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STABILITY INDICATING METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF TERBINAFINE AND ITRACONAZOLE IN API AND TABLET DOSAGE FORM BY RP-HPLC

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ABSTRACT

A simple, Accurate, precise method was developed for the simultaneous estimation of the Terbinafine and Itraconazole in tablet dosage form. Chromatogram was run through Phenomenex C18 4.6 x 250mm, 5µm. Mobile phase containing Buffer 0.01N Kh2po4: acetonitrile taken in the ratio 65:35v/v was pumped through column at a flow rate of 1.0 ml/min. Temperature was maintained at 30°C. Optimized wavelength selected was 270 nm. Retention time of Terbinafine and Itraconazolewere found to be 2.221 min and 2.819min. %RSD of the Terbinafine and Itraconazole were and found to be 0.8 and 0.9 respectively. %Recovery was obtained as 100.36% and 100.70% for Terbinafine and Itraconazole respectively. LOD, LOQ values obtained from regression equations of Terbinafine and Itraconazole were 0.78,2.37 and 0.11, 0.34 respectively. Regression equation of Terbinafineis y =21293x + 9314.3, and y = 21074x + 5342.7 of Itraconazole Retention times were decreased and that run time was decreased, so the method developed was simple and economical that can be adopted in regular Quality control test in Industries.

KEYWORDS: Terbinafine, Itraconazole, RP-HPLC.

INTRODUCTION

Terbinafine hydrochloride (Lamisil) is a synthetic allylamine antifungal. It is highly lipophilic in nature and tends to accumulate in skin, nails, and fatty tissues. Like other allylamines, terbinafine inhibits ergosterol synthesis by inhibiting the fungal squalene monooxygenase (squalene 2,3-epoxidase), an enzyme that is part of the fungal cell wall synthesis pathway**.**it is chemically called as [(2E)-6,6-dimethylhept-2-en-4-yn-1-yl](methyl)[(naphthalen-1-yl)methyl]amine.

hypothesized to act by inhibiting squalene monooxygenase, thus blocking the biosynthesis of ergosterol, an essential component of fungal cell membranes. This inhibition also results in an accumulation of squalene, which is a substrate catalyzed to 2,3-oxydo squalene by squalene monooxygenase. The resultant high concentration of squalene and decreased amount of ergosterol are both thought to contribute to terbinafine's antifungal activity.
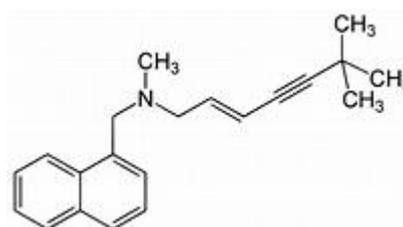
Fig 1: Structure of Terbinafine.

Itraconazole
One of the triazole antifungal agents that inhibits cytochrome P-450-dependent enzymes resulting in impairment of ergosterol synthesis. It has been used against histoplasmosis, blastomycosis, cryptococcal meningitis & aspergillosis**.** It chemically called as 1-(butan-2-yl)-4-{4-[4-(4-{[(2R,4S)-2-(2,4 dichlorophenyl) - 2 - (1H-1,2,4-triazol-1-ylmethyl)-1,3-dioxolan-4-yl] methoxy}phenyl)piperazin-1-yl]phenyl}-4,5 - dihydro-1H-1,2,4-triazol-5-one. it interacts with 14-α demethylase, a cytochrome P-450 enzyme necessary to convert lanosterol to ergosterol. As ergosterol is an essential component of the fungal cell membrane, inhibition of its synthesis results in increased cellular permeability causing leakage of cellular contents. Itraconazole may also inhibit endogenous respiration,

interact with membrane phospholipids, inhibit the transformation of yeasts to mycelial forms, inhibit purine uptake, and impair triglyceride and/or phospholipid biosynthesis.
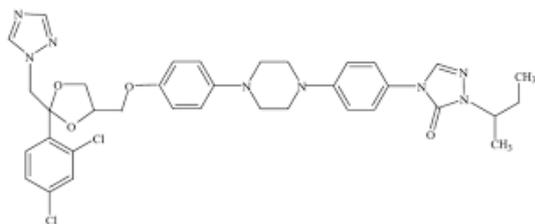

Figure2: chemical structure of Itraconazole.

Experimental work
MATERIALS AND METHODS

Materials
Terbinafine and Itraconazole pure drugs (API), Combination Terbinafine and Itraconazole tablets (IGFORCE FT), Distilled water, Acetonitrile, Phosphate buffer, Methanol, Potassium dehydrogenate ortho phosphate buffer, Ortho-phosphoric acid. All the above chemicals and solvents are from Rankem.

Instruments
Electronics Balance-Denver, p^H meter -BVK enterprises, India, Ultrasonicator-BVK enterprises, Waters HPLC 2695 system equipped with TUV detector with Empower 2 Software., UV-VIS spectrophotometer PG Instruments T60 with special bandwidth of 2 mm and 10mm and matched quartz cells integrated with UV win 6 Software was used for measuring absorbances of Terbinafine and Itraconazole solutions.

Methods
Diluent: Based up on the solubility of the drugs, diluent was selected, Acetonitrile and HPLC grade water taken in the ratio of 50:50.

Preparation of Standard stock solutions: Accurately weighed 62.5 mg of Terbinafine, 25 mg of Itraconazole and transferred to 50 ml volumetric flasks separately. 3/4 th of diluents was added to the flask and sonicated for 10 minutes. Flask were made up with diluents and labeled as Standard stock solution. (1250μg/ml of Terbinafine and 500μg/ml of Itraconazole).

Preparation of Standard working solutions (100% solution): From above solution 1ml stock solution was pipetted out and taken into a 10ml volumetric flask and made up with diluent. (125μg/ml Terbinafine of and 50μg/ml of Itraconazole).

Preparation of Sample stock solutions: 10 tablets were weighed and the average weight of each tablet was calculated, then the weight equivalent to 1 tablet was transferred into a 100 ml volumetric flask, 25ml of diluents was added and sonicated for 25 min, further the volume was made up with diluent and filtered by HPLC filters (2500μg/ml of Terbinafine and 1000μg/ml of Itraconazole).

Preparation of Sample working solutions (100% solution): 0.5ml of filtered sample stock solution was transferred to 10ml volumetric flask and made up with diluent. (125μg/ml of Terbinafine and 50μg/ml of Itraconazole).

Preparation of buffers
0.1% OPA Buffer: 1ml of Conc Ortho Phosphoric acid was diluted to 1000ml with water.

0.01N KH_2PO_4 Buffer (potassium di hydrogen phosphate): Accurately weighed 1.36gm of Potassium dihyrogen Ortho phosphate in a 1000ml of Volumetric flask add about 900ml of milli-Q water added and degas to sonicate and finally make up the volume with water then PH adjusted to 4.0 with dil. Orthophosphoric acid solution.

Method Validation
System Suitability: The system suitability parameters were determined by preparing standard solutions of Terbinafine 125 μg/ml and Itraconazole 50 μg/ml. The solutions were injected six times and the parameters like peak tailing, resolution and USP plate count were determined. The % RSD for the area of six standard injections results should not be more than 2%.

Specificity: Specificity of a method was determined by testing standard substances against potential interferences. There should not find interfering peaks in the blank and placebo at retention times of these drugs in this method. So this method was said to be specific.

Linearity
Preparation of Standard stock solutions: Accurately weighed 62.5 mg of Terbinafine, 25 mg of Itraconazole and transferred to 50 ml volumetric flasks separately. 3/4 th of diluents was added to the flask and sonicated for 10 minutes. Flask were made up with diluents and labeled as Standard stock solution. (1250μg/ml of Terbinafine and 500μg/ml of Itraconazole).

25% Standard solution: 0.25ml each from two standard stock solutions was pipetted out and made up to 10ml.(31.25μg/ml of Terbinafine and 12.5 μg/ml of Itraconazole).

50% Standard solution: 0.5ml each from two standard stock solutions was pipetted out and made up to 10ml. (62.5μg/ml of Terbinafine and 25μg/ml of Itraconazole).

75% Standard solution: 0.75ml each from two standard stock solutions was pipetted out and made up to 10ml. (93.75μg/ml of Terbinafine and 37.5μg/ml of Itraconazole).

100% Standard solution: 1.0ml each from two standard stock solutions was pipetted out and made up to 10ml. (125μg/ml of Terbinafine and 50μg/ml of Itraconazole).

125% Standard solution: 1.25ml each from two standard stock solutions was pipetted out and made up to 10ml. (156.25μg/ml of Terbinafine and 625μg/ml of Itraconazole).

150% Standard solution: 1.5ml each from two standard stock solutions was pipettede out and made up to 10ml (187.5μg/ml of Terbinafine and 75μg/ml of Itraconazole).

Accuracy
Preparation of Standard stock solutions: Accurately weighed 62.5 mg of Terbinafine, 25 mg of Itraconazole and transferred to 50 ml volumetric flasks separately. 3/4 th of diluents was added to the flask and sonicated for 10 minutes. Flask were made up with diluents and labeled as Standard stock solution. (1250μg/ml of Terbinafine and 500μg/ml of Itraconazole).

Preparation of 50% Spiked Solution: 0.25ml of sample stock solution was taken into a 10ml volumetric flask, to that 1.0ml from each standard stock solution was pipetted out, and made up to the mark with diluent.

Preparation of 100% Spiked Solution: 0.5ml of sample stock solution was taken into a 10ml volumetric flask, to that 1.0ml from each standard stock solution was pipetted out, and made up to the mark with diluent.

Preparation of 150% Spiked Solution: 0.75ml of sample stock solution was taken into a 10ml volumetric flask, to that 1.0ml from each standard stock solution was pipetted out, and made up to the mark with diluent.

Precision
Preparation of Standard stock solutions: Accurately weighed 62.5 mg of Terbinafine, 25 mg of Itraconazole and transferred to 50 ml volumetric flasks separately. 3/4 th of diluents was added to the flask and sonicated for 10 minutes. Flask were made up with diluents and labeled as Standard stock solution. (1250μg/ml of Terbinafine and 500μg/ml of Itraconazole).

Preparation of Standard working solutions (100% solution): From above solution 1ml stock solution was pipetted out and taken into a 10ml volumetric flask and made up with diluent. (125μg/ml Terbinafine of and 50μg/ml of Itraconazole).

Preparation of Sample stock solutions: 10 tablets were weighed and the average weight of each tablet was calculated, then the weight equivalent to 1 tablet was transferred into a 100 ml volumetric flask, 25ml of diluents was added and sonicated for 25 min, further the volume was made up with diluent and filtered by HPLC

filters (2500μg/ml of Terbinafine and 1000μg/ml of Itraconazole).

Preparation of Sample working solutions (100% solution): 0.5ml of filtered sample stock solution was transferred to 10ml volumetric flask and made up with diluent. (125μg/ml of Terbinafine and 50μg/ml of Itraconazole).

The repeatability of the method was verified by calculating the % RSD of six replicate injections of 100% concentration (125μg/ml of Terbinafine and 50μg/ml of Itraconazole) on the same day and for intermediate precision % RSD was calculated from repeated studies on different days.

Limit of Detection (LOD) and Limit of Quantitation (LOQ): The LOD and LOQ were calculated from the slope(s) of the calibration plot and the standard deviation (SD) of the peak areas using the formulae LOD = 3.3 σ/s and LOQ = 10 σ/s.

Robustness: Robustness of the method were verified by altering the chromatographic conditions like flow rate, mobile phase ratio and temperature are made, but there were no recognized change in the result and all are within range as per ICH guidelines. Robustness conditions like flow minus (0.9 ml/min), flow plus (1.1 ml/min), 60:40mobile phase minus 50:50 mobile phase plus, temperature minus (25°C) and temperature plus (35°C) were maintained and samples were injected in duplicate manner. System suitability parameter was passed. % RSD was within the limit.

Degradation Studies
Acid degradation: To 1 ml of stock solution Terbinafine and Itraconazole, 1ml of 2N Hydrochloric acid was added and refluxed for 30 mins at 60 °C. The resultant solution was diluted to obtain 125μg/ml and 50μg/ml solutions and 0.5μl solutions were injected into the system and the chromatograms were recorded to assess the stability of the sample.

Oxidative Degradation: To 1 ml of stock solution of Terbinafine and Itraconazole, 1 ml of 20% hydrogen peroxide (H_2O_2) was added separately. The solutions were kept for 30 min at 60°C. For HPLC study, the resultant solution was diluted to obtain 125μg/ml and 50μg/ml solution and 0.5μl were injected into the system and the chromatograms were recorded to assess the stability of the sample.

Alkali Degradation: To 1 ml of stock solution Terbinafine and Itraconazole, 1 ml of 2N sodium hydroxide was added and refluxed for 30 mins at 60°C. The resultant solution was diluted to obtain 125μg/ml and 50μg/ml solution and 0.5μl were injected into the system and the chromatograms were recorded to assess the stability of the sample.

Thermal Degradation: The standard drug solution was placed in oven at 105°C for 6Hrs to study dry heat degradation. For HPLC study, the resultant solution was diluted to 125µg/ml and 50µg/ml solution and 0.5µl were injected into the system and the chromatograms were recorded to assess the stability of the sample.

Photo Degradation: The photochemical stability of the drug was also studied by exposing the 1250µg/ml and 500µg/ml solution to UV light by keeping the beaker in UV chamber for 7 days or 200 Watt hrs/m2 in photo stability chamber. For HPLC study, the resultant solution was diluted to obtain 125µg/ml and 50µg/ml solutions and 0.5µl were injected into the system and the chromatograms were recorded to assess the stability of the sample.

RESULTS AND DISCUSSION
Optimized conditions
Chromatographic conditions
Mobile phase   **:**   **65**%   0.01N   Kh2po4:   35% Acetonitrile
Flow rate   **:**   1 ml/min
Column   **:** Phenomenex C18 (4.6 x 250mm, 5µm)
Detector wave length   **:**   270nm
Column temperature   **:**   30°C
Injection volume   **:**   10µL
Run time   **:**   **10** min
Diluent   **:**   Water and Acetonitrile in the ratio 50:50
Results   **:**   In this Trail by changing the mobile phase both peaks were eluted with good peak shape and as per ICH guidelines all system suitability parameters was within the limit. So this method was optimized.
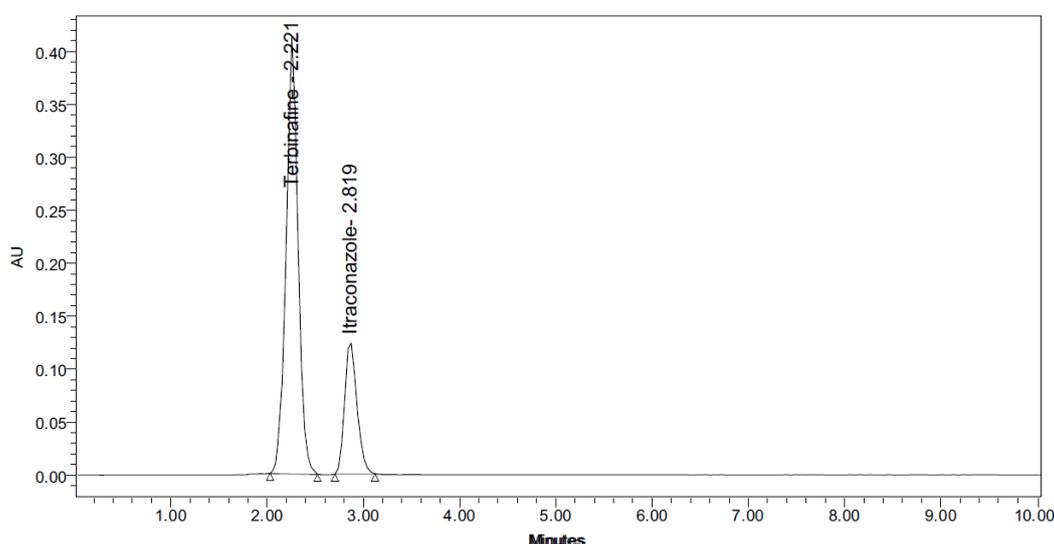

Fig 6.7 Optimised chromatogram 4.

Observation: Terbinafine and Itraconazole were eluted at 2.221 min and 2.819 min respectively with good resolution. Plate count and tailing factor was very satisfactory, so this method was optimized and to be validated.

System suitability: All the system suitability parameters were within the range and satisfactory as per ICH guidelines.

Table 6.1: System suitability parameters for Terbinafine and Itraconazole.

S no	Terbinafine			Itraconazole			
Inj	RT(min)	USP Plate Count	Tailing	RT(min)	USP Plate Count	Tailing	Resolution
1	2.218	7732	1.28	2.879	9400	1.2	6.0
2	2.222	7779	1.28	2.883	9980	1.19	5.8
3	2.224	7158	1.24	2.884	10319	1.19	5.8
4	2.224	7015	1.28	2.886	11105	1.18	5.9
5	2.234	7728	1.21	2.924	9808	1.21	6.3
6	2.289	7216	1.3	2.998	9587	1.21	6.0

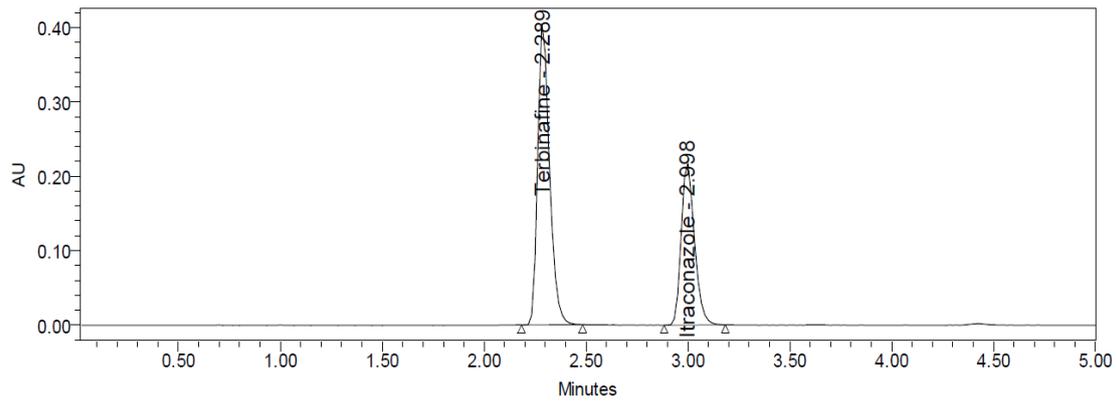**Fig 6.8 System suitability Chromatogram.**

Discussion: According to ICH guidelines plate count should be more than 2000, tailing factor should be less than 2 and resolution must be more than 2. All the system suitable parameters were passed and were within the limits.
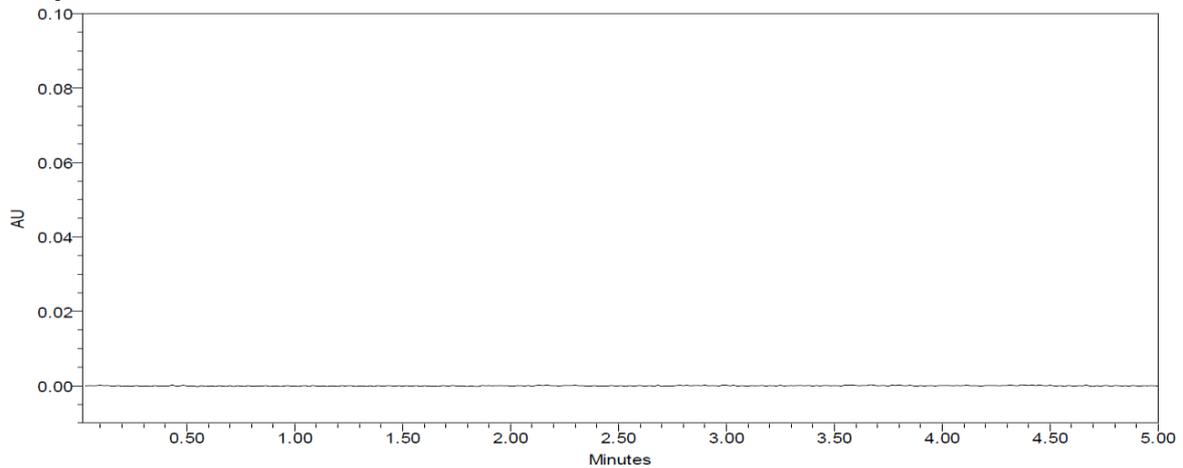
Validation
Specificity
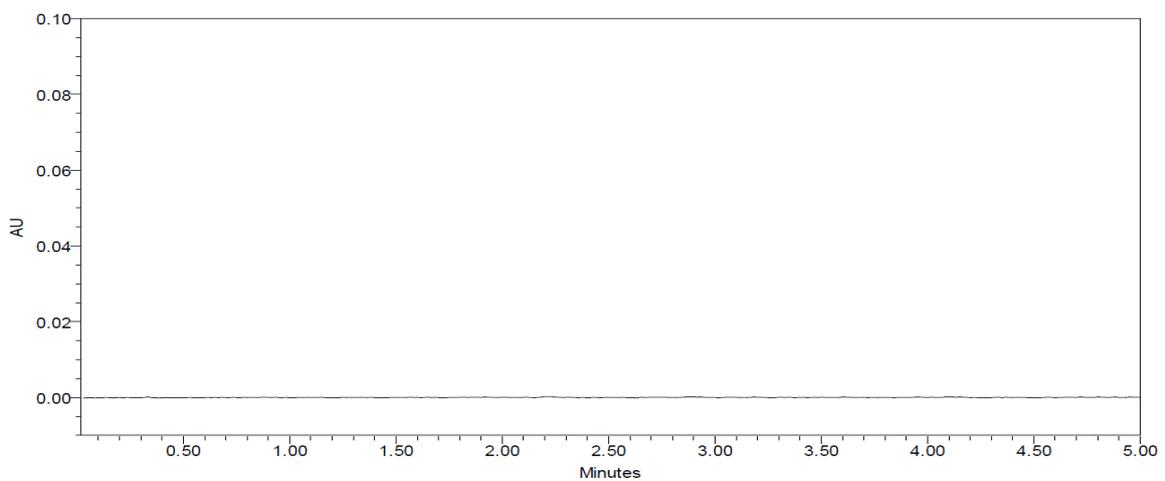

Figure No. 6.9. Chromatogram of blank.
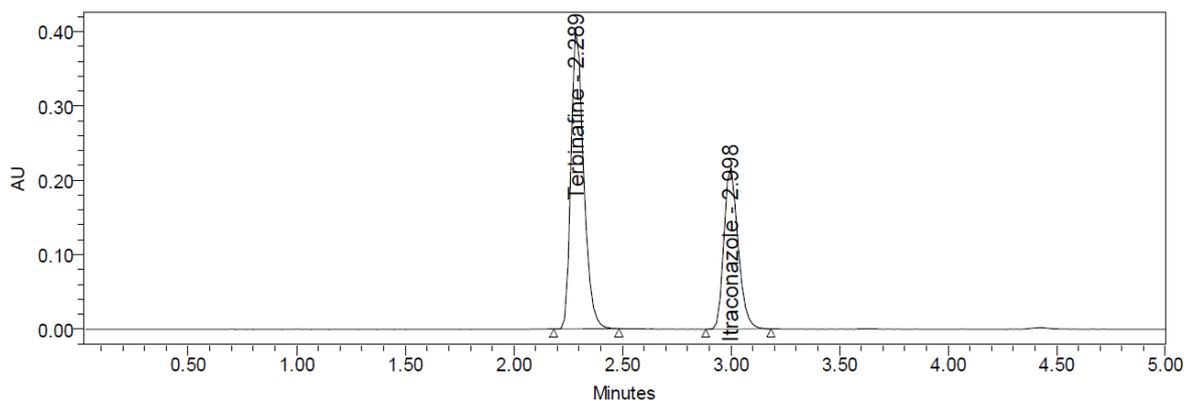

Figure No. 6.10 Chromatogram of placebo.

Figure No. 6.11 Optimized chromatogram.

Discussion: Retention times of Terbinafine and Itraconazole were 2.289 min and 2.998 min respectively. We did not found and interfering peaks in blank and placebo at retention times of these drugs in this method. So this method was said to be specific.

Linearity
Table 6.2: Linearity table for Terbinafine and Itraconazole.

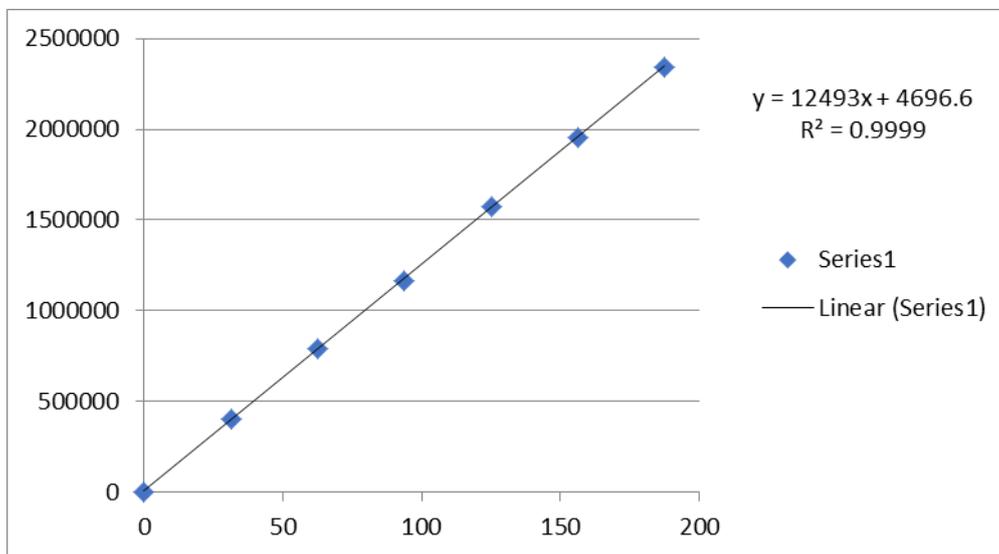| Terbinafine		Itraconazole	
Conc (µg/mL)	Peak area	Conc (µg/mL)	Peak area
0	0	0	0
31.25	402410	12.5	241770
62.5	786734	25	474925
93.75	1165461	37.5	706333
125	1575220	50	956078
156.25	1957570	62.5	1192093
187.5	2344330	75	1411241

$$y = 12493x + 4696.6$$
$$R^2 = 0.9999$$

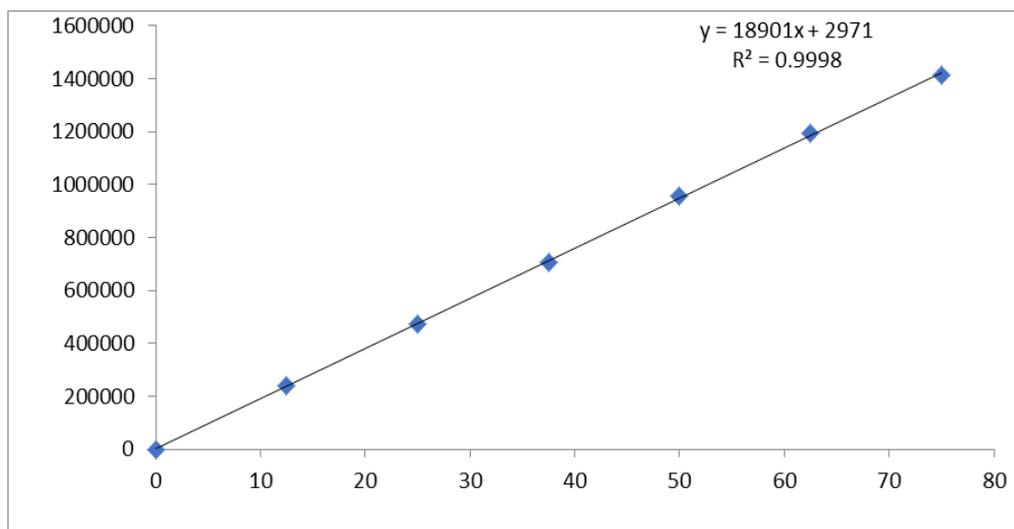**Fig No. 6.12 Calibration curve of Terbinafine.**

Fig No. 6.13 Calibration curve of Itraconazole.

Discussion: Six linear concentrations of Terbinafine (31.25-187.5μg/ml) and Itraconazole (12.5-75μg/ml) were injected in a duplicate manner. Average areas were mentioned above and linearity equations obtained for Terbinafine was $y = 12493x + 4696$ and of Itraconazole was $y = 18901x + 2971$. Correlation coefficient obtained was 0.999 for the two drugs.

Precision
System Precision
Table 6.3 System precision table of Terbinafine and Itraconazole.

S. No	Area of Terbinafine	Area of Itraconazole
1.	1615103	977378
2.	1624387	985152
3.	1600006	970599
4.	1601721	971171
5.	1600097	963379
6.	1631506	983380
Mean	1611543	975177
S.D	13672.1	8340.7
%RSD	0.8	0.9

Discussion: From a single volumetric flask of working standard solution six injections were given and the obtained areas were mentioned above. Average area, standard deviation and % RSD were calculated for two drugs. % RSD obtained as 0.8% and 0.9% respectively for Terbinafine and Itraconazole .As the limit of Precision was less than "2" the system precision was passed in this method.

Repeatability
Table 6.4: Repeatability table of Terbinafine and Itraconazole.

S. No	Area of Terbinafine	Area of Itraconazole
1.	1629420	992058
2.	1626262	984877
3.	1636635	986859
4.	1635464	990697
5.	1630581	983667
6.	1609817	976901
Mean	1628030	985843
S.D	9722.0	5456.1
%RSD	0.6	0.6

Discussion: Multiple sampling from a sample stock solution was done and six working sample solutions of same concentrations were prepared, each injection from each working sample solution was given and obtained areas were mentioned in the above table. Average area, standard deviation and % RSD were calculated for two drugs and obtained as 0.6% and 0.6% respectively for Terbinafine and Itraconazole. As the limit of Precision was less than "2" the system precision was passed in this method.

Intermediate precision (Day_ Day Precision)
Table 6.5: Intermediate precision table of Terbinafine and Itraconazole.

S. No	Area of Terbinafine	Area of Itraconazole
1.	1615166	983465
2.	1560931	969094
3.	1585653	947490
4.	1555232	947142
5.	1606725	962106
6.	1562963	944093
Mean	1581112	958898
S.D	25466.7	15526.2
%RSD	1.6	1.6

Discussion: Multiple sampling from a sample stock solution was done and six working sample solutions of same concentrations were prepared, each injection from each working sample solution was given on the next day of the sample preparation and obtained areas were mentioned in the above table. Average area, standard deviation and % RSD were calculated for two drugs and obtained as 1.6% and 1.6% respectively for Terbinafine and Itraconazole. As the limit of Precision was less than "2" the system precision was passed in this method.

Accuracy
Table 6.6: Accuracy table of Terbinafine.

% Level	Amount Spiked (µg/mL)	Amount recovered (µg/mL)	% Recovery	Mean %Recovery
50%	62.5	63.75	102.00	
	62.5	61.81	98.90	
	62.5	62.91	100.65	
100%	125	126.21	100.97	
	125	124.50	99.60	100.36%
	125	125.60	100.48	
150%	187.5	186.54	99.49	
	187.5	189.70	101.17	
	187.5	187.49	99.99	

Table 6.6: Accuracy table of Itraconazole.

% Level	Amount Spiked (µg/mL)	Amount recovered (µg/mL)	% Recovery	Mean %Recovery
50%	25	24.54	98.17	
	25	25.26	101.05	
	25	25.20	100.79	
100%	50	50.97	101.94	
	50	50.62	101.25	100.70%
	50	50.81	101.62	
150%	75	76.18	101.58	
	75	74.82	99.76	
	75	75.11	100.15	

Discussion: Three levels of Accuracy samples were prepared by standard addition method. Triplicate injections were given for each level of accuracy and mean %Recovery was obtained as 100.36% and 100.70% for Terbinafine and Itraconazole respectively.

Sensitivity
Table 6.7: Sensitivity table of Terbinafine and Itraconazole.

Molecule	LOD	LOQ
Terbinafine	0.78	2.37
Itraconazole	0.11	0.34

Robustness
Table 6.8: Robustness data for Terbinafine and Itraconazole.

S.no	Condition	%RSD of Terbinafine	%RSD of Itraconazole
1	Flow rate (-) 0.9ml/min	0.3	0.3
2	Flow rate (+) 1.1ml/min	0.8	0.6
3	Mobile phase (-) 60B:40A	0.2	0.2
4	Mobile phase (+) 50B:50A	0.4	0.4
5	Temperature (-) 25°C	0.4	0.4
6	Temperature (+) 35°C	1.2	1.3

Discussion: Robustness conditions like Flow minus (0.9ml/min), Flow plus (1.1ml/min), mobile phase minus (60B:40A), mobile phase plus (50B:50A), temperature minus (25°C) and temperature plus(35°C) was maintained and samples were injected in duplicate manner. System suitability parameters were not much affected and all the parameters were passed. %RSD was within the limit.

Assay: (Itrav-T), bearing the label claim Terbinafine 250mg, Itraconazole 100mg. Assay was performed with the above formulation.

Preparation of Sample Preparation: 10 tablets were weighed and the average weight of each tablet was calculated, then the weight equivalent to 1 tablet was transferred into a 100 ml volumetric flask, 25ml of diluents was added and sonicated for 25 min, further the volume was made up with diluent and filtered by HPLC filters (2500µg/ml of Terbinafine and 1000µg/ml of Itraconazole) 0.5ml of filtered sample stock solution was transferred to 10ml volumetric flask and made up with diluent. (125µg/ml of Terbinafine and 50µg/ml of Itraconazole).

Inject equal volumes of Blank (diluent), Standard (6 replicate) and sample solution (duplicate).

Average % Assay for Terbinafine and Itraconazole obtained was 99.95% and 100.22% respectively.

Table 6.9: Assay Data of Terbinafine.

S.no	Standard Area	Sample area	% Assay
1	1615103	1629420	100.67
2	1624387	1626262	100.47
3	1600006	1636635	101.11
4	1601721	1635464	101.04
5	1600097	1630581	100.74
6	1631506	1609817	99.46
Avg	1611543	1628030	100.58
Stdev	13672.1	9722.0	0.60
%RSD	0.8	0.6	0.6

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Table 6.10: Assay Data of Itraconazole.

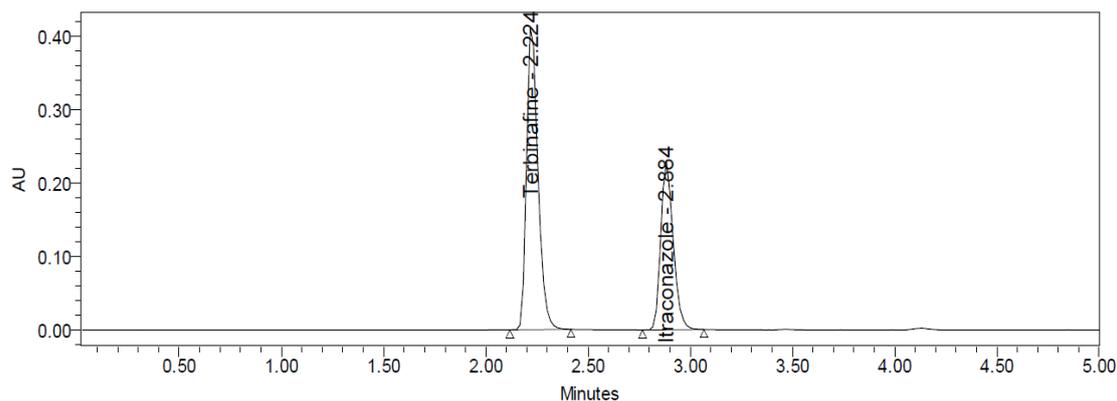| S. no	Standard Area	Sample area	% Assay
1	977378	992058	101.32
2	985152	984877	100.59
3	970599	986859	100.79
4	971171	990697	101.19
5	963379	983667	100.47
6	983380	976901	99.78
Avg	975177	985843	100.69
Stdev	8340.7	5456.1	0.56
%RSD	0.9	0.6	0.6

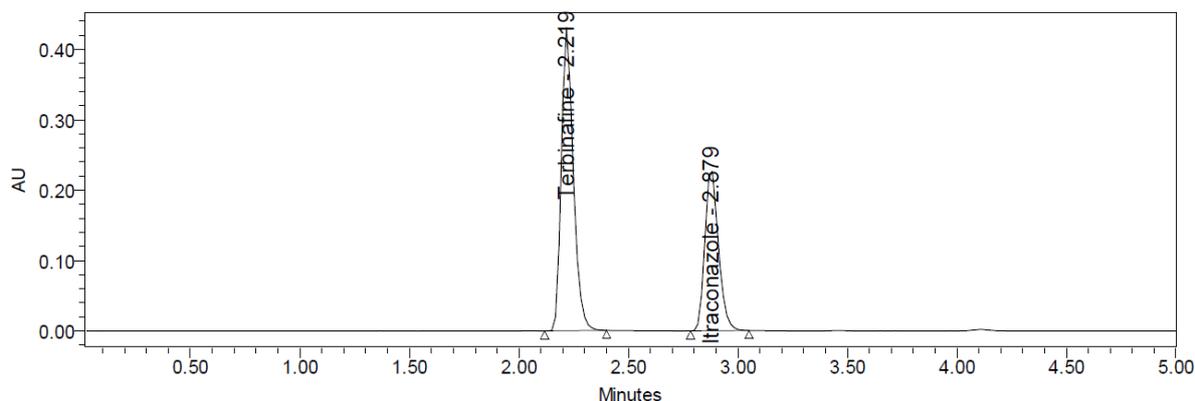**Fig 6.34: Chromatogram of working standard solution.**

Fig No. 6.35: Chromatogram of working sample solution.

6.8. Degradation data

Type of degradation	Terbinafine			Itraconazole		
	AREA	%RECOV ERED	% DEGRADED	AREA	% RECOVERED	% DEGRADED
Acid	1539445	95.11	4.89	931267	95.12	4.88
Base	1541514	95.24	4.76	933054	95.30	4.70
Peroxide	1530109	94.53	5.47	919870	93.95	6.05
Thermal	1578882	97.55	2.45	953392	97.38	2.62
Uv	1592287	98.37	1.63	962283	98.28	1.72
Water	1602600	99.01	0.99	969983	99.07	0.93

CONCLUSION

Retention time of Terbinafine and Itraconazolewere found to be 2.221 min and 2.819min. %RSD of the Terbinafine and Itraconazole were and found to be 0.8 and 0.9 respectively. %Recovery was obtained as 100.36% and 100.70% for Terbinafine and Itraconazole respectively. LOD, LOQ values obtained from regression equations of Terbinafine and Itraconazole were 0.78,2.37 and 0.11, 0.34 respectively. Regression equation of Terbinafineis y =21293x + 9314.3, and y = 21074x + 5342.7 of Itraconazole Retention times were decreased and that run time was decreased, so the method developed was simple and economical that can be adopted in regular Quality control test in Industries.

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