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Analytical Assay Method Development and Validation of Itraconazole in Itraconazole Ointment By Reverse Phase-HPLC

Sutapa Biswas[1], Laxmi Sah[2], Akhilesh Shah[3]*

1.Department of Pharmaceutical Chemistry, Manipal College of Pharmaceutical Sciences, Manipal-576104, Karnataka, India

2.School of Pharmacy, Sharada University, Greater Noida, Uttar Pradesh, India

3.Department of Pharmaceutics, Poona College of Pharmacy, Erandwane, Pune-411 038, India

ABSTRACT

This study presents the analytical assay method development and validation of Itraconazole in an ointment dosage formulation by using reverse phase HPLC. The assay method development was carried out by using C18 column. In the beginning, the study was carried on Itraconazole API by taking USP method as a reference. By changing few chromatographic parameters, a symmetrical peak and a satisfactory result could able to achieve. Changes done as per USP chapter "allowable adjustment to United States Pharmacopeia method". The aim was to achieve an advanced chromatographic conditions on HPLC system with C18 column (150 × 4.6 mm, 5µm particle size) using mobile phase composed of acetonitrile and neutral buffer. The separation was achieved at different gradient flows for alternative methods. The ideal wavelength was calibrated by using PDA detector and the same wavelength was used for UV- visible detector. The assay method developed was also validated with full agreement with present regulatory guidelines by applying well developed analytical method validation techniques and means which includes the parameters such as linearity, accuracy, method precision, specificity with force degradation, robustness, solution stability, system suitability. The method shows linearity over a concentration range of 10µg/ml to 250µg/ml with r^2=0.999 and thus this represents the method is efficient to provide good detector response. The lower limit of detection and quantification was achieved by carrying out the studies like LOD and LOQ and the results were found to be 10µg/ml and 5µg/ml respectively.

Keywords: Method validation, reverse phase-HPLC, itraconazole ointment, limit of detection, limit of quantification. forced degradation.

*Corresponding Author Email: direct2akhilesh@gmail.com

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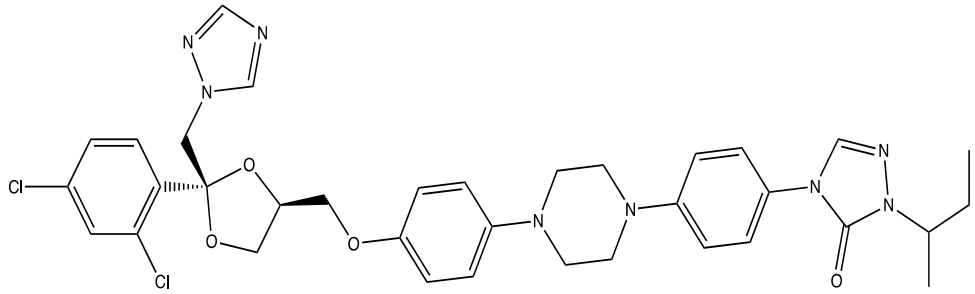
INTRODUCTION

Itraconazole was patented in 1978 and approved for medical use in the United States in 1992. The WHO's listed it as essential medicines, the most needed medications in a basic health system.(1,)



4-(4-(4-(4-(((2*R*,4*S*)-2-((1*H*-1,2,4-triazol-1-yl)methyl)-2-(2,4-dichlorophenyl)-1,3-dioxolan-4-yl)methoxy)phenyl)piperazin-1-yl)phenyl)-2-(*sec*-butyl)-2,4-dihydro-3*H*-1,2,4-triazol-3-one

Figure 1: Itraconazole

Itraconazole is a new triazole drug which is effective against a broad range of superficial and deep fungal pathogens (2). It is lipophilic and is well absorbed after oral administration. It is extremely potent, including its major metabolite, hydroxy-itraconazole(3). The Itraconazole is available presently in three different formulations in the market, that is, in the form of capsule, oral solution and intravenous injection, where it permit a versatile perspective to the therapy of fungal infections(4). As there is no ointment formulation of Itraconazole is available in the world market, a small attempt has been made to formulate the dosage form. After extensive experimentation by Dr. Reddy's Laboratories, Hyderabad, India, as a part of their research, an ointment formulation has been developed. Itraconazole acts by preventing the multiplication of fungi by disrupting the development of ergosterol, an important constituent of the fungal cell membrane. In usual situations, lanosterol, the parent molecule of ergosterol, subjected to 14α-demethylation by fungal cytochrome P450 (CYP). Fungal CYP links with the substrate binding site and obstruct the chemical reaction. As a consequence, other 14α-methyl sterols and lanosterol accumulate in the cell membrane in substitute of ergosterol. The disablement of ergosterol development causes deformities in the fungal membrane penetrability, membrane-bound enzyme activity and in chitin development(5).

No chromatographic methods have been published till date for the quantitation of itraconazole in ointment formulation. Some studies related to itraconazole demonstrating method development for analysis of itraconazole and its associated production impurities by ultra-performance liquid chromatography (UPLC) method were done(6). UV spectroscopy method was developed for analysis of Itraconazole in bulk and capsule dosage forms(7).

MATERIALS AND METHOD

Chemicals and Reagents

The itraconazole API (100.2%) were procured from Smilex Laboratories Limited, Hyderabad. The itraconazole ointment 5% w/w was formulated by Dr Reddy's Laboratories Ltd, Hyderabad. Emplura grade Tetrabutylammonium hydrogen sulphate and HPLC grade methanol were provided by Finar. HPLC grade Acetonitrile and Emplura grade Hydrochloric acid were provided by Standard and Merck respectively. Water was purified using a TKA Genpure.

Instrumentation and Chromatographic Conditions

Mettler electronic analytical balance model AG 245 was used for weighing chemicals and reagents. Lab companion ultrasonic cleanser USC-20 was used for sonicating the sample and standard for a complete extraction of the API form the ointment matrix. 0.45µ PVDF Nylon filter were used for filtration.

The HPLC analysis were performed on a Waters W2690 series HPLC quaternary system with a W2996 PDA detector. An Empower software was used to control the system and data acquisition. An Inertsil ODS 3V column (150x4.6 mm, 5µ) was used. The mobile phase was composed of Tetrabutylammonium hydrogen sulphate and acetonitrile for gradient elution within 25 minutes. The column flow rate was 1 ml/min and the column temperature was maintained at 40ºC. The injection volume was 10 µl and the detection wavelength was 258 nm.

Solution Preparation

Drug stock solution and standard

Itraconazole stock solution was prepared by weighing about 50 mg of the drug in 100 ml of diluent (4.0 ml of hydrochloric acid with methanol to 1ltr). Itraconazole stock solution was prepared for a of concentration of 500µg/ml. From this stock solution standard 100µg/ml was freshly prepared during the analysis day.

Sample preparation

Samples weight was taken of about 200 mg of Itraconazole ointment (5%w/w which is equivalent 10 mg of itraconazole) into a 100 mL volumetric flask, added 70 milli litres of diluent (4mL of hydrochloric acid in one litre of methanol) and sonicated for 10 minutes with occasional shaking. Cool and made up the volume to 100mL with diluent and mixed well. Filter a portion of the sample through 0.45µ Nylon filter.

METHOD VALIDATION

System suitability

The system suitability of a method was determined injecting five injections of the drug standard

solution at a concentration of 100µg/ml. The %RSD of five standard injections must not be more than 2.0%. The USP tailing for Itraconazole peak in standard solution must not be more than 2.0.

Detection and quantitation limits

Lower limit of detection (LOD) and lower limit of quantitation (LOQ) are approximated from the signal-to-noise ratio. Determination of LOD is done by the lowest concentration level resulting in a peak area of three times the baseline noise. The determination of quantitation limit is defined as the lowest concentration level that provided a peak area with a signal-to-noise ratio higher than 10(8).

Linearity

The calibration curve was created with seven concentrations along with the lower limit of quantification (LLOQ) ranging from 10 µg/ml to 250µg/ml. Linearity ascribed of a test procedure is a way to determine how a graph of area vs. concentration provides a straight line. The documentation is processed by using a linear least squares regression. The slope, intercept and correlation coefficient provide knowledge on linearity(8).

Accuracy and Precision

The accuracy of analysis is interpreted as the "closeness of the measured value to the true value". Precision of the assay was determined by repeatability (intra-day). Repeatability refers to the use of the analytical procedure within a laboratory over a short period of time that was evaluated by assaying the QC samples during the same day(8).

Specificity

The method specificity was determined by injecting the standard solution (100 µg/ml) and placebo of the sample to a mixture of known impurities as per USP and checking the interference between the itraconazole peak and its impurities(8).

Force Degradation

Forced degradation of the sample helps to determine the degraded products and the stability of the drug molecule in the sample(8). For degradation studies, about 200 mg of itraconazole ointment was weighed and transferred to a 100 ml volumetric flask added 30 milliliters of diluent and sonicated for 10 minutes with occasional shaking (1st step). To this, 5 ml of 5N sodium hydroxide (for alkaline degradation) or 5N hydrochloric acid (for acid degradation) was added and subjected to 60°C for 1 hr in a water bath (for acid degradation) and subjected to 60°C for 3 hrs in a water bath (for alkaline degradation) . The solution was cooled, dissolved and made to volume with diluent. For oxidative degradation, 5ml of hydrogen peroxide (3%) solution was added in 1st step and allow it to stand for 15minutes at bench top. Made up the volume with diluent and mixed

well(8).

Robustness

Robustness in method validation is a parameter that determine the method's capacity to remain unaffected by small but voluntary variation in method parameters(8).

Change in Flow Rate:

A study was conducted to determine the effect of variation in flow rate of mobile phase. Standard was prepared as per test method and injected into the HPLC system with a flow rate of 0.8 mL/min and 1.2 mL/min (Actual flow rate is 1.0 mL/min). The system suitability parameters were evaluated as per test method with both the flow rates. The results were within the acceptance limits.

Effect of Variation in Column Oven Temperature:

Determination of effect of variation in column oven temperature is done by preparation of standard solution as per the test method and injected into the HPLC system at 35°C and at 45°C column oven temperatures (Actual temperature is 40°C). The system suitability parameters were evaluated as per the test method with both the column oven temperatures. The results were within the acceptance limits.

Solution Stability

The solution stability of a drug solution was done using the sample solution and the standard for short-term stability by keeping at room temperature for 12 h and then analysing. For long-term stability study solution is analyzed by storing for 7 days(8).

RESULTS AND DISCUSSION

Method Development and Optimization

Itraconazole is a non-polar drug, insoluble in aqueous solutions and is freely soluble in organic solvents like methanol and acetonitrile. Overall seven trials have been carried out for optimization of the method. In a first trial, use of tetrabutylammonium hydrogen sulphate in water and acetonitrile as mobile phase in a gradient flow system for a runtime of 20mins resulted in no peak elution for a concentration of 50µg/ml. By making few changes in chromatographic conditions such as increase in runtime and gradient flow, peak got eluted at 24mins in second trial. Further in third trial, two standard injections of different concentrations of 100µg/ml and 250µg/ml concentrations was injected for optimizing the response. 100µg/ml concentration was selected as the peak area was observed to be optimum, but the retention time was too high as shown in the Figure: 2.

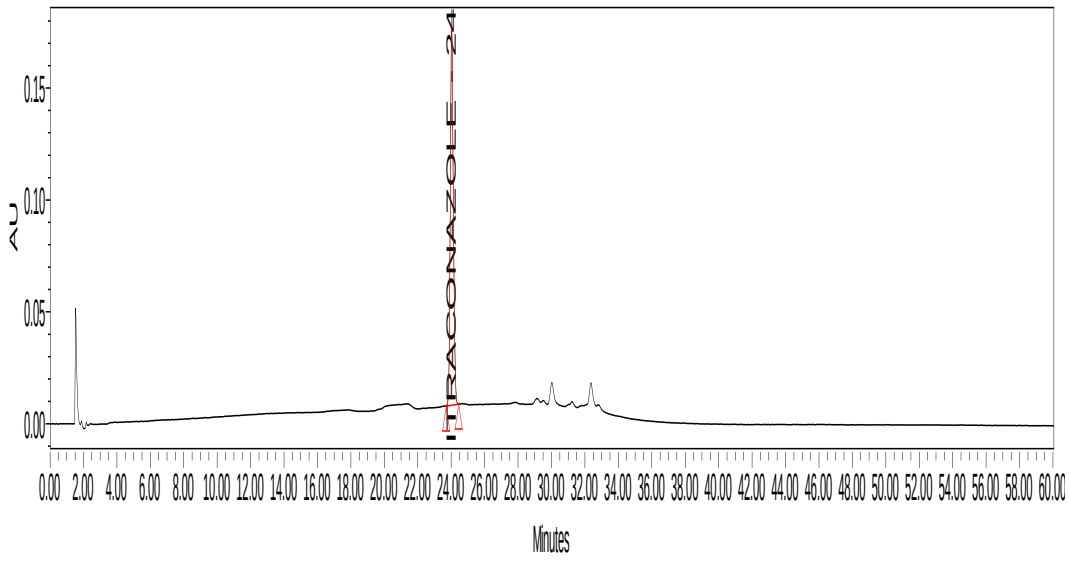


Figure: 2 Retention time

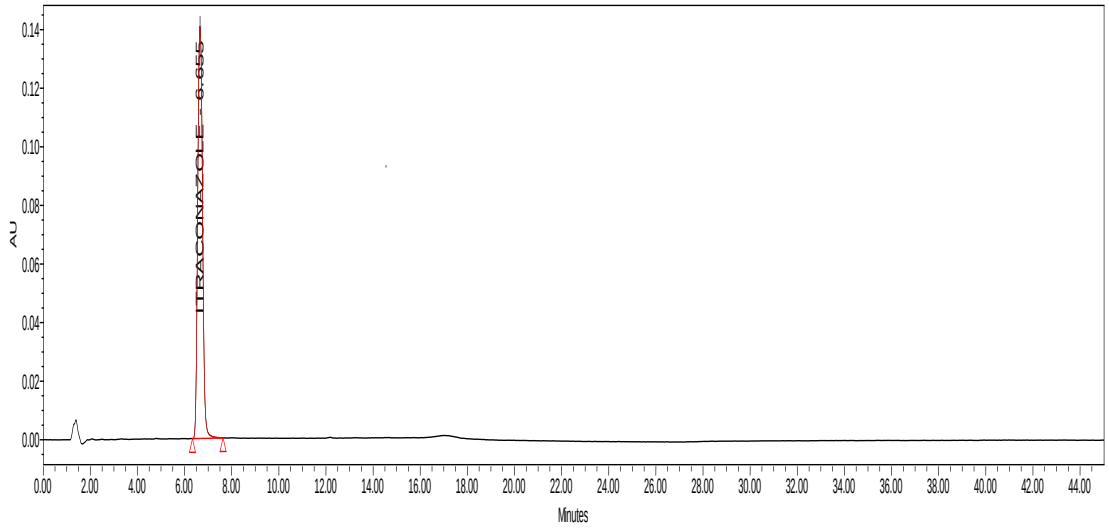


Figure 3: In this trial, a retention time of 6.7 minutes was achieved

In fourth, fifth and sixth trials, did not achieve desirable retention time. But in trial 7, further modification of chromatographic conditions such as runtime and gradient flow where the percentage of aqueous phase has been increased. In this trial, a retention time of 6.7 minutes was achieved as shown in the Figure 3.

Method validation

System Suitability

The %RSD of peak area and retention time for Standard was observed within 2%, indicating the suitability of the system as shown in Table 1. The efficiency of the column as expressed by number of theoretical plates for the five replicate injections was 8624 and the USP tailing for Itraconazole peak in standard solution was 0.8 which should not be more than 2.0.Table 1

Table 1: System Suitability

	Standard (100µg/ml) Retention time (min)	Peak area
Mean (n=5)	6.6	2084160
%RSD		0.36

Detection and quantitation limits (sensitivity)

Table 2 illustrates LLOQ of the drug, standard solution (10µg/ml). The method was found to be sensitive was determined from the six replicate injections of the LLOQ where the %RSD was 0.48.

Table 2: Quantitation limit

Sl. No.	No. of injection	Area	s/n
1	Injection no. 1	213354	86.5
2	Injection no. 2	212374	85.1
3	Injection no. 3	212535	96.9
4	Injection no. 4	212442	100.1
5	Injection no. 5	210339	112.9
6	Injection no. 6	212741	114.9
	Mean	212297.5	
	%RSD	0.48	

Table 2 illustrates LLOD of the drug, standard solution (5µg/ml). The method was found to be sensitive was determined from the three replicate injections of the LLOD where the %RSD was 0.563.

Table 3: Detection limit

Sl. No.	No. of injection	Area	s/n
1	Injection no. 1	107505	50.50
2	Injection no. 2	107703	45.91
3	Injection no. 3	108644	46.98
	Mean	107950.7	
	%RSD	0.563	

Linearity:

The documentation of the calibration curve is processed by using a linear least squares regression. The slope, intercept and correlation coefficient provide knowledge on linearity**.** The peak area ratio of the drug was linear in the range 10µg/ml to 250µg/ml. The correlation coefficient (r^2) of all the calibration curves must be equal to or greater than 0.999 (table 3).

Table 4: Linearity curve data

Linearity Level	Volume of Stock taken	Dilution	Conc.(ppm)	Area
Level-1(10%)	2.0	100	10	201149
Level-2 (25%)	5.0	100	25	514976
Level-3(50%)	5.0	50	50	1024647

Level-4 (100%)	5.0	25	100	2050422
Level-5 (150%)	3.0	10	150	3096626
Level-6 (200%)	4	10	200	4192946
Level-7 (250%)	5	10	250	5236552
Correlation (r^2)				0.99990
Intercept				-22750
Slope				20659.00000
Bias at 100%				-1.110

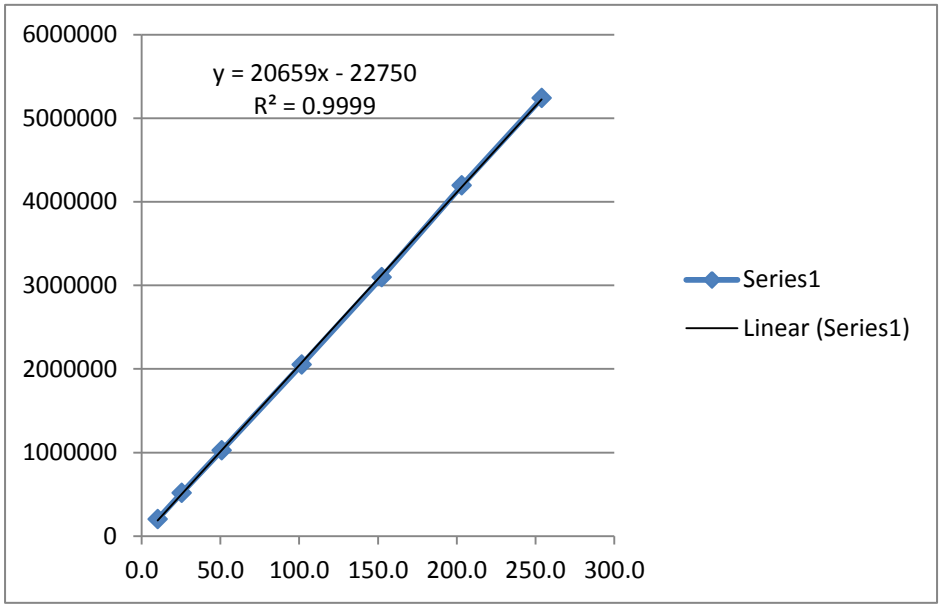


Figure 4: Linearity curve data

Accuracy and Precision:

Accuracy and precision calculated for QC samples during intraday are given in table 4 and table 5 respectively.

Table 4: Accuracy

Accuracy level	Preparation	Sample weight (mg)	Area	Individual % recovery	Mean % recovery (n=3)	% RSD
50%	Preparation 1	113.1	1121436	96.0		
	Preparation 2	110.1	1101480	96.9	96.7	0.688
	Preparation 3	103.6	1040605	97.3		
100%	Preparation 1	216.3	2172802	97.3		
	Preparation 2	203.1	2061708	98.3	97.6	0.591
	Preparation 3	199.8	2007410	97.3		
150%	Preparation 1	306.7	3070782	97.0		
	Preparation 2	317.2	3170154	96.8	96.8	0.259
	Preparation 3	314.3	3130581	96.5		
200%	Preparation 1	405.7	3948120	94.2		
	Preparation 2	410.8	4091875	96.5	95.9	1.51
	Preparation 3	417.7	4186806	97.1		

Table 5: Precision

Sample Name	Wt (mg)	Area	% Assay
Preparation 1	212.61	2126255	97.4
Preparation 2	241.77	2407626	97.1
Preparation 3	226.24	2249789	96.9
Preparation 4	215.77	2146925	97.0
Preparation 5	219.82	2142954	95.0
Preparation 6	220.53	2194362	97.0
		Mean	96.7
		SD	0.852
		%RSD	0.88

The result obtained indicate that the method is accurate and precise.

Specificity:

For the study of specificity few parameters were conducted such as placebo interference, known impurity interference with placebo and known impurity interference with standard. It was also observed that the peak purity angle is always less than purity threshold for itraconazole peak. The specificity of an analytical method indicated where the placebo and impurities must not coelute with the retention time of the drug and the standard.

Table 6: Observation of Specificity:

Name	Retention Time	Purity Angle	Purity Threshold	Area	% Area	USP Resolution	USP Tailing
Impurity-A	2.907	NA	NA	633790	15.33	NA	1.0
Impurity- C	4.590	NA	NA	22371	0.54	8.070	0.9
Impurity-D	5.406	NA	NA	856487	20.72	3.572	0.9
Impurity-E	5.994	NA	NA	408495	9.88	2.363	0.9
Itraconazole	6.612	0.124	0.270	1613239	39.02	2.419	0.8
Impurity-F	6.940	NA	NA	299144	7.24	1.345	1.1
Impurity-G	7.967	NA	NA	300972	7.28	3.866	0.9

Table 7: Observation of interference

Sample	Preparation No.	Interference found at Itraconazole (yes/no)
Placebo preparation	1	No
Placebo with impurities	2	No
Standard with impurities	3	No

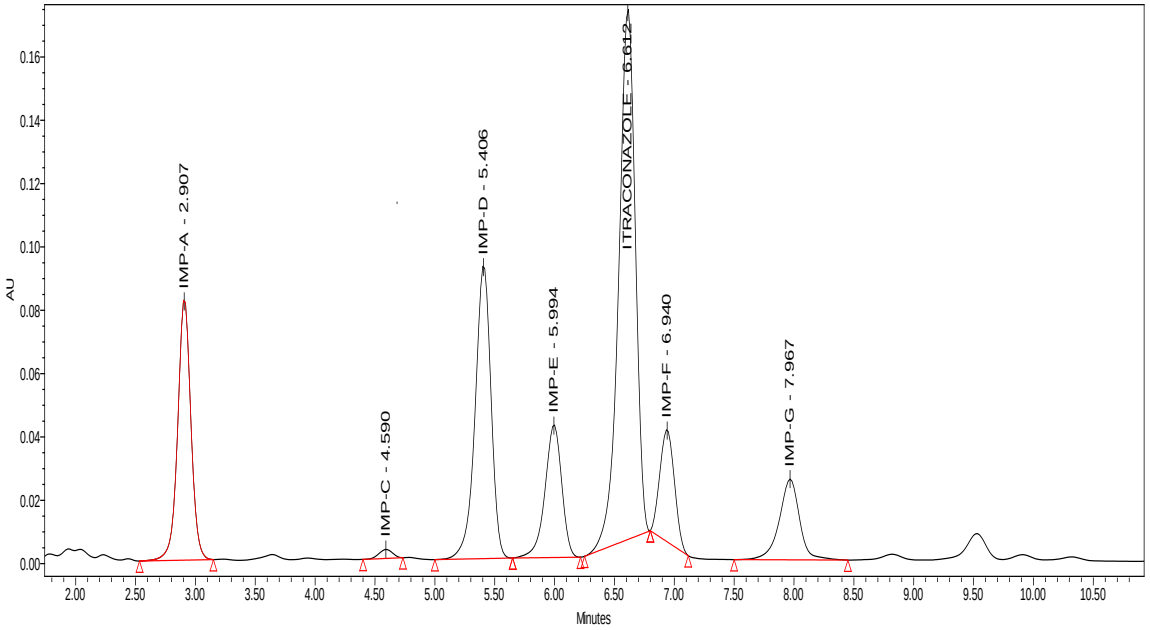


Figure 5: Observation of interference

Force Degradation:

The stress testing involving oxidation, acidic and alkaline degradation states that itraconazole was not completed degraded. However, in oxidation condition, 3% hydrogen peroxide degrades itraconazole drug by 11.39% at bench top condition. In acidic condition, 5N HCl was used to degrade itraconazole by 20.39% at 60°C for 1hr. Further, in alkaline condition, 5N NaOH was used to degrade itraconazole drug by 4.39% at 60°C for 3hrs. In each case, purity angle was found to be less than purity threshold.

Table 8 Drug product degradation result

Conditions	% Assay	% of net degradation	Purity Angle	Purity Threshold
Standard	NA	NA	0.084	0.283
Sample control	97.24	NA	0.069	0.271
Peroxide degradation	85.85	11.39	0.091	0.276
Acid degradation	77.08	20.16	0.171	0.245
Base degradation	92.85	4.39	0.074	0.266

Range:

The method is linear over a range of 10µg/ml -250 µg/ml.

Table 9: Range

Parameter		Acceptance criteria	Itraconazole	
1	Linearity (correlation coefficient)	Should not be less than 0.999.	0.999	
2	Intercept	To report the result	-22750	
3	Slope	To report the result	20659.00000	
4	Y-intercept value	Should not be more than ±2% of the response at 100% level	-1.110	
5	Precision	% RSD should not be more than 2.0%	**Strength**	**%RSD**

			5%	0.88
6	Accuracy	Should be between 80 and 120%.	**Level**	**Mean %Recovery**
			50%	96.72
			100%	97.62
			150%	96.73
			200%	95.92

Robustness:

Table 10: Variation in Flow Rate

System suitability parameters	Observed value			Acceptance criteria
	1.0 mL/min (STP)	1.2 mL/min	0.8 mL/min	
USP Tailing for Itraconazole peak in sample chromatogram	0.8	0.8	0.9	Not more than 2.0
USP Plate count for Itraconazole peak in sample chromatogram	10472	8739	12493	Not less than 3000
Relative standard deviation for Itraconazole peak in five replicate injections of standard	0.92	0.39	0.89	Not more than 2.0%
% Assay of sample	96.02	96.70	97.205	

Table 11: Variation in column oven temperature

System suitability parameters	Observed value			Acceptance criteria
	40°C(STP)	35°C	45°C	
USP Tailing- for Itraconazole peak in standard chromatogram	0.8	0.9	0.8	Not more than 2.0
USP Plate count for Itraconazole peak in standard chromatogram	10477	9396	11323	Not less than 3000
Relative standard deviation for Itraconazole peak in five replicate injections of standard	0.9	0.9	0.8	Not more than 1.0%
% Assay of the sample	97.2	96.4	97.4	

The method is robust by few deliberate changes such as variation in flow rate and variation in column temperature.

Solution Stability

The standard is considered to be stable as the similarity factor is in the range of 0.98 to 1.02. The test solution is considered to be stable as the difference in % assay results from initial to -next time intervals not more than ±2.0.

Table 12: Stability of standard solution

Solution type	Bench top stability	
Standard solution	**Interval**	**Similarity factor**
	Initial	NA
	Day 7	0.98

Table 13: Stability of test solution on bench top

Solution type	Bench top stability		
Test preparation (5%)	Interval	Observed value	%Difference with initial
	Initial	97.28	1.94
	Day 7	99.17	

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