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Research Article

Novel Stability indicating Rp-HPLC Method for the Determination of Assay of Voriconazole in Pharmaceutical Products

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Abstract: A novel stability indicating RP-HPLC method having a very short run time was developed and validated for determination of assay of Voriconazole in bulk solution, lyophilized injectable dosage form and in physiological compatible solutions. The method is novel, rapid, precise, accurate and capable of separating known impurities and degradants from Voriconazole peak proving the stability-indicating nature of the method. The analytical method consists of isocratic elution at a flow rate of 0.8 mL/min on a Devolosil C18 column (100 x 4.6 mm, 3µm) at temperature of 45°C and UV detection wavelength of 254 nm. 10µL of the voriconazole sample was injected and peak was eluted at about 2.5 min. with total run time of 10 minutes. The % recovery was well within the range between 98% and 102%. The RSD for precision and accuracy of the method was found to be less than 2%. The method was validated as per the International Conference on Harmonization (ICH) guidelines and can be successfully applied for routine analysis of Voriconazole in bulk samples and its formulations.

Keywords: Voriconazole, RP-HPLC, Isocratic, Assay Method, Stability indicating

INTRODUCTION

Voriconazole (VCZ) is chemically (2R,3S)-2-(2, 4-difluorophenyl)-3-(5-fluoro-4-pyrimidinyl)-1-(1H-1,2,4triazol-1-yl)-2-butanol. VCZ is a used to treat serious fungal or yeast infections, such as aspergillosis (fungal infection in the lungs), candidemia (fungal infection into the blood) esophageal candidiasis (candida esophagitis) or other fungal infections (infections in the skin, stomach, kidney, bladder or wounds). It inhibits the cytochrome P450 (CYP)-dependent enzyme 14-alpha-sterol demethylase, thereby disrupting the cell membrane and halting fungal growth [1]. VCZ 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 VCZ. Different process related impurities are observed with various synthetic routes. Four of the known VCZ-related substances have been mentioned here; chemical structures for VCZ and its related substances, *viz* imp-B, imp-C, imp-D, and imp-F.

VCZ is official in the United States Pharmacopeia [3]. The USP method of analysis of VCZ suffers from a major limitation, a very long run time which makes it practically impossible to execute for analysis of large number of commercial batches at a time. Moreover, the methods reported so far are not so precise and accurate in separating the known impurities and degradants from VCZ peak within a short time.

The analytical method, which has been reported, are very few for the determination of VCZ in pure drug, pharmaceutical dosage forms and in biological samples using liquid Chromatography [4-16] either in single or in combined forms**.** The present work deals with the development and validation of a novel, simple, rapid and reliable isocratic RP-HPLC method with UV detection for the determination of VCZ in bulk solution, lyophilized injectable dosage forms and physiological compatible solutions. Confirmation of the applicability of the developed method was validated according to the International Conference on Harmonization (ICH) guidelines [17] for the determination of VCZ in bulk solution and lyophilized injectable dosage forms.

The method development was initiated with different C18 columns like Inertsil ODS-3V (250 x 4.6mm, 5µm), Zodiac (250 x 4.6mm, 5µm), Devolosil (100 x 4.6mm, 3µm) and 50 mM sodium phosphate buffer with acetonitrile as organic modifier in different ratios. The method development was conducted with different column temperature like 25°C, 30°C, 45°C and 50°C, and with different injection volume like 5µL, 10µL and 20µL.

The structure of VCZ is represented in Fig. 1(A), while the structures of Impurity B, Impurity C, Impurity D and Impurity F are depicted in Fig. 1(B), Fig. 1(C), Fig. 1(D), and Fig. 1(E), respectively.

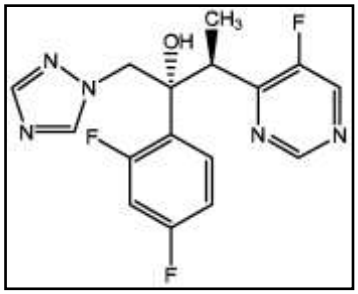

Fig-1: (A): Molecular Structure of Voriconazole

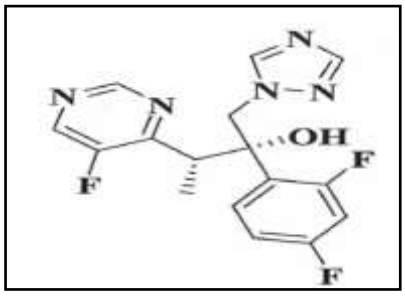

Fig-1: (B): Molecular Structure of VCZ Impurity-B

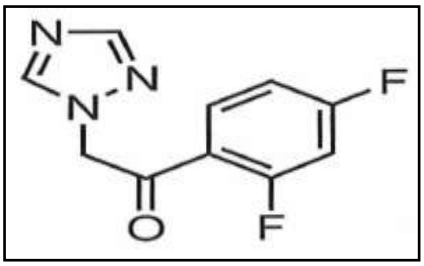

Fig-1: (C): Molecular Structure of VCZ Impurity-C

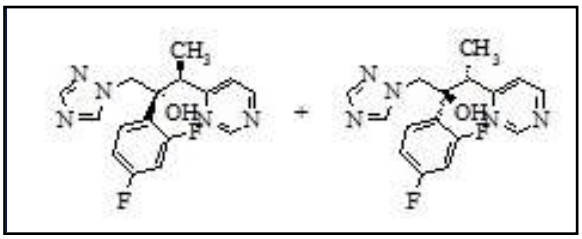

Fig-1: (D): Molecular Structure of VCZ Impurity-D

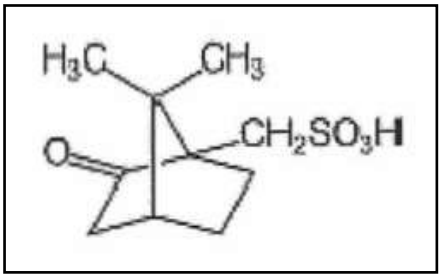

Fig-1: (E): Molecular Structure of VCZ Impurity-F

MATERIALS AND METHODS

Chemicals

Sodium dihydrogen phosphate dihydrate (Analytical grade), Acetonitrile (HPLC grade) were purchased from Merck (Mumbai, India). Milli-Q water produced from Milli- Q system (Merck Millipore, Mumbai, India). Voriconazole working standard (B.No.: VORWS40/14), Voriconazole lyophilized sample (B.No. VZI1501AC) (VLS) & Voriconazole lyophilized placebo (B.No. 30000065-001/P) (VLP) were manufactured at Celon Laboratories Limited (Hyderabad, India). Voriconazole impurity B (B.No. SL-049-033), Voriconazole impurity C (B.No. SP-07409LBV), and Voriconazole impurity F (B.No. SP-002-137) were procured from Simson Pharma (Mumbai, India). Voriconazole impurity D (B.No. TLC/V-0604) was procured from TLC Pharma (Hyderabad, India). 0.9% Sodium Chloride Injection, 0.45% Sodium Chloride Injection, 0.9% Sodium Chloride & 5% Dextrose Injection, 0.45% Sodium Chloride & 5% Dextrose Injection were procured from Nirma Limited (Sachana, India). 5% Dextrose Injection and Ringer Lactate Solution for Injection were procured from Claris Otsuka Limited (Ahmedabad, India). 5% Dextrose in 20mEq of Potassium Chloride Injection and 5% Dextrose & Ringer Lactate Solution for Injection were prepared at Celon Laboratories Limited (Hyderabad, India).

Equipment

The chromatographic system consisted of Agilent 1260 Infinity series model equipped with quaternary pump (DEABJ02269), vacuum degasser (DEABJ02269), auto injector with variable injection valve (DEABE04741), Diode Array Detector (DEAAX01521) and column oven (DEAAK05395). The system was connected through a HP interface (CN40490LKY) to open lab software (ver.2.0) in a computer system for data collection and processing.

Prepared Solutions

Working standard solutions of VCZ (50 ppm) was prepared by weighing and transferring 25 mg of VCZ to 50 mL volumetric flask, dissolved and diluted to volume with diluent (Milli-Q water and Acetonitrile in the ratio of 1:1 % v/v). 5 mL of above solution was pipetted into 50 mL volumetric flask and diluted to volume with diluent.

Pharmaceutical dosage forms (50 µg/mL)

Two vials of VLS were reconstituted with 19 mL of water in each vial and transferred the solution into 200 mL volumetric flask, mixed and made up to the volume with diluent. 5 mL of above solution was pipetted into 200 mL volumetric flask and diluted to volume with diluent (Milli-Q water and Acetonitrile in the ratio of 1:1 % v/v).

Placebo preparation

Two vials of formulation placebo was reconstituted with 19 mL of water in each vial and transferred the solution into 200 mL volumetric flask, mixed and made up to the volume with diluent. 5 mL of above solution was pipetted into 200 mL volumetric flask and diluted to volume with diluent.

Chromatographic conditions

The chromatographic system consisted of Agilent 1260 Infinity series model equipped with Diode Array Detector. Separation was performed on a 100 x 4.6 mm i.d., 3µm particle size Devolosil C18 column. Mobile phase consisted of a phosphate buffer 0.05M (pH 4.5) and Acetonitrile, Buffer and Acetonitrile in the ratio 800:200 % v/v as Mobile Phase A and Acetonitrile and water in the ratio 800:200 % v/v as Mobile Phase B in the ratio of 35:65; and degassed before use. Flow rate and injector volume were 0.8 mL/min. and 10µL respectively. Column oven temperature and wavelength were set at 45°C and 254 nm respectively.

Assay method Development

The method development was initiated with different C18 columns such as Inertsil ODS-3V (250x4.6mm, 5µm), Zodiac (250x4.6mm, 5µm), Devolosil (100x4.6mm, 3µm) and 50 mM sodium phosphate buffer with acetonitrile as organic modifier in different ratios. As a part of development trials, 50 mM sodium phosphate buffer and acetonitrile (80:20%v/v) as a mobile phase-A, water and acetonitrile (20:80%v/v) as a mobile phase-B was used. As a part of method finalization, the column temperature was optimized with 45°C and injection volume with 10µL for better peak shape and symmetry.

To ensure the system efficiency and method repeatability, it is essential that there is no blank interference at the retention time of VCZ peak, % Relative Standard Deviation for five replicate Standard injections should not be more than 2.0%, Similarity factor for Standard preparation-1 and Standard preparation -2 should be in a range of 0.98-1.02 and tailing factor of VCZ peak should be not more than 2.0.

To prove that the developed analytical method is appropriate for the determination of VCZ in pharmaceutical dosage form and physiological compatibility solution, precision, linearity, specificity, accuracy, forced degradation, solution stability and robustness were performed.

Application

The method was intended to be used to determine the VCZ concentration in bulk solution, lyophilized injectable formulations and in physiological compatible solutions.

Statistical Analysis

All the data were analyzed using Graph Pad Prism 6 software (Graph Pad software Inc., La Jolla, CA, USA). The results were expressed as Mean $\pm$ SD and % RSD. A value of $p<0.5$ was considered as significant, $p<0.05$ as highly significant while $p<0.005$ was considered as extremely significant. A value of $p>0.5$ was considered to be statistically insignificant.

VALIDATION OF THE METHOD

Assay performance

The proposed HPLC method is validated as per ICH Q2 (R1). System suitability, method precision, intermediate precision, linearity, accuracy, specificity and forced degradation, solution stability, temperature and flow variation were performed as a part of validation. The results for the validated parameters were discussed in the following sections.

Precision

To demonstrate the reproducibility of the method, six samples from the same batch of VLS were analyzed individually and the assay content (VCZ) of each sample was estimated. The average assay for six determinations was calculated along with % RSD for each determination (Table 1). The % Assay of all the samples (n=12) in the method precision and intermediate precision lies in the range of 98.0 to 100.0. There was no significant change ($p>0.05$) observed in the % Assay of VCZ in method precision and intermediate precision for the individual and average determination proving that the method is highly precise for the determination of assay of VCZ. Table 1contains the precision analysis of VCZ in pharmaceutical dosage form of different analyst on different days (n=6) for each analyst with mean, variance and relative standard deviation.

Linearity

To demonstrate the linearity of the test method concentration of the analyte with respect to detector response was studied. To carry out the study, series of VCZ solutions were prepared with concentration ranging from 25% to 200% to the target assay concentration (50 ppm). The peak area of the VCZ was considered for plotting the graph between concentration and response. The linearity was evaluated by linear regression analysis and was calculated by the least square regression method (Table 2). Based on the correlation coefficient value (0.9997), the concentration versus response is linear throughout the range and also the % Y intercept results (1.4) confirms the linearity. Table 2 contains the linearity of VCZ in pharmaceutical dosage form range from 25 % to 200 % level of the target concentration (50 ppm), (n=2) for each specification level. Linearity was established through Correlation Coefficient, Slope, Y-Intercept and % Y-Intercept.

Accuracy

To validate the accuracy of the test method, recovery experiments were conducted at a concentration of 50, 80, 100, 120 and 150% of the sample concentration (50 ppm). Each test solution was prepared in triplicate and analysis was also performed in triplicate. The assay content value at the beginning of validation was considered as the true value (100% level) for recovery calculations. The percentage assay, percentage recovery, mean percentage recovery were calculated from the data obtained (Table 3). Based on the results, the recovery values was observed in the range of 98.0 to 102.0 for all the preparations (n=15), also % RSD results (1.3) confirms the accuracy of the test method. Table 3 contains the average recoveries of VCZ after spiking VCZ standard with placebo (n=3 spiked samples at each concentration level)

Specificity

A study was conducted to demonstrate the interference of placebo and known impurities at the retention time of VCZ peak. Placebo was prepared in duplicate and injected into HPLC system. The impurities were prepared as per specification level and the results shows there was no placebo interference (Table 4) at the retention time of VCZ peak. Impurity-F was not detected in the assay method, Impurity-C & D were eluted before VCZ peak confirming the specificity. Table 4 contains the specificity of the VCZ peak in the presence of placebo (n=2 preparations) and known impurities.

Forced degradation

The forced degradation study was conducted to ensure that the analytical method is stability indicating and capable of separating degradants from the main analyte peak (Table 5). VLS was found to be unstable when exposed to acid, base, peroxide and thermal stress. The analytical method is capable of separating all the degradants from the VCZ peak when exposed to stress conditions confirmed by peak purity evaluation. Table 5 contains the peak purity establishment for VCZ analyte in acid stress, base stress, oxidation and thermal stress (n=1 for each condition)

Solution stability

VLS and standard solutions of 50 ppm were prepared and stored at ambient temperature and refrigerated conditions. The analysis of each solution was repeated at periodic intervals covering a time period of 24 hrs. The areas from each of the experiment were taken and the percentages were calculated (Table 6 & 7). The results obtained confirm that the VLS and standard solutions are stable up to 24 hrs at room temperature and refrigerated conditions. Table 6 & 7 contains the establishment of standard and sample stability at room temperature and refrigerated conditions for 24 hrs.

Robustness

The deliberate changes in the flow rate (+0.2 mL/min) and column temperature (+5°C) were made to establish that system suitability will be unaffected by meeting the acceptance criteria (Table 8). The observed % RSD, similarity factor and tailing factor values showed that there is no significant variation with the change in flow rate or column temperature. Table 8 contains the establishment of robustness for analytical method with changes in actual flow rate and actual column temperature.

Physiological solution compatibility study

VCZ pharmaceutical injectable dosage form was reconstituted initially with 19 mL of water for injection then diluted with physiological solutions (0.9% Sodium Chloride Injection, Sodium Lactate (Ringer Lactate) Solution for Injection, 5% Dextrose & Ringer Lactate Solution for Injection, 0.45% Sodium Chloride & 5% Dextrose Injection, 5% Dextrose Injection, 5% Dextrose in 20mEq of Potassium Chloride Injection, 0.45% Sodium Chloride Injection and 0.9% Sodium Chloride & 5% Dextrose Injection) at a concentration of 0.5mg/mL and 5mg/mL. The diluted solutions were stored at 2° to 8°C for a period of 24 hrs and analyzed (Table 9). The results were indicative that VLS is compatible with physiological solutions and were stable when diluted upto 24 hrs at different concentrations. Table 9 contains the establishment of Physiological solution compatibility study for 24 hrs in different diluents.

RESULTS AND DISCUSSION

Table-1: Method precision results

Sample No.	Method precision % Assay	Intermediate Precision % Assay
Sample No-1	99.2	100.0
Sample No-2	98.8	99.3
Sample No-3	98.6	99.5
Sample No-4	98.7	99.6
Sample No-5	98.1	100.0
Sample No-6	99.2	100.0
Average	**98.8**	**99.7**
Standard deviation	**0.413**	**0.308**
% RSD	**0.42**	**0.31**

Table-2: Linearity regression summary results

S. No	Level	Concentration (mg/mL)	Area
1	25%	0.0126	3208685
2	50%	0.0252	6331076
3	75%	0.0377	9268225
4	100%	0.0503	12352043
5	150%	0.0755	18888705
6	200%	0.1006	24466810
Correlation Coefficient			**0.9997**
Slope			**243377581.92**
Y-Intercept			**-173584.625**
% Y-Intercept			**1.40**

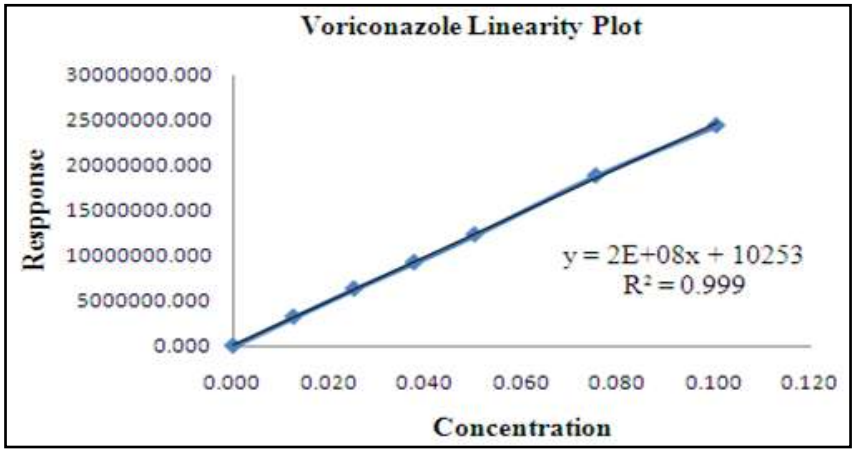

Fig-2: Linearity regression summary results

Table-3: Recovery of VCZ summary table

Sample No.	Concentration (mg/mL)	Recovered (%)
50%	0.025	98.1
80%	0.040	99.9
100%	0.050	99.2
120%	0.060	101.3
150%	0.075	98.2
Mean	**99.3**	
Standard	**1.32**	
% RSD	**1.3**	

Table-4: Specificity of VCZ summary table

S.No	Standard/Impurity Name	Retention Time
1	Placebo	Not Detected
2	Voriconazole	2.50
3	Voriconazole Impurity-C	1.76
4	Voriconazole Impurity-D	2.00
5	Voriconazole Impurity-F	Not detected

Table-5: Forced degradation data of VCZ summary table

Stress Type	Stress Conditions	% Assay	% Degradation	Peak Purity
Unstressed	---	101.4	---	Pass
Acid Stress	1 N HCl at RT for 1 hr	96.4	5.0	Pass
Base Stress	0.1 N NaOH at 5 min	86.7	14.7	Pass
Oxidation	1 % H_2O_2 at RT for 1 hr	96.7	4.7	Pass
Thermal	60°C for 6 hours	93.4	8.0	Pass

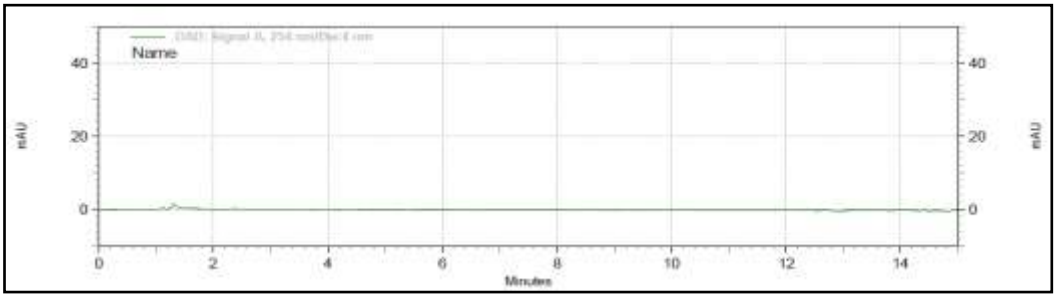

Fig-3: Chromatogram of blank

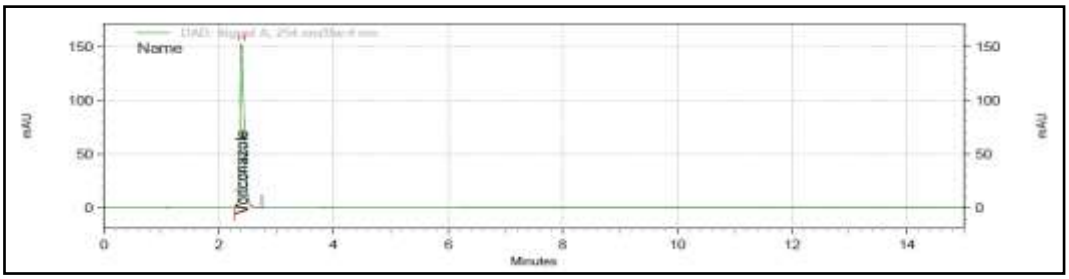

Fig-4: Chromatogram of standard

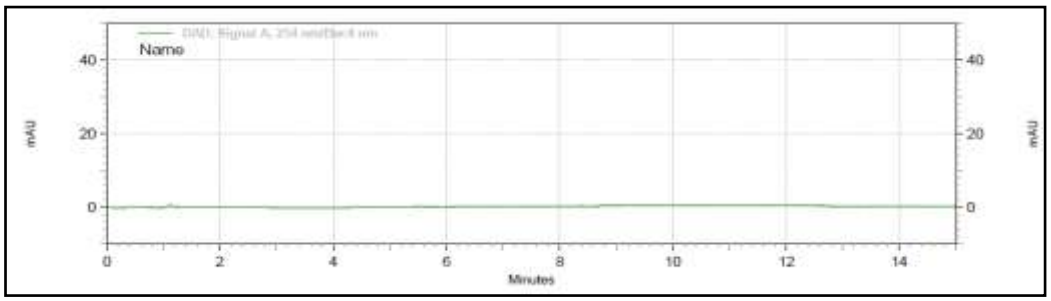

Fig-5: Chromatogram of placebo

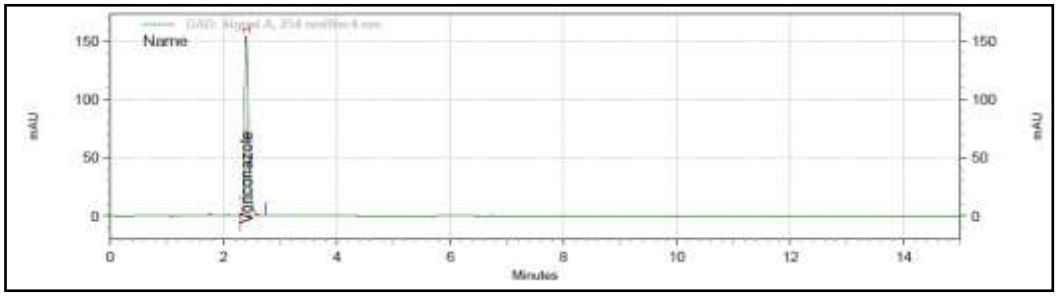

Fig-6: Chromatogram of sample

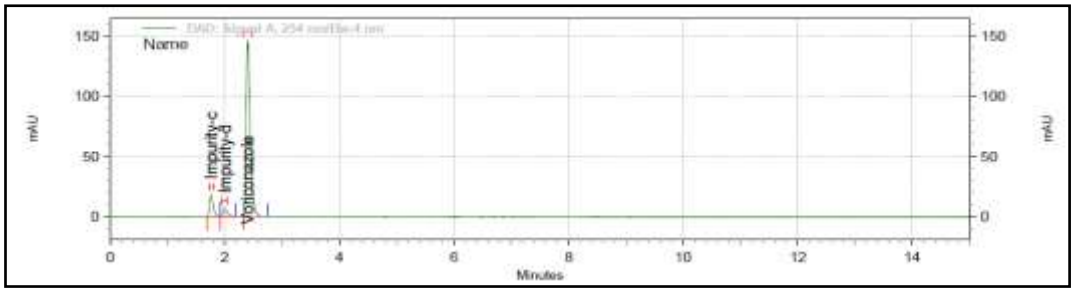

Fig-7: Chromatogram of spiked sample

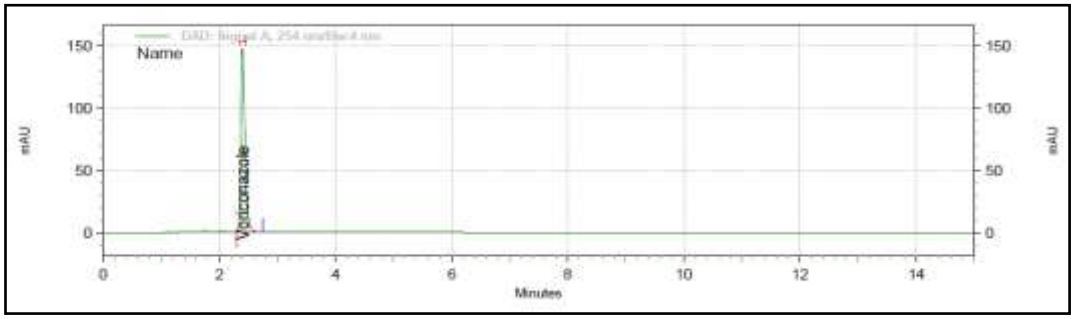

Fig-8: Chromatogram of acid degradation sample

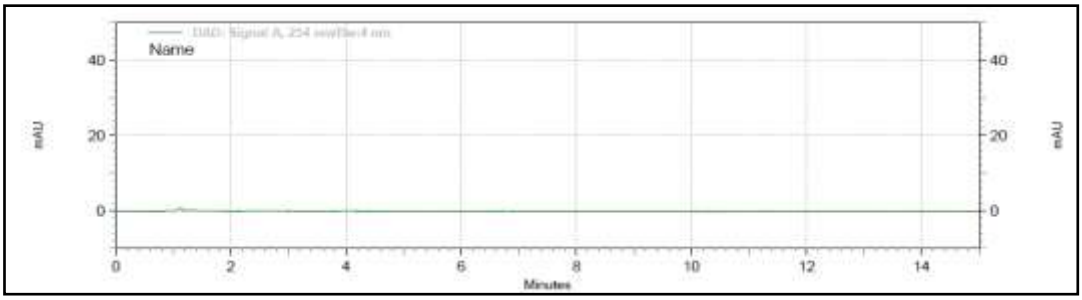

Fig-9: Chromatogram of acid degradation placebo

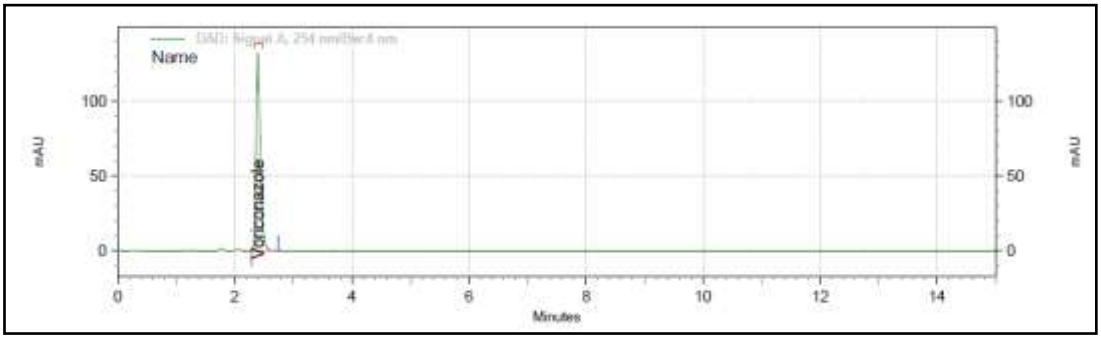

Fig-10: Chromatogram of alkali degradation sample

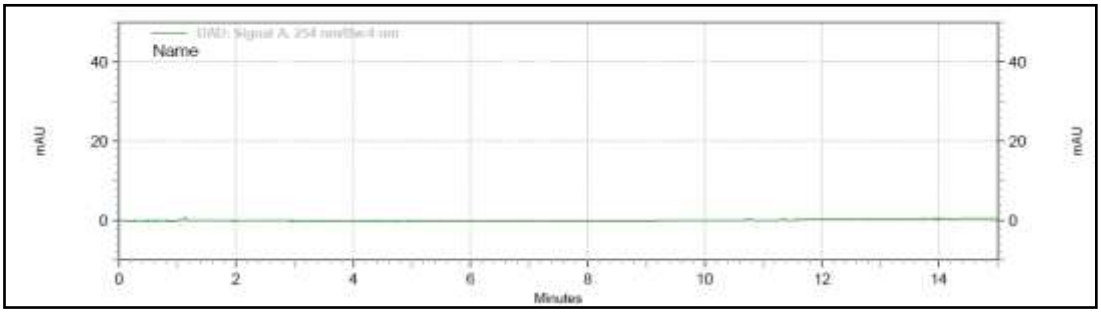

Fig-11: Chromatogram of alkali degradation placebo

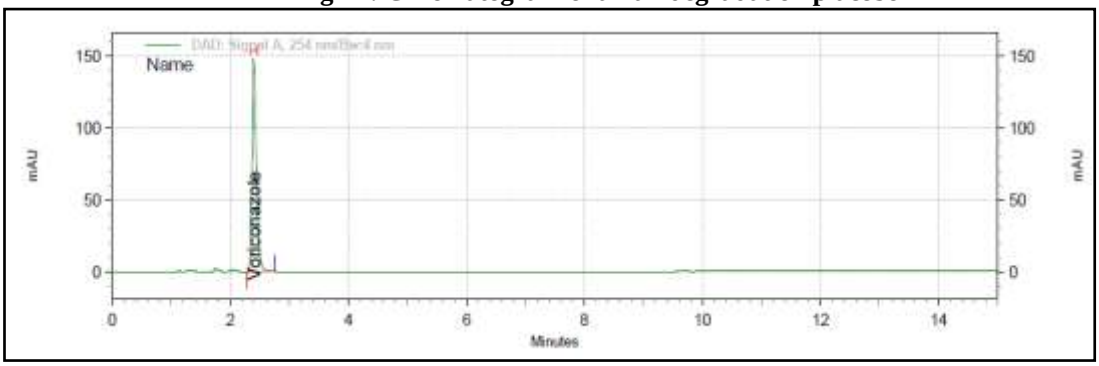

Fig-12: Chromatogram of oxidation degradation sample

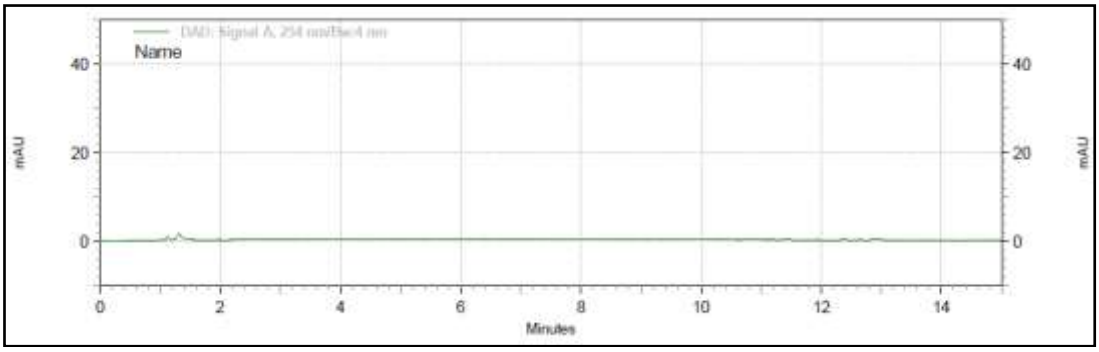

Fig-13: Chromatogram of oxidation degradation placebo

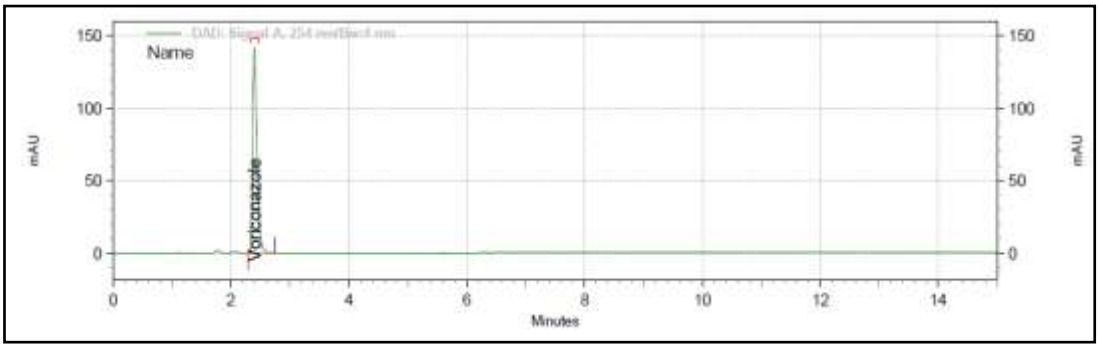

Fig-14: Chromatogram of thermal degradation sample

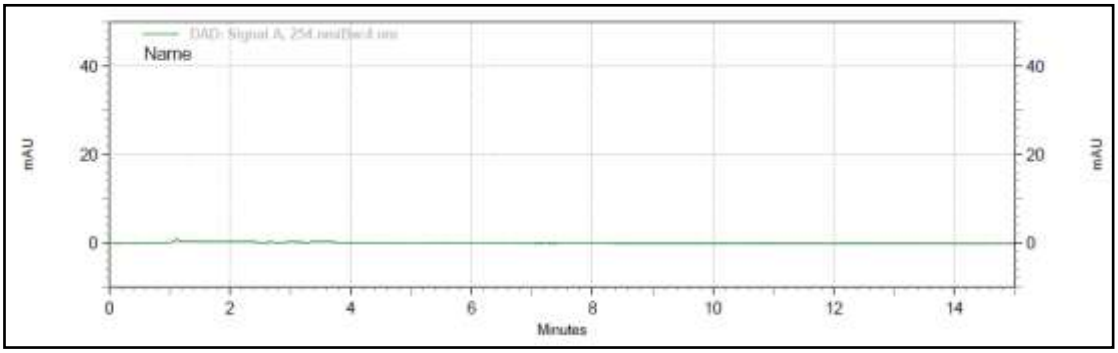

Fig-15: Chromatogram of thermal degradation placebo

Table-6: Solution stability of standard summary table

S. No	Condition	Time Point	Similarity factor
1	NA	Initial	NA
2	Room Temperature	24 Hours	1.00
3	Refrigerator	24 Hours	0.98

Table-7: Solution stability of sample summary table

S. No	Name	Condition	Time Point	% Assay	% Difference
1	Sample	Room Temperature	Initial	101.7	NA*
			24 Hours	100.9	0.8
2	Sample	Refrigerator	Initial	101.7	NA
			24 Hours	100.9	0.8

Table-8: Robustness parameters summary table

Parameter	Flow Rate			Temperature		
	Actual	Change-1	Change-2	Actual	Change-1	Change-2
	0.8 mL/min	0.6 mL/min	1.0 mL/min	45˚C	40˚C	50˚C
Blank Interference	Nil	Nil	Nil	Nil	Nil	Nil
% RSD with 5 Injections of Standard Solution-1	0.09	0.12	0.05	0.12	0.16	0.12
Similarity Factor of Standard Solution-2	1.00	0.99	0.99	1.00	1.01	1.01
Tailing factor of VCZ peak	1.37	1.39	1.30	1.41	1.41	1.39

Table-9: Physiological solution compatibility study data summary table

Diluent	Tests			
	Assay (0.5 mg/mL)		Assay (5.0 mg/mL)	
	Initial	24 hr	Initial	24 hr
0.9% Sodium Chloride Injection	102.2	98.1	99.2	98.7
Sodium Lactate (Ringer Lactate) Solution for Injection	101.2	96.6	99.1	98.1
5% Dextrose & Ringer Lactate Solution for Injection	101.2	99.4	99.7	99.3
5% Dextrose & 0.45% Sodium Chloride Injection	99.6	96.6	100.4	96.6
5% Dextrose Injection	99.0	95.4	96.6	100.0
5% Dextrose in 20mEq of Potassium Chloride Injection	103.4	97.2	100.2	96.3
0.45% Sodium Chloride Injection	100.5	95.0	100.0	96.1
5% Dextrose & 0.9% Sodium Chloride Injection	102.0	96.9	99.5	96.7

CONCLUSION

The analytical method developed to determine the assay of VCZ in bulk and lyophilized pharmaceutical dosage form is precise, accurate, linear and stability indicating. The above method is novel, rapid and cost effective when compared with other analytical methods and can be employed in commercial scale for the assay of VCZ in bulk, lyophilized pharmaceutical dosage form and in different physiological solutions for parenteral administration to patients.

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