, imp-B, imp-C, and imp-D were determined for every 6 h interval up to the study period. The mobile phase stability was also carried out for 48 h by injecting the freshly prepared sample solutions for every 6 h interval. Contents of imp-A, imp-B, imp-C, and imp-D were checked in the test solutions.

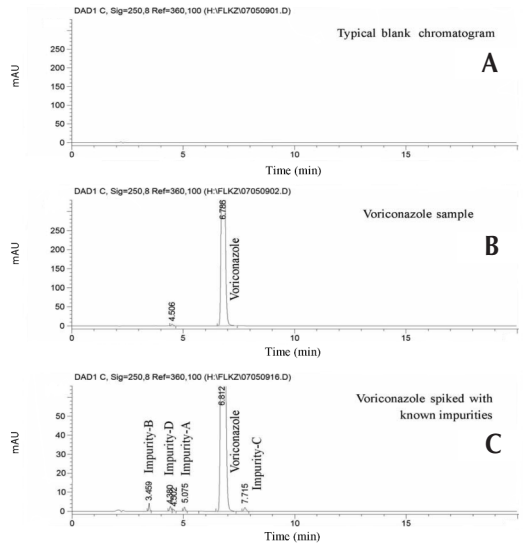

Figure 2. HPLC chromatogram of blank (A), unspiked, and spiked (imp-A, imp-B, imp-C, and imp-D were spiked at 0.15% level in pure voriconazole) samples.

Results and Discussion

Optimization of chromatographic conditions

The main objective of the chromatographic method is to separate voriconazole from imp-A, imp-B, imp-C, and imp-D. Impurities were coeluted using different stationary phases such as C18, C8, phenyl, and cyano as well as different mobile phases containing buffers like phosphate, sulphate, and acetate with different pH (4–8) and using organic modifiers like acetonitrile, methanol, and ethanol in the mobile phase. The chromatographic separation was achieved on an Agilent Zorbax SB-C18 (250 mm × 4.6 mm) column with 5-µm particles. The mobile phase consists of a mixture of ammonium phosphate dibasic buffer (pH adjusted to 6.0 using diluted orthophosphoric acid; 50 mM)–acetonitrile (52:48, v/v). The flow rate of the mobile phase was 1.0 mL/min at 25ºC column temperature. The peak shape of the voriconazole was found to be symmetrical. In optimized chromatographic conditions, voriconazole, imp-A, imp-B, imp-C, and imp-D were separated with resolution greater than 4.0, typical retention time were ~ 3.5, 4.4, 5.1, 6.8, and 7.7 min, respectively (Figure 2). The system suitability results are given in Table I, and the developed LC method was found to be specific for voriconazole and its four impurities, namely imp-A, imp-B, imp-C, and imp-D (Figure 2).

Results of forced degradation studies

Degradation was not observed in the voriconazole sample when subjected to stress conditions like light and deoxidization (Figure 3). Voriconazole was degraded to imp-A and imp-D under acid hydrolysis, base hydrolysis, and water hydrolysis (heat at 60°C), and they were confirmed by co-injection with the qualified imp-A and imp-D standards. Under oxidation stress, voriconazole was degraded to imp-D and other unknown degradants. Peak purity test results obtained from DAD confirm that the voriconazole peak is homogenous and pure in all the analyzed stress samples. The assay of voriconazole is unaffected in the presence of imp-A, imp-B, imp-C, and imp-D,

Table I. System-Suitability Report

Compound (n = 3)*	USP resolution	Asymmetry factor	Capacity factor	Selectivity factor	RSD % (for retention time)	Number of theoretical plates (USP tangent method)
Imp-B	—	1.09	0.65	—	0.09	7439
Imp-D	5.5	1.06	1.09	1.68	0.14	10526
Imp-A	4.1	1.17	1.42	1.30	0.19	12330
Voriconazole	8.2	1.11	2.24	1.58	0.18	12889
Imp-C	4.2	1.18	2.67	1.19	0.15	27294

* n = number of determinations.

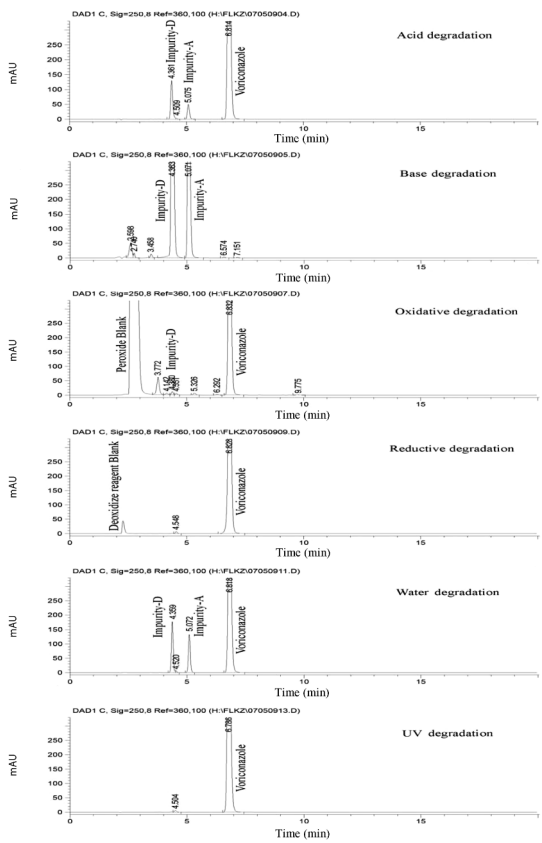

Figure 3. Typical HPLC chromatograms of stressed test samples of voriconazole.

and its degradation products confirm the stability-indicating power of the method. The summary of forced degradation studies is given in Table II.

Precision

The % RSD of assay of voriconazole during the assay method precision study was within 0.7%, and the % RSD for the area of imp-A, imp-B, imp-C, and imp-D in related substance method precision study was within 3%. The % RSD of the assay results obtained in the intermediate precision study was within 0.8%, and the % RSD for the area of imp-A, imp-B, imp-C, and imp-D were well within 4%, confirming good precision of the method.

LOD and LOQ

The LOD of imp-A, imp-B, imp-C, and imp-D were 0.01%, 0.02%, 0.02%, and 0.02% (of analyte concentration; i.e., 250 µg/mL) for 20 µL injection volume. The LOQ of imp-A, imp-B, imp-C, and imp-D were 0.04%, 0.05%, 0.05%, and 0.06% (of analyte concentration; i.e., 250 µg/mL) for 20 µL injection volume. The method precision for imp-A, imp-B, imp-C, and imp-D at LOQ level was below 5% RSD.

Linearity

The linearity calibration plot for the assay method was obtained over the calibration ranges tested (i.e., 10–100 µg/mL), and correlation coefficient obtained was greater than 0.999. Linearity was checked for the assay method over the same concentration range for three consecutive days. The % RSD values of the slope and Y-intercept of the calibration curves were 1.9 and 2.8, respectively. The result shows that an excellent correlation exists between the peak area and concentration of the analyte.

Linear calibration plot for the related substance method was obtained over the calibration ranges tested [i.e., LOQ (0.05%) to 0.3%] for impurity imp-A, imp-B, imp-C, and imp-D. The correlation coefficient obtained was greater than 0.997. Linearity was checked for the related substance method over the same concentration range for three consecutive days. The % RSD values of the slope and Y-intercept of the calibration curves were 3.4 and 4.5, respectively. These results show that an excellent correlation exists between the peak area and the concentration of imp-A, imp-B, imp-C, and imp-D.

Accuracy

The percentage recovery of voriconazole in bulk drug samples ranged from 98.8% to 100.4% (Table III). The percentage recovery of impurities in voriconazole samples varied from 96.3% to 103.2%. The HPLC chromatograms of blank, unspiked, and spiked sample at 0.15% level of all four impurities in voriconazole bulk drug sample are shown in Figure 2.

Robustness

In all the deliberate varied chromatographic conditions (flow rate, column temperature, pH, and composition of organic solvent), the resolution between voriconazole, imp-A, imp-B, imp-C, and imp-D was greater than 4.0, illustrating the robustness of the method.

Table II. Summary of Forced Degradation Results

Stress condition	Time	% Assay of active substance	% Mass balance (% assay + impurities)	Remarks
Acid hydrolysis (0.5N HCl)	48 h	87.9	99.7	Degraded to imp-A and imp-D
Base hydrolysis (0.5N NaOH)	48 h	0	99.8	Degraded to imp-A, imp-D, and other unknown degradants
Oxidation (3% H_2O_2)	48 h	91.9	99.6	Degraded to imp-D and other unknown degradants
Deoxidization (3.0% $NaHSO_3$)	10 days	99.6	99.6	No degradation products formed
Water hydrolysis at 60°C	48 h	77.5	99.7	Degraded to imp-A and imp-D
UV (254 nm)	48 days	99.6	99.6	No degradation products formed

Table III. Recovery Results of Voriconazole Sample

Added (µg) (*n* = 3)*	Recovered (µg)	% Recovery	% RSD
25.3	25.0	98.8	0.9
50.6	50.8	100.4	0.6
75.2	74.8	99.5	0.7

* *n* = number of determinations.

Solution stability and mobile phase stability

The % RSD of the assay of voriconazole during solution stability experiments were within 1.0%. No significant changes were observed in the content of imp-A, imp-B, imp-C, and imp-D during solution stability and mobile phase stability experiments when performed using the related substance method. The solution stability and mobile phase stability experiment data confirms that the sample solutions and mobile phases used during assay and the related substance determination were stable for at least 48 h.

Conclusion

The RP-HPLC method developed for quantitative and related substance determination of voriconazole is precise, accurate, rapid, and specific. The method was fully validated, showing satisfactory data for all the method validation parameters tested. The developed method is stability-indicating and can be very useful for quality monitoring of regular production samples and can also be employed to check the quality during stability studies.

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