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Stability Indicating Reverse Phase Chromatographic Method for Estimation of Related Substances in Voriconazole Drug Substance by Ultra Performance Liquid Chromatography

Maheswara Reddy Musirike*, Hussain Reddy K and Useni Reddy Mallu

Department of Chemistry Sri Krishnadevaraya University, Ananthapur, India

Abstract

A simple, sensitive and accurate analytical method for related substances of Voriconazole has been developed and validated by using RP-UPLC technique. The developed analytical procedure was validated as per ICH recommendations. Analysis was performed on a HALO C18 (100 × 2.1 2.7 µ) column. 20 mM ammonium formate adjusted the pH to 4.5 with formic acid was selected as buffer. Acetonitrile was used as organic modifier in the mobile phase composition. A simple gradient was applied in the method. Flow rate of mobile phase was kept at 0.4 ml per min. Column compartment temperature was maintained at 45°C. Injection volume was set at 1 µL with an auto sampler maintained the temperature at 10°C. Detection of all the components was monitored at 254 nm by photodiode array detector. Developed method satisfies the system suitability criteria, peak integrity, and resolution for the parent drug and its related substances. The proposed method was validated for Specificity, precision, accuracy, linearity, limit of detection and quantification. Forced degradation studies were conducted to assess stability indicating nature of the method under acidic, basic, oxidative and photolytic conditions. Run time less than 7.0 minutes indicating that the method is cost effective and productive; it can be successfully applied for testing of related substances in drug substance and assay of drug substance in routine quality control analysis and stability testing.

Keywords: Voriconazole; Ultra performance liquid chromatography; Fungal infections; Related substances, Method development and validation

Introduction

Voriconazole is a second-generation triazole antifungal agent, designated chemically as (2R,3S)-2-(2,4- difluorophenyl)-3-(5-fluoro-4-pyrimidinyl)-1-(1H-1,2,4-triazol-1-yl)- 2-butanol. Voriconazole has been widely used for the treatment of invasive fungal diseases, particularly invasive aspergillosis. In addition, voriconazole is also approved for the treatment of invasive candidosis, as well as for less frequent fungal infections such as fusariosis and scedosporiosis. Its fungicidal action is due to inhibition of fungal cytochrome P450-dependent 14α-sterol demethylase, a key enzyme of ergosterol biosynthesis. Inhibition of ergosterol biosynthetic pathway leads to a disruption of the integrity and the function of the fungal membrane.

As per the literature Voriconazole estimation was done by chiral capillary electrophoresis method [1] and HPTLC [2] methods. For the determination of voriconazole concentrations in the biological fluids, the main methods presently used are high-performance liquid chromatographic techniques coupled with mass spectrometry [3-9]. High performance liquid chromatographic techniques coupled with ultraviolet spectroscopy were proposed for estimation voriconazole concentration in human plasma [10-14].

Since this drug is being marketed in domestic and international market the present investigation by the author was to develop a rapid, accurate and precise RP-UPLC method [15-17] for the determination of related substances. The objective was to develop a cost effective ultra fast reverse phase UPLC method for estimation of related substances in Voriconazole, the developed method was validated as per regulatory guidelines and successfully transferred to quality control lab. The innovative approach of using stationary phase with sub 2 µ particles [18-21] provides a comprehensive combination of selectivity and speed. The validation parameters [22,23] provide valuable information on precision accuracy, limit of detection, limit of quantification and linearity of related substances. The method was subjected to validation according to ICH requirements [24,25].

Figure 1 represents chemical structures of Voriconazole and its four related substances.

Materials and Methods

Instrumentation and reagents

The Waters ultra performance liquid chromatograph equipped with photodiode array detector .The output signal was monitored and processed using the Empower software. Acetonitrile was purchased from Rankem. MilliQ purification system was used to get HPLC grade water. Analytical column was a 100-mm × 2.1-mm Halo C18 (advanced materials technology) with 5-µm spherical particles. Test samples and reference standards of Voriconazole were donated by Apotex India pvt ltd.

Chromatographic conditions

Mobile phase consists of 20 mM ammonium formate buffer and pH of the solution was adjusted to 4.5 with formic acid. The chromatographic separation was achieved on a Halo C18, 100 × 2.1

***Corresponding author:** Maheswara Reddy Musirike, Department of Chemistry Sri Krishnadevaraya University, Ananthapur, Andhra Pradesh- 515003, India, Tel: 085542 55700; E-mail: musirikemahesh@gmail.com

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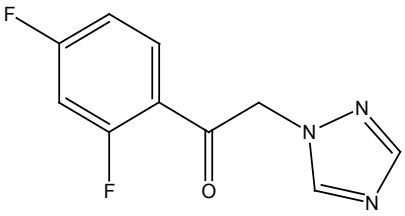
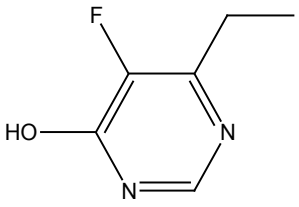


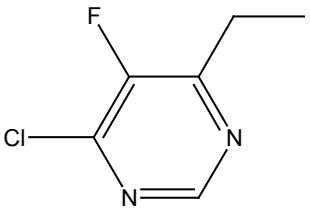
1-(2,4-Difluorophenyl)-2-(1,2,4-triazol-1-yl)ethanone

(a) Voriconazole impurity A (Process/Degradation impurity)



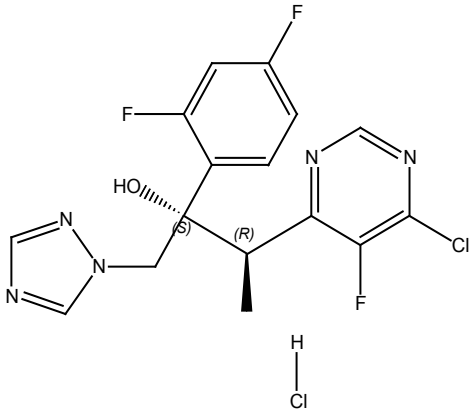
6-Ethyl-5-fluoropyrimidin-4-ol

(b) Voriconazole impurity B (Process impurity)



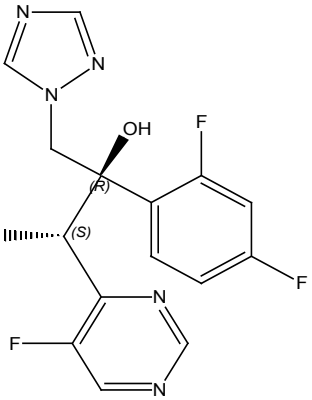
4-Chloro-6-ethyl-5-fluoropyrimidine

(c) Voriconazole impurity C (Process/Degradation impurity)



(2R, 3S/2S, 3R)-3-(4-chloro-5-fluoropyrimidin-6-yl)-2-(2, 4-difluorophenyl)-1-(1H-1, 2, 4-triazol-1-yl)butan-2-ol Hydrochloride

(d) Voriconazole impurity D (Process impurity)



(2R,3S) -2- (2, 4- difluorophenyl)-3-(5-fluoro-4-pyrimidinyl)-1-(1H-1, 2, 4-triazol-1-yl)-2-butanol

(e) Voriconazole

Figure 1: Chemical structures of Voriconazole and its related substances.

mm 2.7 µm Column. HPLC grade acetonitrile was used as organic modifier. Mobile phase flow rate was kept at 0.4 mL/min. Gradient program was set as Time/ % of solution B: 0/10, 1/20, 1.5/40, 4/50, 5/70, 5.5/10, 7/10. Column temperature was maintained at 45°C and detection was carried at 254 nm. Sample compartment was maintained at 10°C with an injection volume of 1 µL.

Preparation of standard and sample

A mixture of standard solution was prepared by weighing Voriconazole and its related compounds to yield a final concentration 0.10% each of Voriconazole, Impurity A, Impurity B, Impurity C and Impurity D with respect to the test sample concentration of 0.5 mg/ml. Buffer and acetonitrile in the ratio 8:2 was used as the diluent for preparation of sample and standard solution.

System suitability

A standard solution was injected on to the chromatographic system to ensure system suitability by verifying Retention times (RT), Peak Tailing factor (T), column efficiency (N) and resolution (R) for all related substances of Voriconazole. For confirmation of co- elution and interference of other impurities, peak purity testing was done.

Tailing factor should be not less than 0.9 and not more than 2.0. Plate count should be minimum 5000 and resolution should not be less than 2.0 among all the components. Peak purity evaluation should be done to evaluate homogeneity of peak.

System precision

Precision of the method was reported by injecting six replicates of

standard solution consecutively under the same analytical conditions. Standard deviation and % of residual standard deviations of all the components were reported. Intermediate precision of the method was also evaluated using different analyst, different day and different make of instrument in the same laboratory.

Report standard deviation and % RSD of all the related substances and Voriconazole. % RSD should not be more than 5.0%.

Limit of detection (LOD) and limit of quantification (LOQ)

The Limit of detection and Limit of quantification for Voriconazole and its related substances were determined from linear regression plot. Residual standard deviation and slope of the standard solution were considered for calculation of LOQ and LOD. Precision, Linearity and Accuracy were proved at LOQ level.

Residual standard deviation multiplied with 10 and divided by slope can be used for calculation of quantification limit and residual standard deviation multiplied with 3 and divided by slope can be used for calculation of detection limit.

Linearity

Solutions for Linearity of related substances method was prepared by serially diluting the impurity stock solution to required concentration levels. The solutions were prepared at six different concentration levels ranging from LOQ to 160% with respect to specification limits. Calibration curve was drawn by plotting the peak response of related substances versus its corresponding concentrations.

Correlation coefficient of the calibration curve should not be less than 0.990, slope, intercept and relative response factors were established.

Accuracy

Voriconazole sample solution was spiked with impurity standard solutions at three concentration levels corresponding to LOQ, 100% and 160% of impurity concentration. The % recovery of three levels was reported.

% Recovery was calculated based on amount of standard addition and amount of recovery in the test sample solution. % Recovery should be not less than 80% and not more than 120%.

Specificity

Demonstration of method specificity is to measure the analyte resolution in the presence of its potential impurities and degradants. The specificity of the developed RP-UPLC method was conducted in presence of sample diluent and its four potential impurities. Test samples were exposed to forced degradation studies under various acid, base, oxidative and photolytic conditions.

Test ample should be free of interferences from sample diluent and degradation impurities. Peak purity should be evaluated to assess homogeneity of peak under all the stress conditions.

Results and Discussion

Method development and optimization

The main objective of the chromatographic method development was to separate Voriconazole impurities from the main peak with an adequate resolution and symmetry. The initial method scouting started with an Isocratic mobile phase. It was observed that the impurity C is closely eluting with main peak in isocratic mode and also peak symmetry of impurities and voriconazole was poor. Adequate resolution and symmetrical peak shapes were achieved in gradient elution with a shorter run time. 20 mM Ammonium formate was selected as a buffer by considering mass compatibility. The wavelength maximum for voriconazole and its related substances was in the range of 245-260 nm. Selection of 254 nm as detection of all impurities provides good response. Resolution of all the related substances and symmetrical peaks were noticed in Halo C18 column when compared to Acquity BEH C18 column. Selection of acetonitrile as organic modifier provides stable baseline and eliminates interference from blank.

System suitability

All the related substances of voriconazole were eluted as per the retention given below. Adequate resolution was found between all the related substances. Voriconazole drug substance was found symmetric and well separated by its potential process impurities. A typical system suitability chromatogram of sample diluent, standard solution and test solution chromatograms are shown in Figure 2a-2c.

In the optimized conditions, Voriconazole and its related substances were well resolved with a resolution of more than 2.0. The tailing factor is in the range of 1.0-1.2 which indicates symmetry of peaks. Theoretical plates more than 10000 show the efficiency of the column. System suitability results are tabulated in Table 1.

System precision

System precision was evaluated by performing six replicate injections of standard solution at specification level. The % relative standard deviation of 6 injections was within the acceptable limit.

The obtained % RSD results are in the range of 1.2-3.8 which indicates the precision of the instrument to proceed for analysis. Results are tabulated in Table 2.

Limit of detection (LOD) and Limit of quantification (LOQ)

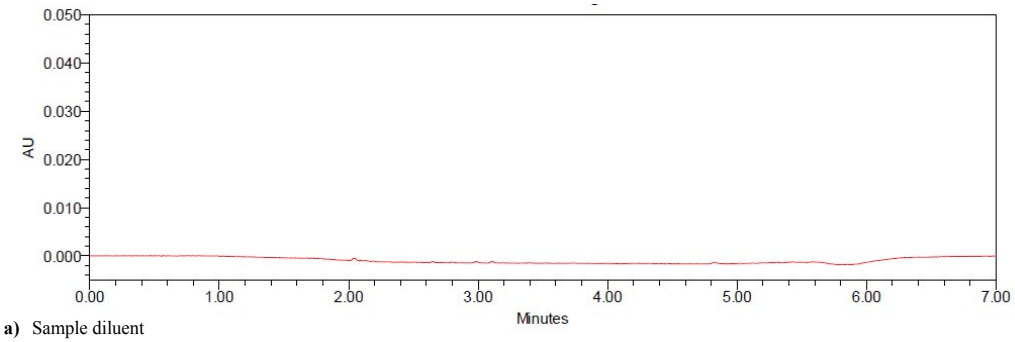
Limit of detection (LOD) and limit of quantification (LOQ) of Voriconazole drug substance and its related substances were established from linear regression curve by measuring residual standard deviation and slope of the curve. Results are tabulated in Table 3. Sensitivity is the ability of method to detect and quantify the impurities present in the sample accurately.

Precision at LOQ level was determined for 6 consecutive injections and the obtained % RSD was 4.8 %. Accuracy at LOQ level was in the range of 89 to 105.2%. The obtained correlation coefficient of 0.999 from regression statistics declares linear relationship of the method.

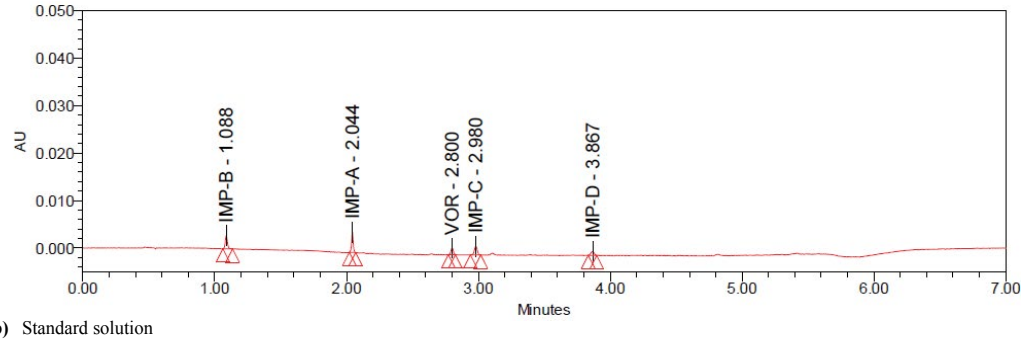
Linearity

Linearity of the method was to establish a linear relationship of concentration over response. Solutions of Voriconazole and its related substances are prepared from LOQ level to 160% of the specification limit. The correlation coefficient obtained was greater than 0.99. The regression statistics for Voriconazole drug substance and its related substances are tabulated in Table 4.

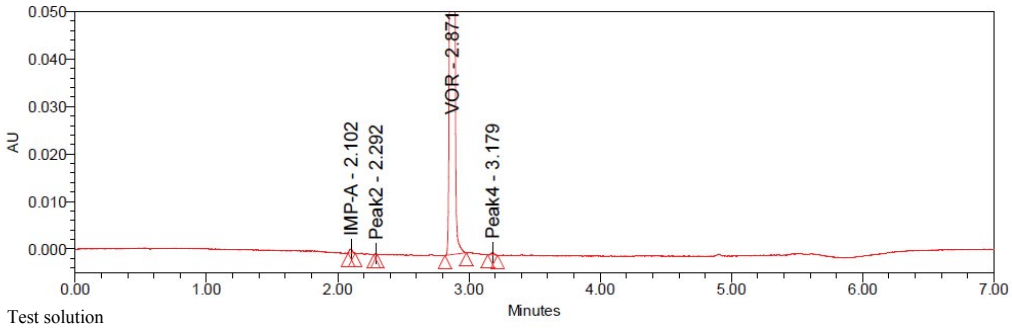
The obtained data stated that an excellent correlation was established between the peak response and concentration of the analyte and impurities. Relative response factor was also calculated to estimate the response factor of impurity against drug the response of substance to measure the accurate content of impurity present in the drug substance. Graphical representation of linear regression is shown in Figure 3.



a) Sample diluent



b) Standard solution



c) Test solution

Figure 2: A typical chromatogram showing the Voriconazole (a) sample diluent (b) Standard solution.

Component	RT (Min)	Resolution	Tailing factor	Plate count	Peak angle	Peak threshold
Impurity B	1.1	-	1.1	12500	2.4	6.1
Impurity A	2.0	37.0	1.0	13339	5.0	5.3
Voriconazole	2.8	26.1	1.1	45666	10.2	16
Impurity C	3.0	4.7	1.2	125212	8.8	13.5
Impurity D	3.9	19.6	1.1	113331	10.8	21.3

Table 1: System suitability results.

Injection	Response				
	Voriconazole	Impurity A	Impurity B	Impurity C	Impurity D
1	1856	3750	3136	2767	1505
2	1811	3741	3037	2520	1373
3	1861	3671	2961	2544	1406
4	1803	3729	3096	2505	1354
5	1886	3797	3103	2630	1380
6	1811	3791	3020	2596	1396
Mean	1838	3746	3059	2594	1403
STDEV	34	46	65	97	53
% RSD	1.9	1.2	2.1	3.7	3.8

Table 2: System precision results.

Inj.#	Voriconazole	Impurity A	Impurity B	Impurity C	Impurity D
LOQ (µg/ml)	124	81.4	60.8	76.8	146.7
LOD (µg/ml)	41	27.1	20.3	25.6	48.9

LOQ-Limit of Quantitation; LOD-Limit of Detection

Table 3: Limit of quantitaion and detection.

Level	Voriconazole		Impurity A		Impurity B		Impurity C		Impurity D	
	Conc. (µg/mL)	Area (µV*sec)	Conc. (µg/mL)	Area (µV* sec)	Conc. (µg/mL)	Area (µV* sec)	Conc. (µg/mL)	Area (µV* sec)	Conc. (µg/mL)	Area (µV* sec)
LOQ	123.6	350	81.3	460	60.8	220	76	210	146.7	246
40%	424	810	432	1780	428	1357	412	1190	436	695
80%	848	1498	864	3240	856	2565	824	2200	872	1295
100%	1060	1790	1080	3920	1070	3120	1030	2650	1090	1570
120%	1272	2096	1296	4640	1284	3750	1236	3190	1308	1820
160%	1696	2769	1728	5995	1712	4954	1648	4176	1744	2380
Slope	1.5271		3.3493		2.8483		2.4975		1.3257	
Intercept	173.85		279.40		92.38		93.99		97.75	
R	0.9997		0.9994		0.9998		0.9993		0.9990	
RRF	1.00		2.19		1.87		1.64		0.87	

RRF-Relative Response factor, R- Correlation Coefficient

Table 4: Linearity results.

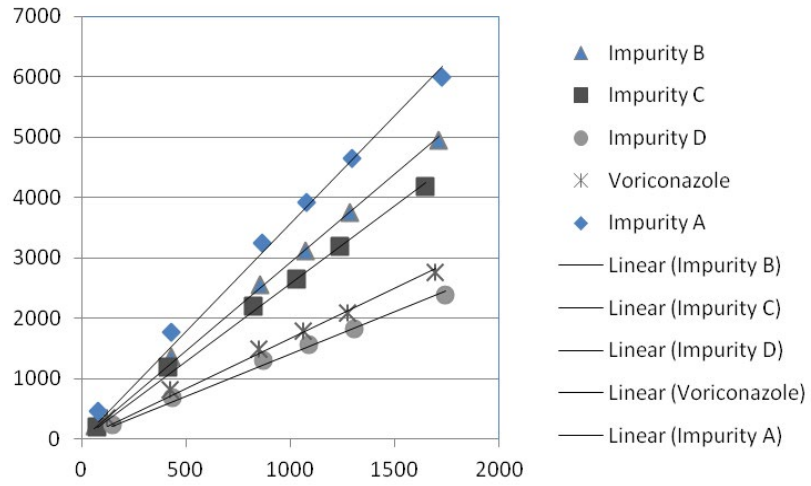


Figure 3: Graphical representation of regression plot.

Accuracy

Accuracy of the method was determined by addition of known concentrations of standard solution to the test sample. The obtained recovery value indicates the trueness of the method to estimate impurities. Related substances of voriconazole spiked to the sample over a concentration range varying from QL to 160% of their respective target analyte concentrations. Acceptance criteria were in the range of 80% to 120%.

The obtained percentage recovery value of related substances is in the range of 86.7% to 105.2% which declares the method accuracy. Accuracy results are reported in Table 5. A chromatogram of spiked sample is shown in Figure 4.

Forced degradation studies

Degradation studies were performed to demonstrate stability indicating nature of the method. Voriconazole test sample was exposed under various stress conditions like heat and humidity (40°C and 70% RH for 7 days), thermal (60°C for 7 days) and photolytic conditions of fluorescent light (1.2×10^6 LUX hours), UV light for a total exposure of 200 W·hr/m^2, acid hydrolysis (1 N HCl 80°C for 24 Hrs), base hydrolysis (0.1 N NaOH, 80°C for 30 min) and oxidative stress. Testing of peak purity concludes the homogeneity and interference of unidentified impurities with peak of interest. The obtained peak purity value gives a clear indication of separation between stressed impurities with related substances of Voriconazole.

Sample prone to more degradation in basic and acidic conditions. Deschloro and Impurity A were observed in acid and basic hydrolysis conditions. A slight degradation was observed in heat and peroxide conditions. No degradation was observed in photolytic condition. Peak obtained in all the stress conditions was homogenous and unaffected by the presence of its degradation impurities, confirming the stability indicating nature of the method. Mass balance also established to

S.NO	Accuracy Level	Impurity A	Impurity B	Impurity C	Impurity D
1	LOQ	105.2%	96.1%	89.0%	93.2%
2	100%	94.9%	90.1%	88.7%	100.0%
3	160%	86.7%	93.2%	88.9%	96.8%

Table 5: Accuracy results.

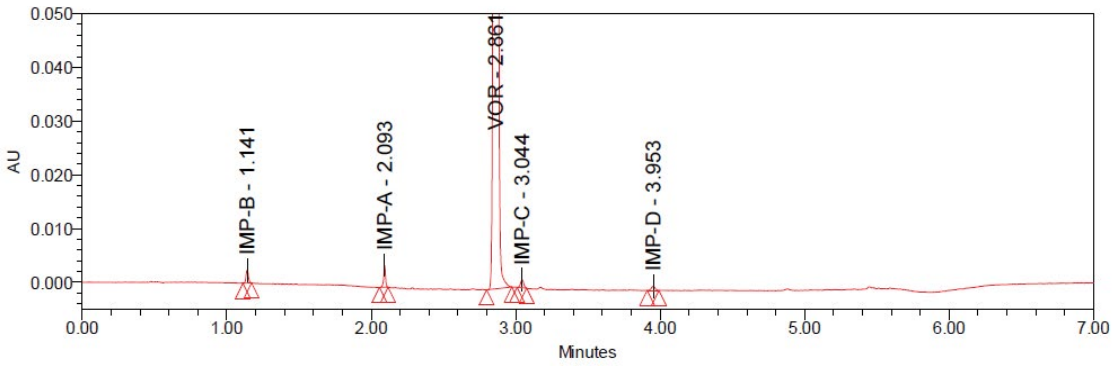


Figure 4: Test sample spiked with standard solution.

Stress condition	Conc. µg/mL	%Degradation	Major degradant	Purity angle	Purity Threshold	Mass balance
Non stressed	512	-	-	0.10	2.2	100
Acid hydrolysis	495	12.6%	Imp-A&C	0.15	2.45	99.2
Base hydrolysis	502	18.1%	Imp-A&C	0.22	3.22	98.8
Oxidation	515	3.2%	Imp-A	0.25	2.66	99.1
Heat and humidity	501	No significant	9.11	0.19	1.72	99.2
Photo stability	511	No significant	8.22	0.26	3.55	99.4
Dry heat	509	5.4%	Imp-A	`0.13	3.75	99.7

Table 6: Forced degradation studies.

match up the sum of impurities with its assay value against reference unstressed sample. The results from forced degradation studies are summarized in Table 6.

Conclusion

This paper describes a simple, accurate and reproducible fast Reverse phase-UPLC method for estimation of related substances in Voriconazole drug substance. Method validation was executed as per the guidelines recommended by ICH. Forced degradation studies were established to prove stability indicating nature of the method. The primary objective of the method was achieved by demonstrating that the method was cost effective, time effective and aiming towards green chemistry. The developed method was specific, precise, accurate and linear to estimate accurate amount of impurities present in the sample. Degradation studies confirmed the homogeneity and free of interferences with the peak of interest.

The method can be adopted to determine the related substances of drug substance in quality control labs. The same procedure can also be used to perform assay of drug substance.

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