EUROPEAN JOURNAL OF BIOMEDICAL AND PHARMACEUTICAL SCIENCES
http://www.ejbps.com

ISSN 2349-8870
Volume: 6
Issue: 10
161-170
Year: 2019

DEVELOPMENT & VALIDATION OF STABILITY INDICATING UPLC METHOD FOR QUANTITATIVE ESTIMATION OF VORICONAZOLE AND ITS IMPURITIES IN DRUG SUBSTANCE

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Article Received on 27/06/2019 **Article Revised on 16/07/2019** **Article Accepted on 06/08/2019**

ABSTRACT
Simple and sensitive RP-UPLC method for the separation and quantification of voriconazole and its impurities are described. Samples are analyzed by means of reverse phase (RP-UPLC) using an Acquity UPLC BEH Shield RP18 (2.1mm x 100mm, 1.7µ), and the mobile phase consists of two Channels A and B. Channel-A 0.05% TFA in water and Channel-B: 0.05% TFA in methanol. The flow rate is 0.2 ml/min. The column temperature was maintained at 30°C and sample temperature was maintained at ambient and wavelength fixed at 255nm UV-detection. It is found that the method of RP-UPLC with UV-detection system for the analysis of voriconazole impurities are straight forward and applied in qualitative and quantitative analysis. The developed UPLC method was validated with respect to specificity, precision, linearity, ruggedness and robustness. Validation study compared as per ICH guideline.

KEYWORDS: UPLC, Voriconazole, Stability Indicating, Validation.

1.0 INTRODUCTION
Voriconazole is a triazole antifungal medication used to treat serious fungal infections. It is used to treat invasive fungal infections that are generally seen in patients who are immuno compromised. These include invasive candidiasis, invasive aspergillosis, and emerging fungal infections. Voriconazole[1] is chemically (2R, 3S)-2-(2, 4-difluorophenyl)-3-(5-fluoropyrimidin-4-yl)-1-(1H-1, 2, 4-triazol-1-yl) butan-2-ol. It is a new[2] antifungal agent mainly used in immunocompromised patients for treatment of invasive aspergillosis, candidaemia in non-neutropenic patients, Flucanozole resistant serious invasive candidiasis, esophageal candidiasis and serious fungal infections[3] due to Scedosporium and Fusarium species.

Literature survey reveals that few UV[4], HPLC[5-7] and LC-MS[8] methods have been reported for estimation of voriconazole in pharmaceutical formulations and biological fluids. The proposed RP-HPLC method was simple, accurate and precise for estimation of voriconazole in tablet dosage forms. The objective of the present work is to develop a stability indicating UPLC method and validated as per ICH and USP validation guidelines[9-11] for the estimation of voriconazole in quality control laboratories with respect to specificity, limit of detection.

The chemical structure of voriconazole shown in **(Fig. 1.1).**
Impurity profiling of active pharmaceutical ingredients (API) in both bulk material and formulations is one of the most challenging tasks. The presence of unwanted or in certain cases unknown chemicals, even in small amounts, may influence not only the therapeutic efficacy but also the safety of the pharmaceutical products. For these reasons, all major international pharmacopoeias have established maximum allowed limits for related compounds for both bulk and formulated APIs. As per the requirements of various regulatory authorities, the impurity profile study of drug substances and drug products has to be carried out using a suitable analytical method in the final product.

Hence, the objective of this study is to develop simple, sensitive and accurate RP-UPLC method for estimation of related substances in voriconazole in pharmaceutical dosage forms. The developed method was validated according ICH[9-11] guidelines including various stability parameters proved for its accuracy.

2.0 EXPERIMENTAL

2.1 Reagents and chemicals

Trifluoroacetic acid, Methanol, Hydrochloric acid, Sodium hydroxide, Potassium dihydrogen phosphate and Hydrogen peroxide was procured from Merck. Water (Milli-Q). All chemicals were of an analytical grade and used as received.

2.2 Instrumentation

The analytical separations were carried out on Waters UPLC system with PDA detector. The analytical column was waters (RP-UPLC) using an Acquity UPLC BEH Shield RP18 (2.1mm x 100mm, 1.7µ), and the mobile phase consists of two Channels A and B. Channel-A 0.05% TFA in water and Channel-B: 0.05% TFA in methanol and (water : acetonitrile)(80:20) v/v was used as diluent. The flow rate was 0.2 ml/min. The column temperature was maintained at 30°C and sample temperature was maintained at ambient and wavelength fixed at 255nm UV-detection. The control of UPLC system and data collection was Empower[3] software.

2.3 Preparation of Diluted standard solution and sample solution

2.3.1. Diluted standard solution preparation

A working standard stock solution of Voriconazole was prepared by dissolving standard 25mg of Voriconazole into 100 ml volumetric flask, to this added 50 ml of diluent and sonicated for 10 minutes at a temperature not exceeding 20°C. Allowed the solution to attain room temperature and then diluted to the volume with diluent.

Further diluted 1ml of above stock solution in to 100ml volumetric flask to this added 50 ml of diluent mixed well and then diluted to the volume with diluent. (Concentration 5µg/ml).

2.3.5. Sample preparation

Weigh and transfer accurately 25mg of Voriconazole into 50ml volumetric flask and added 25ml of diluent and sonicated in ultrasonic bath for 20minutes with intermediate shaking at a temperature not more than 20°C. Allowed the flask to attain room temperature and diluted to the volume with diluent.

2.3.6. Spiked sample preparation

Impurity Stock solution

Weighed accurately 3 mg of each impurity (Impurity-A, B and C) transferred into a 25 ml volumetric flask 10ml of diluent was added and sonicated. Then volume was made up with diluent.

Weighed accurately 25 mg of voriconazole sample transferred into a 50 ml volumetric flask, added 20ml of diluent and sonicated to dissolve, further added 1.0 ml of impurity stock solution mixed well. Then volume was made up with diluent.

3.0 DISCUSSION

3.1 Method optimization parameters

An understanding of the nature of API (functionality, acidity, or basicity), the synthetic process, related impurities, the possible degradation pathways and their degradation products are needed for successful method development in reverse-phase UPLC. In addition, successful method development should result a robust, simple and time efficient method that is capable of being utilized in manufacturing setting.

3.2 Selection of wavelength

The sensitivity of the UPLC method depends upon the selection of detection wavelength. An ideal wavelength is one that gives good response for related substances and the drugs to be detected. The wavelength for measurement was selected as 255 nm from the absorption spectrum.

3.3. Selection of stationary phase

Proper selection of the stationary phase depends up on the nature of the sample and chemical profile. The drug selected for the present study was polar compound and could be separated either by normal phase chromatography or reverse phase chromatography. From literature survey, it was found that different C_{18} columns could be appropriately used for the separation of related substances for Voriconazole.

3.4. Selection of mobile phase

Different mobile phase and stationary phases were employed to develop a suitable LC method for the quantitative determination of impurities in Voriconazole. A number of column chemistries supplied by different manufacturers and different mobile phase composition were tried to get good peak shapes and selectivity for the impurities present in Voriconazole.

4.0 RESULTS

Poor base line and distorted peak shape and resolution was observed when BEH C18 RP Shield (100mm x 2.1mm, 1.7µ) and gradient mobile phase programmed of Mobile Phase: A 0.1% OPA in water and Mobile Phase: B 0.1% OPA in Acetonitrile. There was no proper resolution of impurities and analyte peak and efficiency of the peak is also not achieved and peak interferences are present.

Distorted peak shape and no proper resolution was observed when BEH C18 RP Shield (100mm x 2.1mm, 1.7µ) and gradient mobile phase programmed of Mobile Phase: A 0.1% TFA in water and Mobile Phase: B 0.1% TFA in Acetonitrile. There was no proper resolution of impurities and analyte peak and efficiency of the peak is also not achieved and peak interferences are present.

In further attempt made using BEH C18 RP Shield (100mm x 2.1mm, 1.7µ) Channel-A 0.05% TFA in water and Channel-B: 0.05% TFA in Methanol. The resolution of both drug and impurities was achieved. These

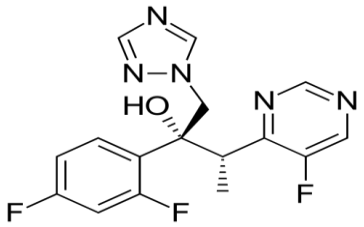
chromatographic conditions were selected for validation studies.

Chromatographic conditions

Mobile phase consists of 0.05% TFA in water as mobile phase A and 0.05% TFA in methanol as mobile phase B. Water and acetonitrile (80 : 20)v/v was used as diluent. The flow rate was kept at 0.20 ml/min with injection volume 2.0 µL. The separation was carried on Acquity UPLC BEH Shield RP18 (2.1mm x 100mm,1.7µ) column. The column temperature was kept at 30 ± 2 °C. The gradient program was 0.01/30,5/40,7/70,8/80,10/80,10.1/30,12/30.

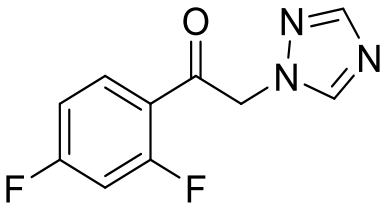
TFA: Trifluoroacetic acid

OPA: Orthophosphoricacid



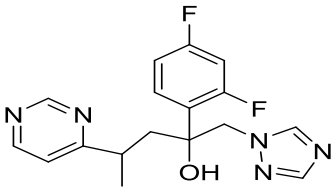
Chemical name : (2R, 3S)-2-(2, 4-Difluorophenyl)-3-(5-fluoropyrimidin-4-yl)-1-(1H-1,2,4- triazol-1-yl) butan-2-ol.

Fig. 1.1: Chemical structural of Voriconazole.



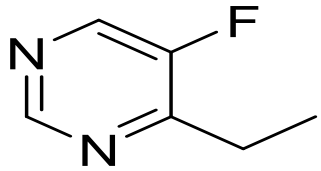
Chemical name: 2,4-Difluoro-α-(1H-1,2,4-triazolyl) acetophenone (Impurity-A)

Fig. 1.2: Voriconazole Impurity-A.



Chemical name: (R*, S*)-α-(2, 4-Difluorophenyl)-β-methyl-α-(1H-1, 2, 4-triazol-1-ylmethyl)-4-pyrimidineethanol (Impurity-B).

Fig. 1.3: Voriconazole Impurity-B.



Chemical name: 4-Ethyl-5-fluoropyrimidine (Impurity-C)

Fig. 1.4: Voriconazole Impurity- C.

5.0 METHOD VALIDATION

5.1 Specificity

Blank interference

A study to establish the interference of blank was conducted. Diluent was injected as per the test method.

Spiked Sample Preparation:

Impurity Stock solution:

Weighed accurately 3 mg of each impurity (Impurity-A, B and C) transferred into a 25 ml volumetric flask 10ml of diluent was added and sonicated. Then volume was made up with diluent.

Weighed accurately 25 mg of voriconazole sample transferred into a 50 ml volumetric flask, added 20ml of diluent and sonicated to dissolve, further added 1.0 ml of impurity stock solution mixed well. Then volume was made up with diluent.

It was observed that known impurities are not co eluting with each other and main analyte peak. Voriconazole standard solution preparation and in spiked test preparation was calculated and found to be within the acceptable limit.

5.2 Forced degradation studies

Forced degradation studies were performed to establish the stability indicating power of the method. In this study Voriconazole raw material, related impurities and final product were subjected to acidic, basic, peroxide, thermal and photolytic stress studies on sample concentration of 0.5 mg/ml in diluent. Weigh and transfer 25 mg of Voriconazole sample into 50 ml volumetric flask added 25 ml of diluent and sonicated for 20 min with intermediate shaking at a temperature not more than 20 °C and then added respective degradant (Acid, Alkali, Oxidant) and performed the stress study. Samples were neutralized after degradation and then diluted to the volume with diluent and injected to verify the stability indicating power of the analytical method. Stress conditions under which the study was performed, the amount of Voriconazole remains, %impurities generated and mass balance results were tabulated.

5.3 Precision

5.3.1 System Precision

Standard solution was injected in six replicate injections to check the Relative Standard Deviation (% RSD) for finding the precision of the system to be used for validation.

Acceptance Criteria: RSD should not be more than 5.0%.

The %RSD of peak area for Voriconazole was found to be 0.54 which is below 5.0% indicates that the system gives precise result.

5.3.2 Method Precision

Precision of the impurities and degradants method was determined by injecting six sample solutions spiked with impurities (Impurity-A, B and C) at specification level.

The samples were prepared as per the method and the result for precision study is tabulated in **Table: IV.**

The method precession was performed with six replicate solutions of standard solutions prepared and the system suitability parameters found were within the acceptance criteria.

5.3.3 Precision at Limit of Quantitation

Inject the blank, precision at LOQ solutions six times into UPLC. Record the peak area of Impurity-A, Impurity-B, Impurity-C and Voriconazole in each injection. Calculate the % RSD for the area of impurities and analyte peak. The % RSD for area of impurities and analyte peak from six preparations should be not more than 10.0.

The Precision at Limit of quantitation parameter results met the acceptance criteria.

5.4 Limit of detection (LOQ) & Limit of Quantitation (LOD)

For the present developed UPLC method Limit of Detection was found to be 0.16µg/ml for impurity-A, 0.16µg/ml for impurity-B, 0.16µg/ml for impurity-C and 0.17µg/ml for Voriconazole and Limit of Quantification was found to be 0.53µg/ml for impurity-A, 0.52µg/ml for impurity-B, 0.54µg/ml for impurity-C and 0.55µg/ml for Voriconazole respectively. LOD and LOQ were determined based on signal to noise ratio.

The limit of limit of quantitation and detection of quantitation values obtained for each impurity and Voriconazole are within the acceptance criteria.

5.5 Linearity and Range

Voriconazole and Impurities (impurities-A, B and C) in the concentration levels from LOQ to 200 % standard solution were injected into UPLC system. The linearity graph was plotted from LOQ to 200% of drug concentration. Report the linearity range as the range for determining the impurities. Results obtained are in the tables (Table VIII, Table IX, Table X and Table XI) & figures show the line of best fit for peak area versus concentration for each impurity.

The method for the estimation of Impurity-A, B, C and Voriconazole was found to be linear and the correlation coefficient was found to be 0.999, 1.000, 1.000, 1.000 and 1.000. The method was found to be linear in the range of LOQ to 200%.

5.6 Accuracy

Recovery of Voriconazole impurities in Voriconazole was performed. The sample was taken and varying amounts of Voriconazole impurities representing 50 to 150 % of specification level were added to the flasks. The spiked samples were prepared as per the method and the results are tabulated in **Table XII.**

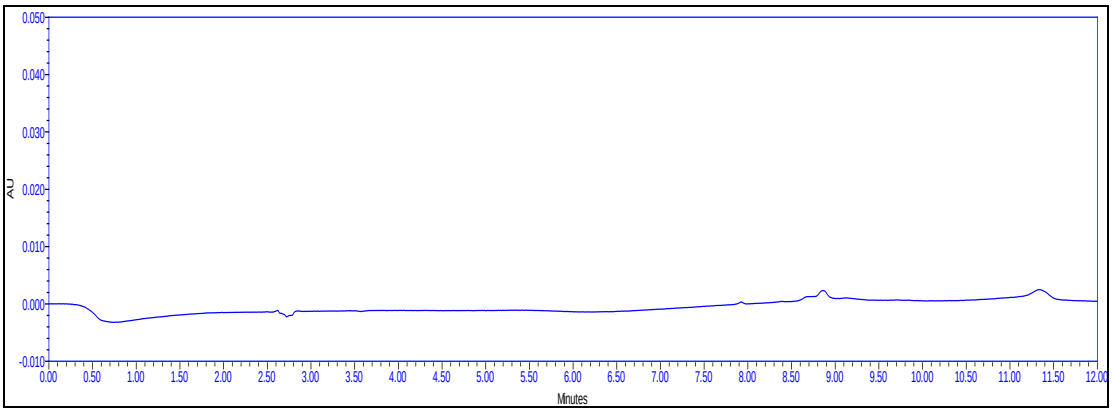


Figure: 1.5 typical chromatogram of Blank.

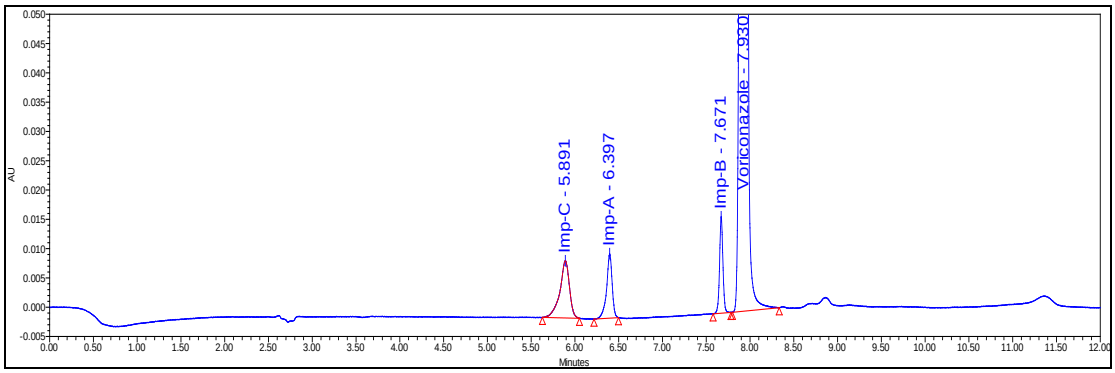


Figure: 1.6 typical chromatogram Spiked Sample.

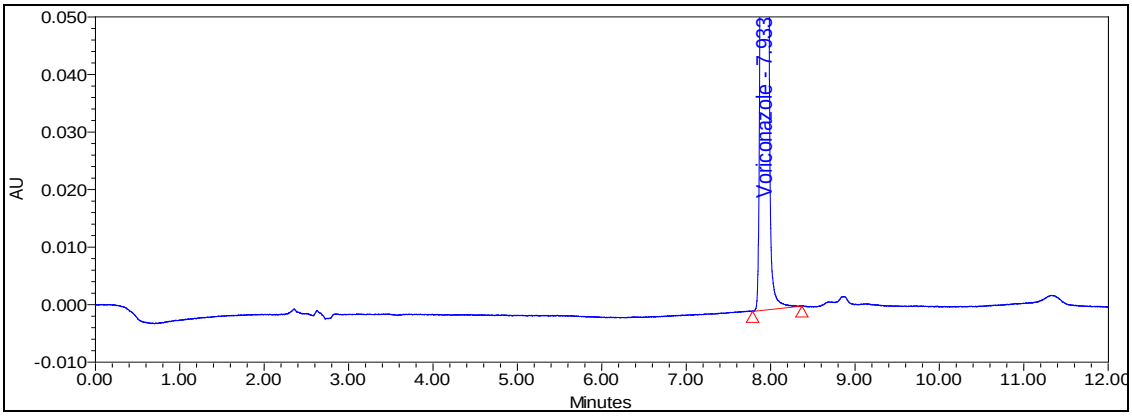


Figure: 1.7 Typical chromatogram of control sample.

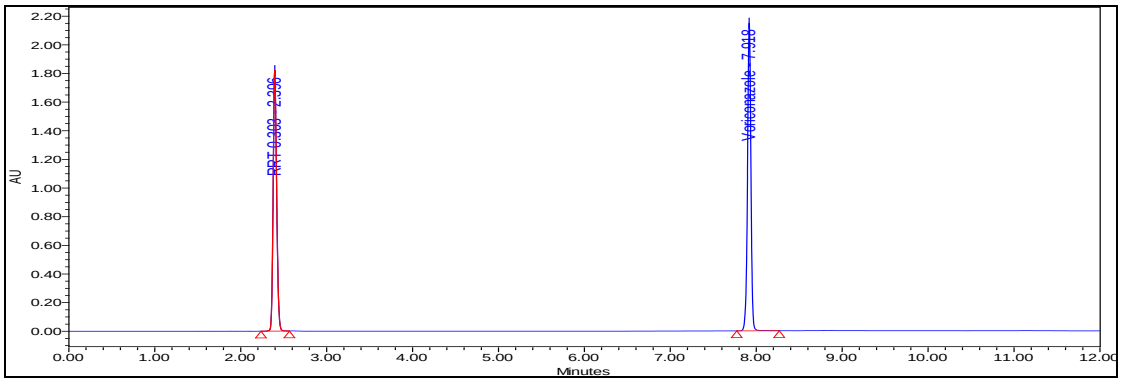


Figure: 1.8 Typical chromatogram of acid degradation sample.

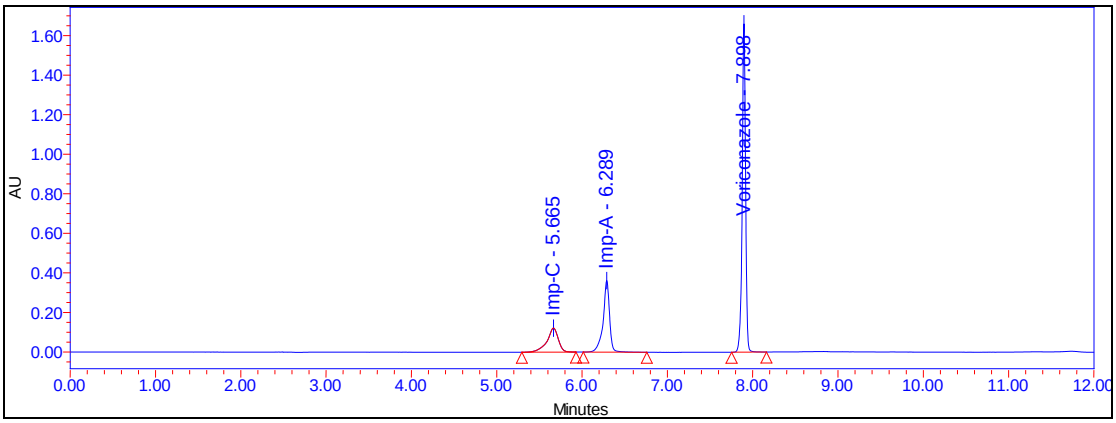


Figure: 1.9 Typical chromatogram of alkali degradation sample.

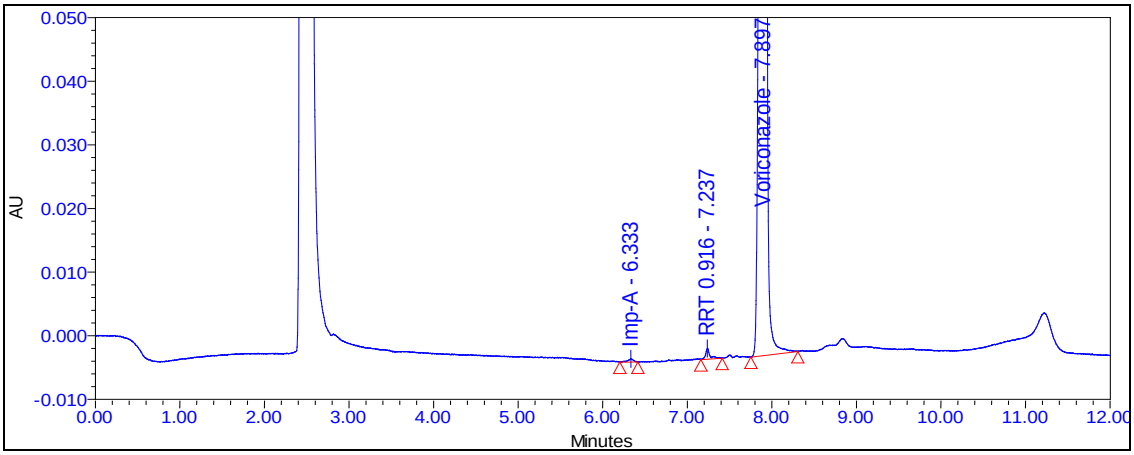


Figure: 2.0 Typical chromatogram of oxidation degradation sample.

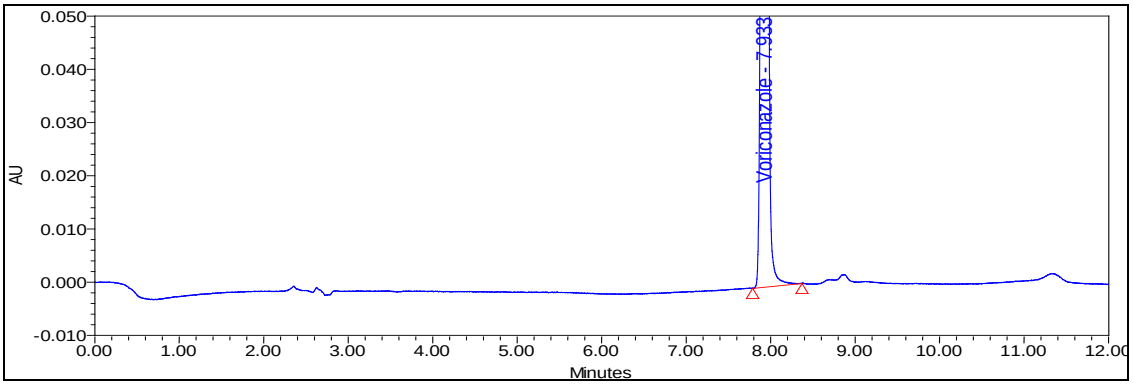


Figure: 2.1 Typical chromatogram of thermal degradation sample.

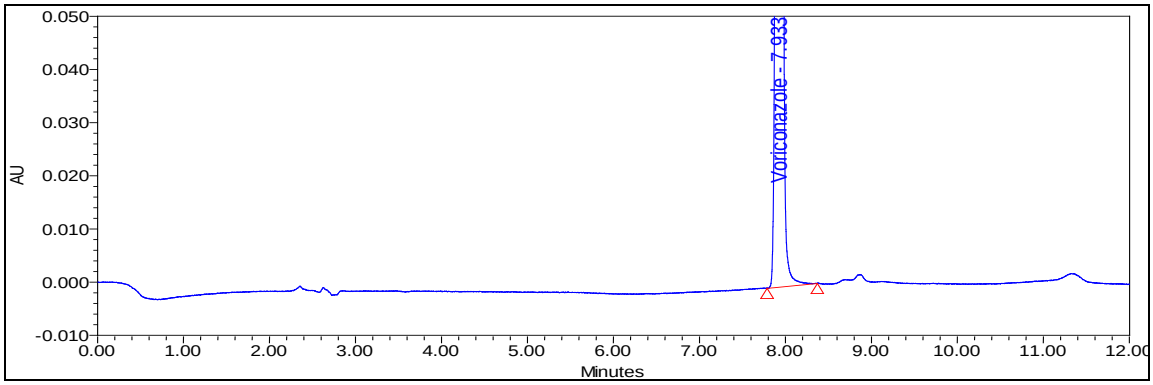


Figure: 2.2 Typical chromatogram of photolytic degradation sample.

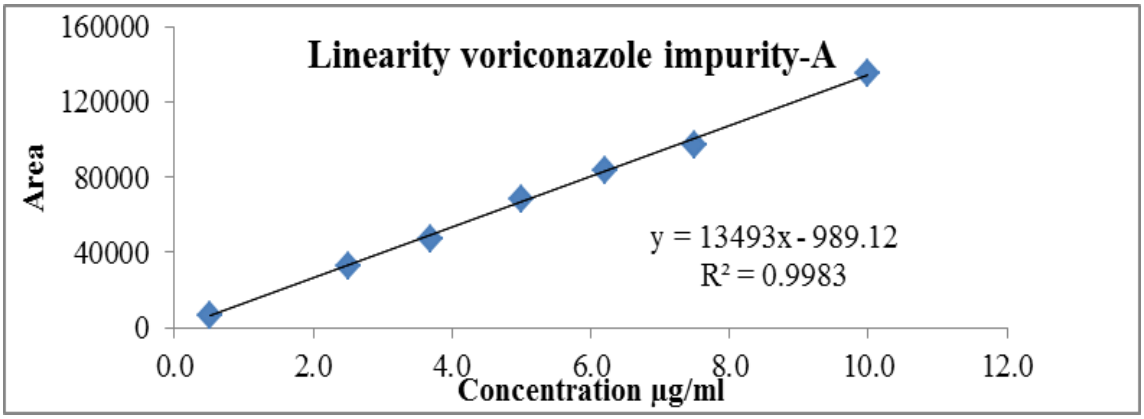


Fig. 2.3: linearity of detector response for Impurity-A.

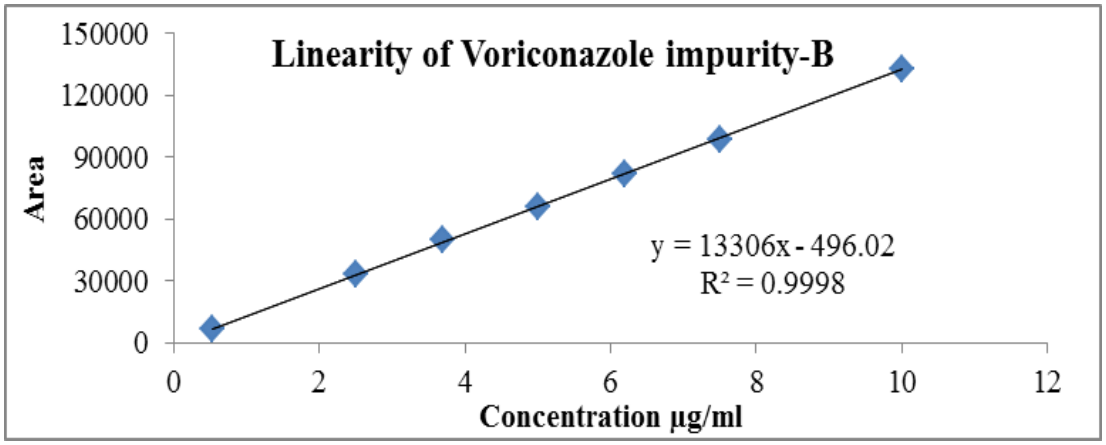


Fig. 2.4: linearity of detector response for Impurity-B.

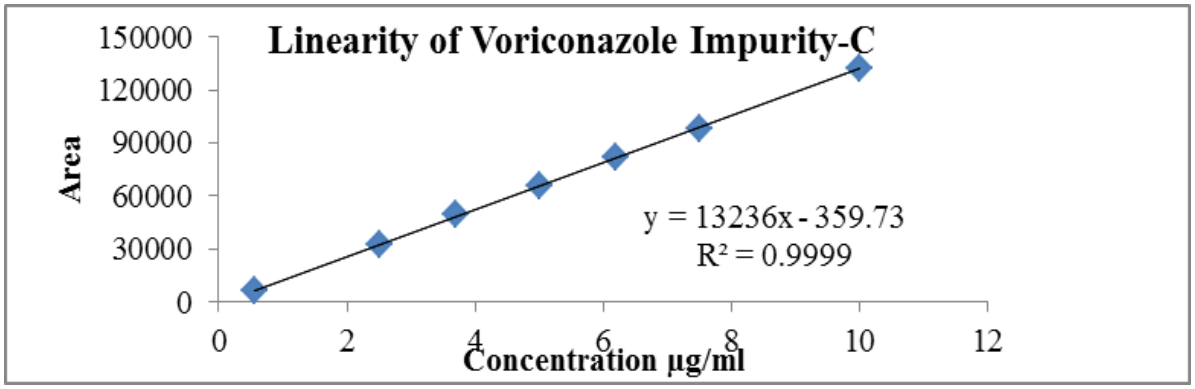


Fig. 2.5: linearity of detector response for Impurity-C.

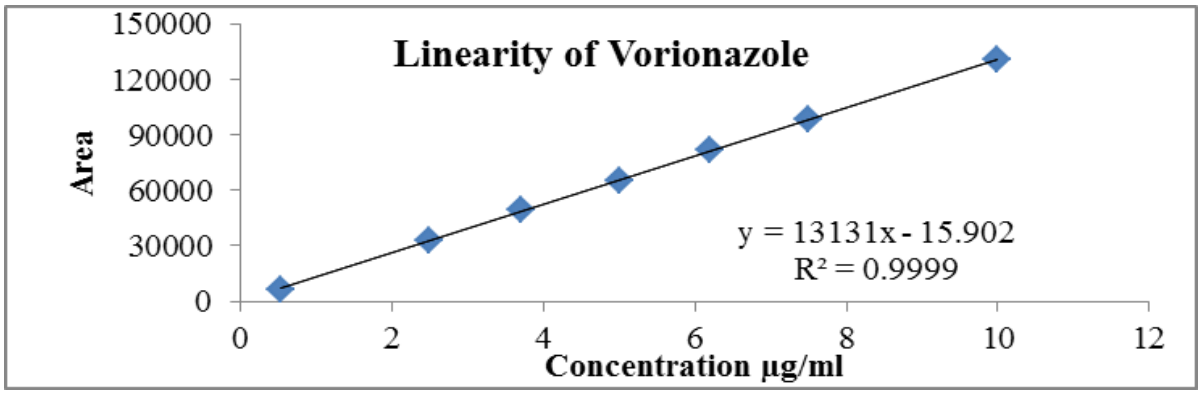


Fig. 2.6: linearity of detector response for Voriconazole.

I. Impurity interference data

Preparation	RT	Peak response
Blank	NA	ND
Sample with spike impurities		
Impurity-A	5.88	68500
Impurity-B	6.39	65800
Impurity-C	7.66	65700
Voriconazole	7.99	13100000

II. Stress condition and its results

S. No	Stress condition	% Drug remained	% impurities
1	As such sample	100.3	ND
2	1N HCl_60°C_2Hrs	74.8	23.1
3	1 N NaOH_60°C_2Hrs	98.8	1.3
4	3% H_2O_2_Bench top on 6hours	99.5	0.6
5	Thermal_105 °C_48 Hrs	100.1	ND
6	Photolytic stability	100.0	ND

III. System Precision data for Voriconazole

Injection	Area of standard
1	65470
2	64875
3	65412
4	64998
5	65001
6	65789
Avg	65258
%RSD	**0.54**

IV. Results of method precision

Sample name	Imp-A	Imp-B	Imp-C
sample-1	0.513	0.533	0.522
sample-2	0.515	0.515	0.514
sample-3	0.517	0.518	0.521
sample-4	0.523	0.511	0.513
sample-5	0.522	0.509	0.522
sample-6	0.517	0.507	0.517
Mean	**0.518**	**0.516**	**0.518**
%RSD	**0.76**	**1.84**	**0.79**

V. Results of precision at LOQ level

S.No.	Imp-A	Imp-B	Imp-C	Voriconazole
1	0.052	0.053	0.051	0.053
2	0.055	0.054	0.055	0.052
3	0.055	0.052	0.053	0.057
4	0.052	0.054	0.053	0.053
5	0.056	0.052	0.057	0.054
6	0.055	0.056	0.054	0.056
Mean	0.054	0.054	0.054	0.054
%RSD	3.18	2.83	3.79	3.58

VI. LOD for Voriconazole and impurities

Impurity/Compound name	Concentration in ppm	S/N
Impurity-A	0.16	4.13
Impurity-C	0.16	5.16
Impurity-B	0.16	4.65
Voriconazole	0.17	8.25

VII.LOQ for Voriconazole and impurities

Impurity/Compound name	Concentration in ppm	S/N
Impurity-A	0.53	10.35
Impurity-C	0.52	40.52
Impurity-B	0.54	23.05
Voriconazole	0.55	15.65

VIII. Linearity of detector response Impurity-A

Levels (%)	Concentration (µg/ml)	Area response of Impurity-A
LOQ	0.5	6450
50	2.5	32754
100	3.7	47125
125	5.0	68500
150	6.2	83877
200	7.5	97254
Correlation Coefficient:		0.999

IX. Linearity of detector response Impurity-B.

Levels (%)	Concentration (µg/ml)	Area response of Impurity-B
LOQ	0.54	6350
50	2.5	32854
100	3.7	49725
125	5	65800
150	6.2	81977
200	7.5	98354
Correlation Coefficient:		1.000

X. Linearity of detector response Impurity-C

Levels (%)	Concentration (µg/ml)	Area response of Impurity-C
LOQ	0.55	6450
25	2.5	32854
50	3.7	49325
100	5	65700
125	6.2	81977
150	7.5	98354
200	10	132043
Correlation Coefficient:		1.000

XI. Linearity of detector response Voriconazole

Levels (%)	Concentration (µg/ml)	Area response of Voriconazole
LOQ	0.53	6550
50	2.5	32754
100	3.7	49125
125	5	65500
150	6.2	81877
200	7.5	98254
Correlation Coefficient:		1.000

XII. Accuracy study of Voriconazole.

S.No.	Theoretical (%)	% Mean Recovery		
		Impurity-A	Impurity-B	Impurity-C
1	50	96.7	97.7	101.7
2	100	104.1	107.7	108.4
3	150	104.4	109.4	108.7

6.0 SUMMARY

A simple, accurate and reproducible reverse phase UPLC method was developed for the estimation of Voriconazole in drug substance. The present described impurities two are (Imp-A and Imp-C) related to degradants and one (Impurity-C) related to synthesis. There was one UPLC method was published but no clarity on degradation profile, so here the optimized method was clearly showing degradation with graphical representation, the method consists of 0.05% TFA in water and 0.05%TFA in methanol in gradient elution mode with a run time of 12minutes and a flow rate of 0.2 ml/min. UV detection was carried out at a wavelength of 255 nm with an injection volume of 2.0 µL. Acquity UPLC BEH Shield RP18 (2.1mm x 100mm, 1.7µ). The retention time of Voriconazole was found to be 7.9 minutes. The developed method was validated as per ICH Q2A (R1) guideline. The proposed UPLC method was linear over the range of LOQ-200% level for impurities and analyte peak, the correlation coefficient was found to be 0.999 and 1.000 for all impurities and analyte peak. Recovery of the impurities and Voriconazole was found to be within the limit 85-115 %. Relative standard deviation for system precision was found to be 0.54. Method precision was found to be below 2.0% impurities and analyte peak of Voriconazole. Limit of Detection was found to be 0.16µg/ml for impurity-A, 0.16µg/ml for impurity-B, 0.16µg/ml for impurity-C and 0.17µg/ml for Voriconazole and Limit of Quantification was found to be 0.53µg/ml for impurity-A, 0.52µg/ml for impurity-B, 0.54µg/ml for impurity-C and 0.55µg/ml for Voriconazole respectively. The method developed was statistically validated in terms of Selectivity, accuracy, linearity, precision and robustness. For Selectivity, the chromatograms were recorded for standard and sample solutions of voriconazole and its related substances. Selectivity studies reveal that the peak is well separated from each other.

7.0. CONCLUSIONS

So far published journals are explained about development and validations but not clear information about degradation and potential impurities during stress study. The present paper describes development, validation and stress study. From the results, it was observed that lesser run time, cost effective, ecofriendly and the developed method was proven to be specific, precise, linear, accurate, rugged and robust and is suitable for its intended purpose. Hence it was concluded that this method can be used for the routine estimation of related substances of Voriconazole in drug substances.

8.0. ACKNOWLEDGEMENT

The authors wish to thank to Dr Reddys Laboratories for providing the analytical support to pursue this work.

9.0. COMPLIANCE WITH ETHICAL STANDARDS

*** Disclosure of potential conflicts of interest**

The authors declare no conflicts of interest.

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