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Method Development and Validation of Fluconazole and Itraconazole by Using RP-HPLC

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Abstract

The development and validation of the Fluconazole and Itraconazole is performed by a rapid, sensitive, and reliable RP-HPLC technique using a Waters HPLC System with PDA detection was designed and validated. Chromatography was carried out on an Inertsil -ODS C18 (250 x 4.6 mm, 5) column with a flow rate of 1.0 ml/min. Temperature was held at an ambient (room temperature). The optimized selected wavelength was 257 nm using filtered and mixed Degassed Methanol: Acetonitrile (70:30) as a mobile phase. Fluconazole retention period is 3.049 minutes while Itraconazole retention times are 4.316 minutes. The values of LOD, LOQ obtained for the fluconazole and itraconazole by using the relative equations were 5.06 and 1.69 respectively. This process was validated according to the ICH (International conference for harmonization) guidelines accuracy, precision, linearity, and robustness.

Keywords

Method Development, Validation, Fluconazole, Itraconazole, RP-HPLC

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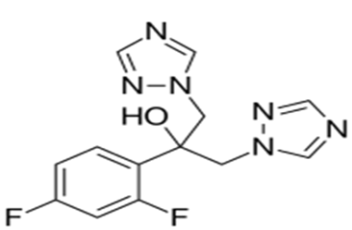
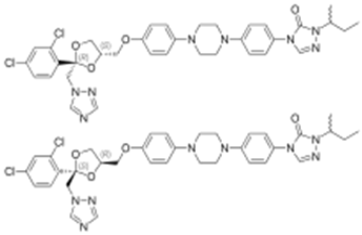
INTRODUCTION

Fluconazole and itraconazole are a highly specific inhibitor of the cytochrome P450-dependent enzyme that binds to the lanosterol 14--demethylase in fungi[1,2,3]. This enzyme typically converts lanosterol to ergosterol, which is required for the formation of fungal cell walls. A single iron atom in the heme group of lanosterol 14--demethylase interacts with a free nitrogen atom on the azole ring of fluconazole[4,5]. This prevents oxygen activation and, as a result, inhibits lanosterol demethylation, putting an end to the ergosterol production process[6]. Methylated sterols then accumulate in the cellular membrane of the fungal cell, halting fungal development[7,8,9].



Fluconazole



Itraconazole

The main aim of the in-process investigation is to develop an accurate, precise, sensitive, selective, reproducible and rapid analytical technique by following ICH guidelines for cost effective estimation of a Fluconazole and Itraconazole.

MATERIALS AND METHODS

The attribution of the Fluconazole and Itraconazole was gifted from the Aurbindo, Methanol (HPLC grade) from Merck specialties limited. HPLC grade water are from the Loba chemicals, Dihydrogen potassium phosphate (Analytical grade) from Finar**,** Acetonitrile from the Hetero private ltd.

METHOD DEVELOPMENT: Preparation of the mobile phase[10]**:** For mobile phase take 700ml methanol and 300ml ACN in a volumetric flask, add buffer to maintain pH. Then the solution poured in beaker and sonicate it for 30minutes so that no entrapping of the bubbles.

Preparation of Fluconazole and Itraconazole standard solution: Stock A[11]**:** Take 10mg of Fluconazole drug in volumetric flask, to it add 7ml of methanol then keep it for sonication about 30 minutes. Then to this add 3ml of methanol make up to the mark and sonicate it for 10minutes(1000µg/ml).

Stock B: Take 10mg of Itraconazole drug in volumetric flask, to it add 7ml of methanol sonicate for 30 minutes. Then add 3ml of methanol make up to the mark and sonicate it for 10minutes.Take 1ml of above stock solution in 10ml volumetric flask then add 10ml of methanol, test the stock solution A and B.

Result and discussion

To improve the method development & validation of Fluconazole and Itraconazole on literature surveys. As a result, the trials detailed below show how the optimization was accomplished. Wavelength that was observed and detected 257nm, flow rate (1ml/min), temperature (°C), injection volume (µl).

Table no 1: TRIALS FOR THE MOBILE PHASE

TRIAL	COLUMN	MOBILE PHASE	Flowrate	Temperature	Injection vol
1	Inertsil-C18 plate ODS	Degassed Acetonitrile: Water 90:10.	1.0	22	20
2	Inertsil-C18 Plate ODS	Degassed Acetonitrile: methanol (45:55) V/V.	1.0	22	20
3	Inertsil-C18 plate ODS	Degassed acetonitrile: Methanol (50:50) V/V.	1.0	22	20

Table no 2: INFERENCE OF MOBILE PHASE

TRIAL	DRUG	RUN TIME	RETENTION TIME	INFERNCE
1	Fluconazole	10min	3.156min	The two summits are entirely blended and cannot be distinguished.
	Itraconazole	10min	4.417min	
2	Fluconazole	10min	2.383min	The forms of the peaks are not appealing.
	Itraconazole	10min	3.602min	
3	Fluconazole	10min	2.902min	The summits are not fully separated.
	Itraconazole	10min	3.618min	

OPTIMIZED METHOD (STANDARD METHOD)[12,13,14]**:** In the standard method the Mobile Phase used was Degassed Methanol and Acetonitrile in the ratio of 70:30 V/V. Chromatographic conditions that have been optimize.

Stationary phase	InertsilC18 plate ODS (250 x 4.6 mm, 5 µ)
Mobile phase	Methanol: acetonitrile (70:30)
Flow rate	1.0ml/min
Column temperature	Ambient temperature
Run time	10minutes
Injection volume	20µl
Wavelength	257nm
Retention time	3.049min for Fluconazole and 4.317 for Itraconazole

Table no 3: STANDARD CHROMATOGRAPHIC CONDITIONS

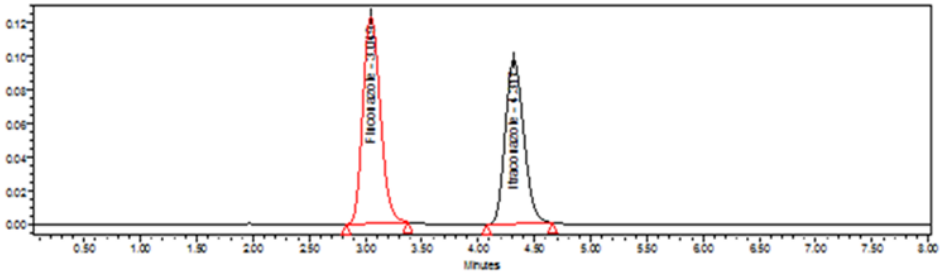


Figure no 1: STANDARD CHROMATOGRAM

Inference: From the chromatogram fluconazole retention time was 3.049min and Itraconazole retention time was 4.317min.

Method validation: From the stock solution prepare the test solutions of 20ppm(20µg/ml), 30ppm, 40ppm, 50ppm. From these solutions consider 40ppm(µg/ml) as the constant i.e., 100% where the peak elute (±2ppm).

SYSTEM SUITABILITY[15,16]**:** For the system suitability, take 40ppm solutions are injected into the column in the system. Acceptance criteria for the system suitability are:

%RSD for retention time is ≤2%, %RSD for peak area is ≤ 2%, Theoretical plates are >3000, USP tailing ≤ 2%.

Table no:4: DATA OF FLUCONAZOLE SYSTEM SUITABILITY

Injection	RT	Peak Area	USP Plate count	USP Tailing
1	3.048	2022356	7823.845712	1.045
2	3.049	2021546	7810.547812	1.087
Mean	3.0459441	2023857	7036.825471	1.061
SD	0.007451	1843.157	----	----
% RSD	0.095741	0.091	----	----

Table no 5: DATA OF ITRACONAZOLE SYSTEM SUITABILITY

Injection	RT	Peak Area	USP Plate count	USP Tailing
1	4.316	322689	8325.874512	1.145
2	4.316	322187	8384.547862	1.165
Mean	4.3176241	322445	8358.8754210	1.152
SD	0.0087541	365.5054	-------	-------
% RSD	0.087541	0.1133	-------	-------

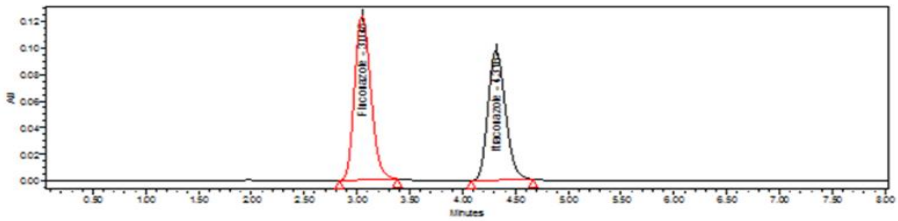


Figure no 2: STANDARD CHROMATOGRAM-1 OF SYSTEM SUITABILITY

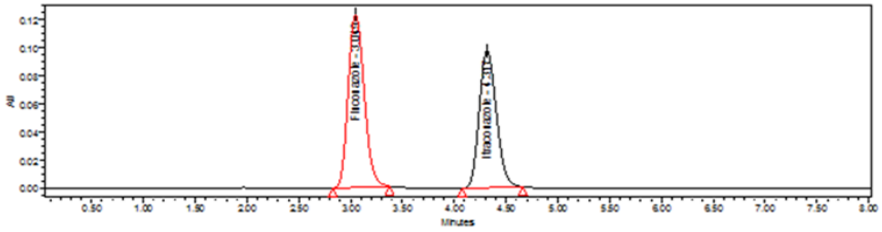


Figure no 3: STANDARD CHROMATOGRAM-2 OF SYSTEM SUITABILITY

SPECIFICITY: Mobile phase blank and working solution of fluconazole and itraconazole at standard 40ppm were injected in to the system.to check the main drug response with respect to the blank[17,18,19]. To check the interference of the peak with respect to the retention times.

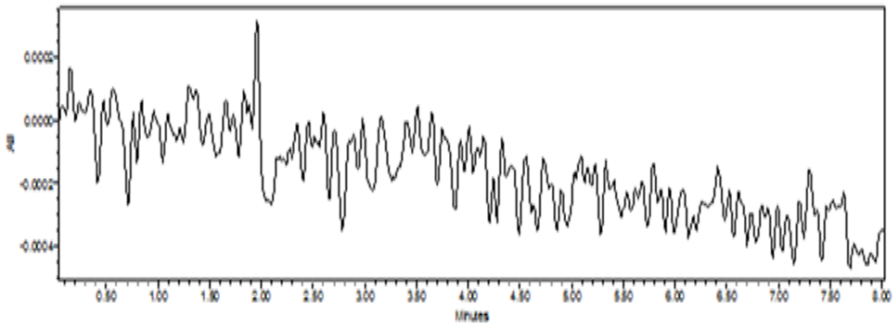


Figure no 4: CHROMATOGRAM OF BLANK SAMPLE

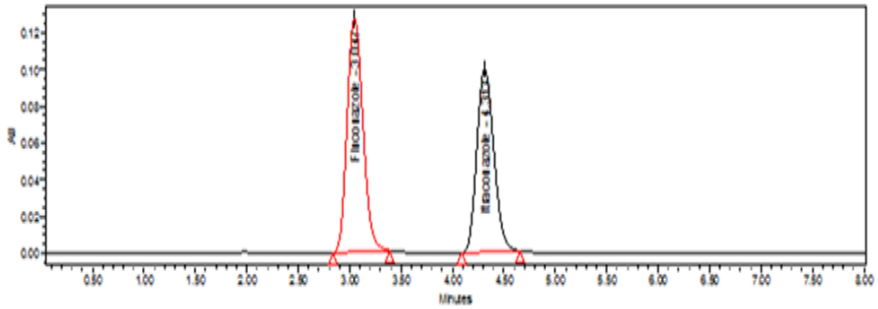


Figure no 5: CHROMATOGRAM OF STANDARD SAMPLE

By the chromatograms, the retention time of the Fluconazole was 3.047 and Itraconazole was 4.137 and no interference occurs in the peaks.

LINEARITY: The linearity of Fluconazole and Itraconazole were determined in the range of the 20ppm, 30ppm, 40ppm, 50ppm, 60ppmone injection of each concentration are injected into the system[20,21]. These concentrations are proportional to the 40ppm working standard.

Table no 6: FLUCONAZOLE DATA LINEARITY

Concentration(ppm)	Peak Area	Statistical Analysis	
0	0	Slope	50529
20	1012458	y-Intercept	-1945
30	1516384	Correlation Coefficient	0.999
40	2022586		
50	2500874		

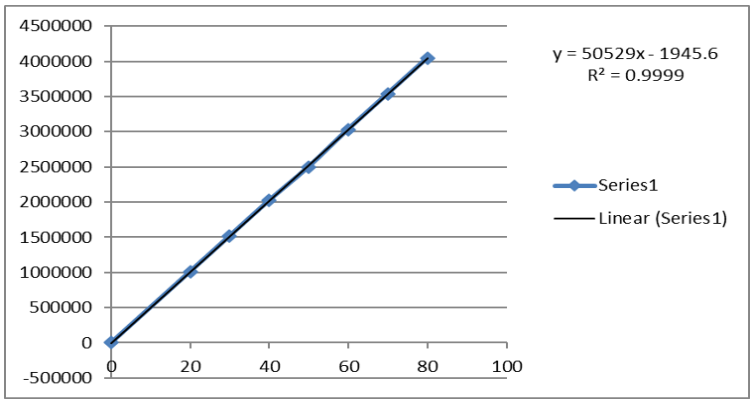


Figure no 6: Fluconazole linearity curve

Table no 7: ITRACONAZOLE DATA LINEARITY

Concentration(ppm)	Peak area	Statistical analysis	
0	0	Slope	8057
20	161274	y-Intercept	-331.8
30	241911	Correlation Coefficient	0.999
40	322548		
50	398456		

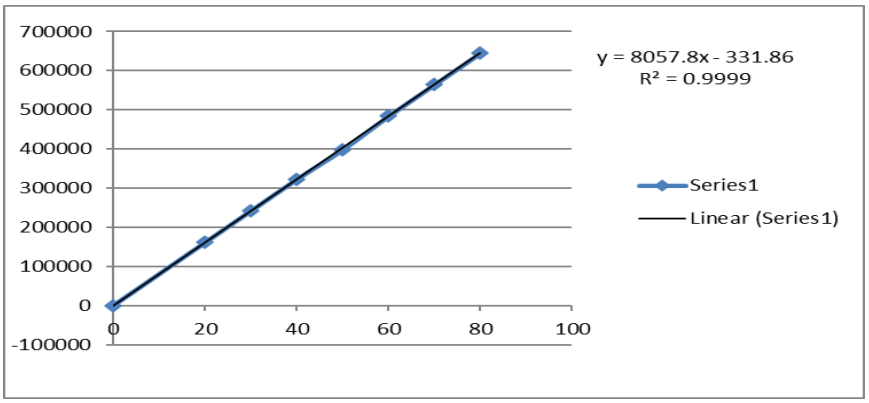


Figure no 7: Itraconazole linearity data

The linearity curve of Fluconazole drug were linear over 1012458 to 4045986 and Itraconazole were linear over 161274 to 645096. And gave a good correlation coefficient value R2=0.9999.

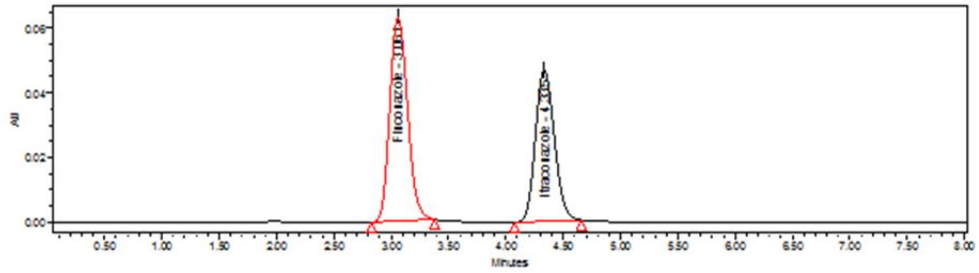


Figure no 8: 0% linearity curve

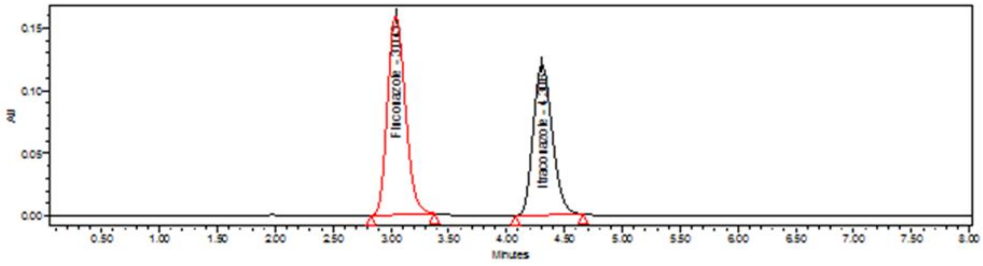


Figure no 9: 20% linearity curve

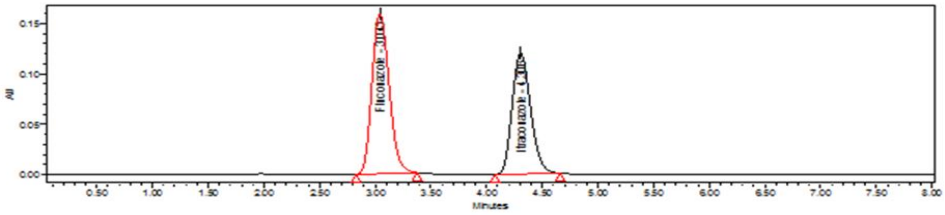


Figure no 10: 30%linearity curve

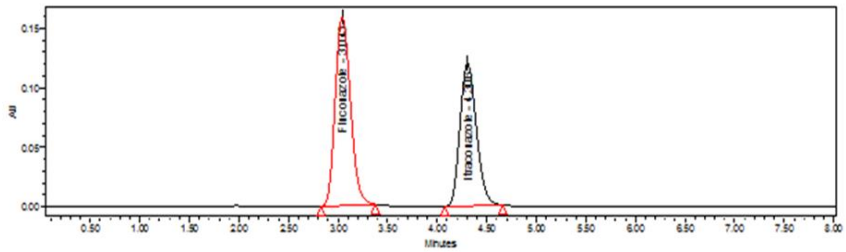


Figure no 11: 40%linearity curve

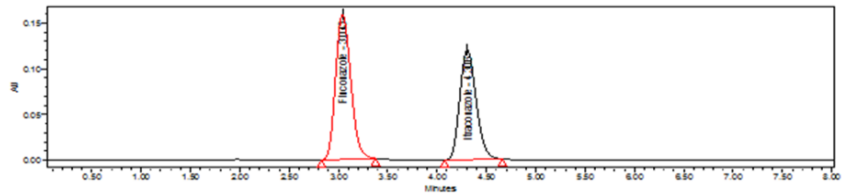


Figure no 12: 50% linearity curve

LIMIT OF DETECTION AND LIMIT OF QUANTIFICATION:

From the linearity plot the LOD and LOQ are calculated:

Fluconazole LOD =0.56 &LOQ=1.69

Itraconazole LOD=0.57& LOQ=1.74

PRECISION[22]: (Repeatability): (a) System precision: For the precision analysis inject 40ppm standard concentration of injections into the system to calculate the peak area and %RSD of Fluconazole and Itraconazole were calculated, Acceptance criteria for the precision:

%RSD = ≤ 2%

Table no 8: FLUCONAZOLE SYSTEM PRECISION

	Injection	Peak Areas of Fluconazole	%Assay
Concentration 40ppm	1	2023987	100.23
	2	2024578	100.26
	3	2028545	100.46
***Statistical* Analysis**	Mean	2023655	100.21
	SD	2025116	100.29
	% RSD	1777.04	0.087

Table no 9: ITRACONAZOLE SYSTEM PRECISION

	Injection	Peak Areas of Itraconazole	%Assay
Concentration 40ppm	1	322124	100.05
	2	322689	100.22
	3	322356	100.12
***Statistical* Analysis**	Mean	322784	100.25
	SD	322500	100.17
	% RSD	237.8406	0.073

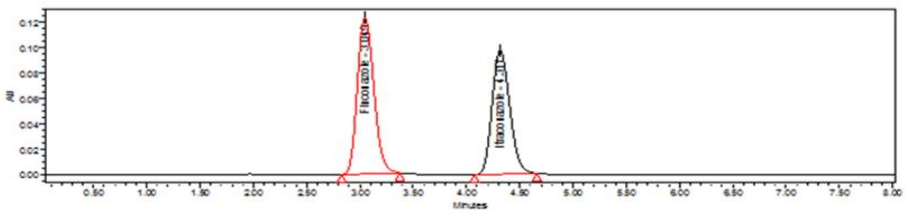


Figure no 13: Standard precision chromatogram 1

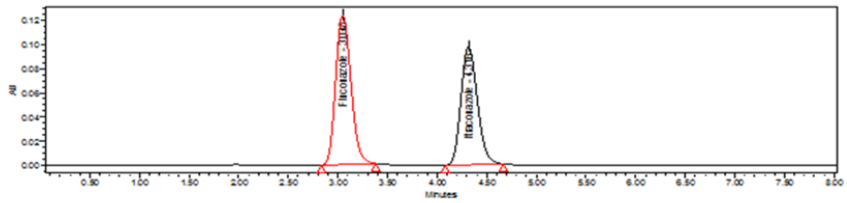


Figure no 14: Standard precision chromatogram 2

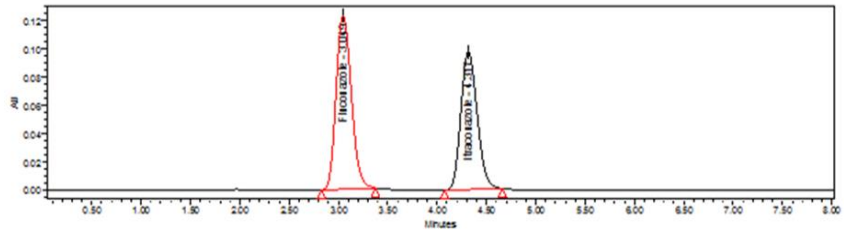


Figure no 15: Standard precision chromatogram 3

(b)METHOD PRECISION:

Table no 10: DATA FOR METHOD PRECISION FOR FLUCONAZOLE

	Injection	Peak Areas of Fluconazole	%Assay
Concentration 40ppm	1	2024568	100.26
	2	2024875	100.28
	3	2026548	100.36
Statistical Analysis	Mean	2026681	100.36
	SD	1725.19	0.014
	% RSD	0.085	1.33

Table no 11: DATA FOR METHOD PRECISION OF ITRACONAZOLE

	Injection	Peak Areas of Itraconazole	%Assay
Concentration 40ppm	1	322546	100.18
	2	322840	100.27
	3	322894	100.29
Statistical Analysis	Mean	322605	100.20
	SD	351.4898	0.109
	% RSD	0.108	0.108

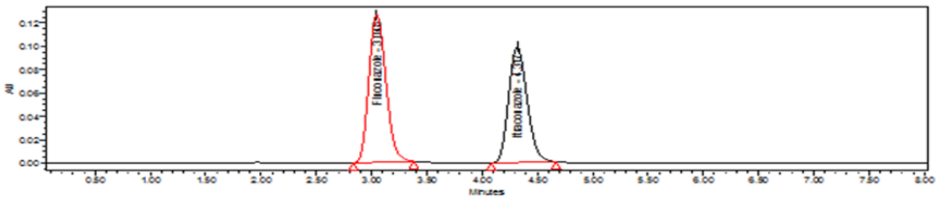


Figure no 16: Standard chromatogram of method precision 1

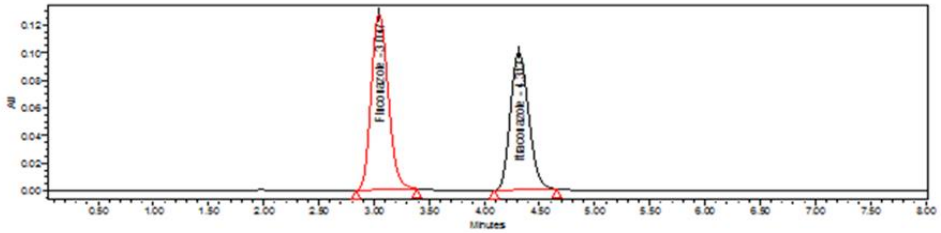


Figure no 17: Standard chromatogram of method precision-2

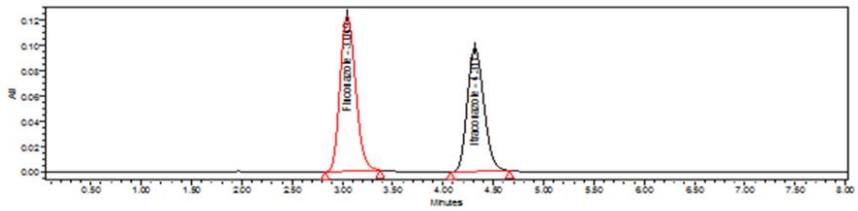


Figure no 18: Standard chromatogram of method precision 3

Chromatograph with high repeatability

Intermediate precision: It is performed by three different analysts.

For Analyst 1 refer table no 8: System precision

Analyst 2

Table no 12: DATA FOR INTERMEDIATE PRECISION OF FLUCONAZOLE

	Injection	Peak Areas of Fluconazole	%Assay
Concentration 40ppm	1	2026885	100.37
	2	2028854	100.47
	3	2027845	100.42
Statistical Analysis	Mean	2026775	100.37
	SD	1852.73	0.091
	% RSD	0.091	0.091

Table no 13: DATA FOR INTERMEDIATE PRECISION OF ITRACONAZOLE

	Injection	Peak Areas of Itraconazole	%Assay
Concentration 40ppm	1	322045	100.03
	2	322265	100.09
	3	322678	100.22
Statistical Analysis	Mean	322365	100.12
	SD	272.6705	0.084
	% RSD	0.084	0.084

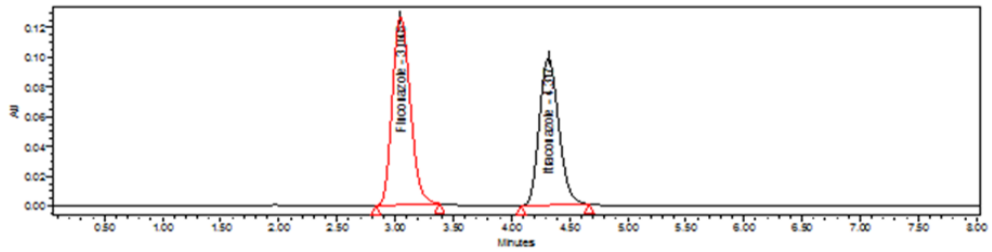


Figure no 19: Chromatographs of intermediate precision 1

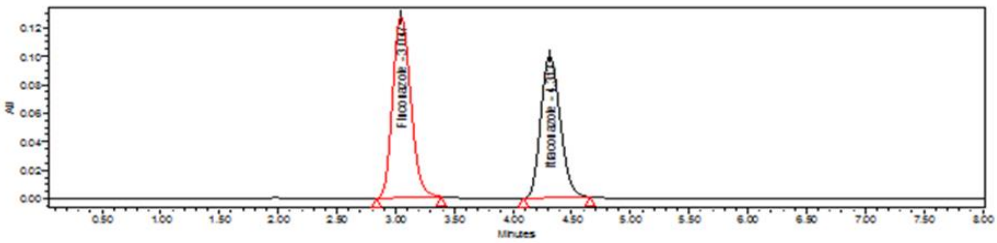


Figure no20: Chromatographs of intermediate precision 2

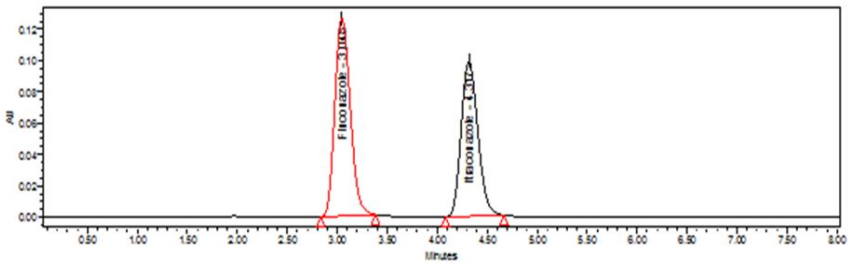


Figure no 21: Chromatographs of intermediate precision

ACCURACY: To check the accuracy of the Fluconazole and Itraconazole inject the solution in 50%, 100%, 150% injections each in to the HPLC system. Acceptance criteria of accuracy = 98 to 102%.

Table no 14: DATA TABLE OF ACCURACY OF FLUCONAZOLE.

Concentration % Of spiked level	Amount added (ppm)	Amount found(ppm)	%Recovery	Statistical Analysis of % Recovery	
50%Injection 1	20	20.13	100.69	**MEAN**	100.73
50%Injection 2	20	20.13	100.68	**%RSD**	0.073
100 %Injection 1	40	40.08	100.21	**MEAN**	100.29
100%Injection 2	40	40.13	100.34	**%RSD**	0.065
150%Injection 1	60	60.07	100.12	**MEAN**	100.21
150%Injection 2	60	60.17	100.29	**%RSD**	0.084

Table no 15: DATA TABLE OF ACCURACY OF ITRACONAZOLE.

Concentration % of spiked level	Amount added (ppm)	Amount found (ppm)	% Recovery	Statistical Analysis of % Recovery	
50%Injection 1	20	20.05	100.27	**MEAN**	100.48
50%Injection 2	20	20.12	100.68	**%RSD**	0.193
100 %Injection 1	40	40.01	100.03	**MEAN**	100.09
100%Injection 2	40	40.06	100.15	**%RSD**	0.062
150%Injection 1	60	60.04	100.07	**MEAN**	100.09
150%Injection 2	60	60.00	100.01	**%RSD**	0.084

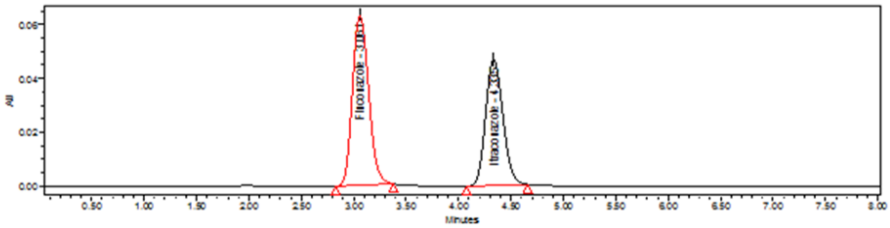


Figure no 21: Chromatograph of accuracy at 50%

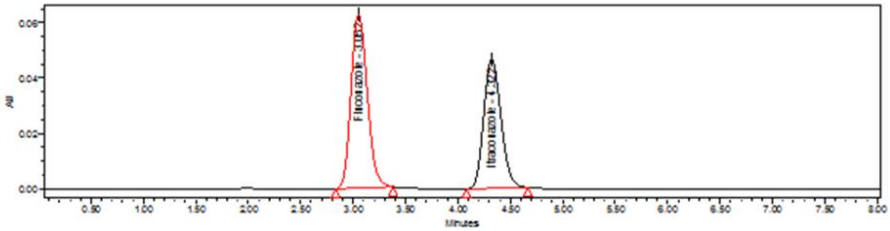


Figure no 22: Chromatograph of accuracy at 50%

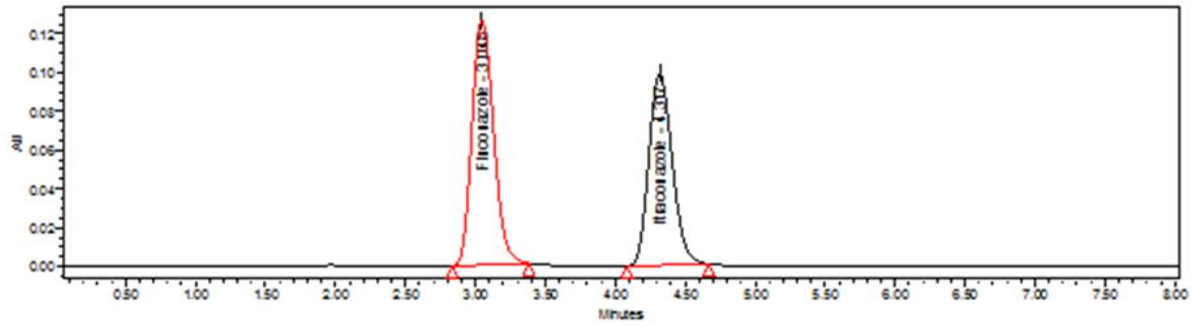


Figure no 23: Chromatograph of accuracy at 100%

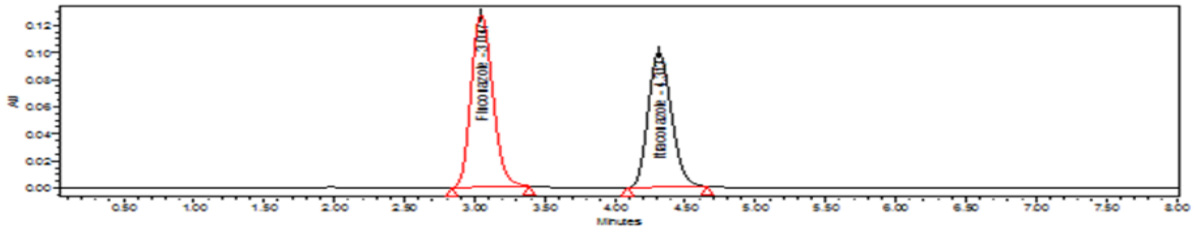


Figure no 24: Chromatograph of accuracy at 100%

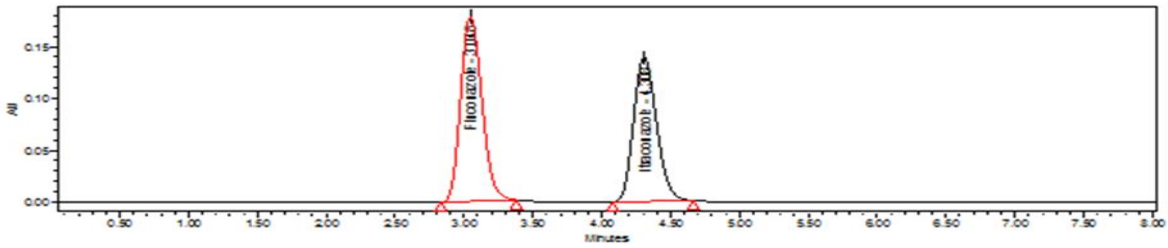


Figure no 25: Chromatograph of accuracy at 150%

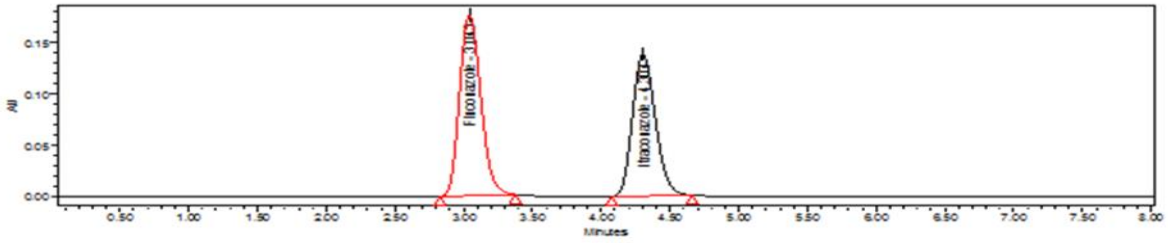


Figure no 26: Chromatograph of accuracy at 150%

RUGGEDNESS: In ruggedness the variability is differ from the system to system.in this system-system variability was checked. For the first system refer system precision table. Now inject 6 injections 40ppm standard solution into another HPLC system and check the variability.

Acceptance criteria of robustness is Assay = ≤ 2%, RSD = ≤2%

Table no 16: FLUCONAZOLESYSTEM VARIABILITY OF SECOND SYSTEM

S.NO:	Peak area	Assay % of Fluconazole
1	2023568	100.21
2	2025689	100.32
3	2028648	100.46
Mean	2026417	100.35
%RSD	0.099	0.099

Table no 17: ITRACONAZOLE SYSTEM VARIABILITY OF SECOND SYSTEM

S.NO:	Peak area	Assay % of Itraconazole
1	321456	99.84
2	321265	99.78
3	321866	99.97
Mean	321516	99.86
%RSD	0.067	0.067

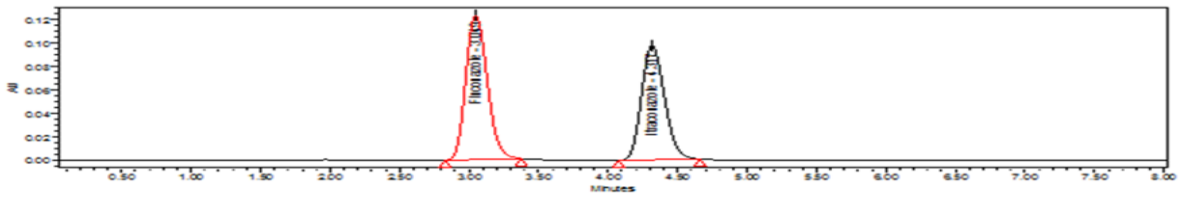


Figure no 27: Chromatograms of system-system variability

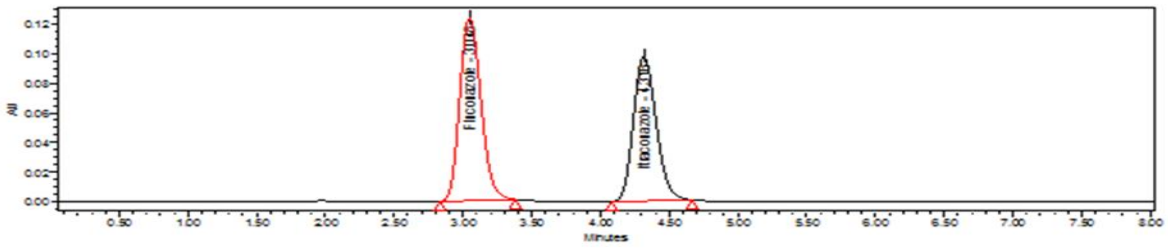


Figure no 28: Chromatograms of system-system variability

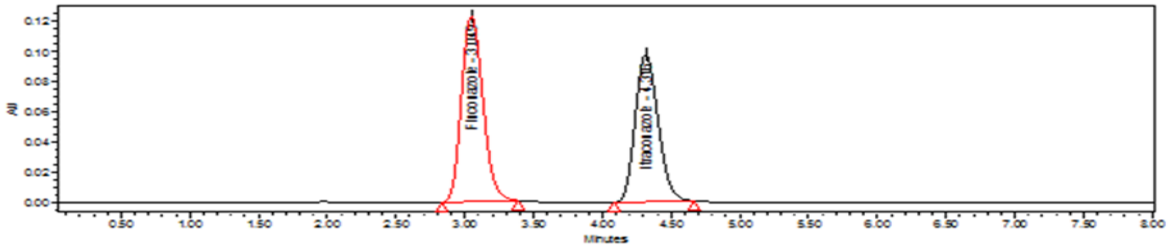


Figure no 29: Chromatograms of system-system variability

ROBUSTNESS[23, 24]**:** To analyzing the robustness of the compound injected 0.8ml, 1ml, 1.2ml of 3 injections each into the HPLC system. Criteria used for acceptance of system suitability are: Plate count - > 300, Resolution - > 2.0, Peak tailing - ≤ 2.0; RSD for peak area - ≤ 2.0 Table

Tableno18: FLUCONAZOLE ROBUSTNESS DATA

Flow 0.8 ml	**Std Area**	**Tailing factor**	**Flow 1.0 ml**	**Std Area**	**Tailing factor**	**Flow 1.2 ml**	**Std Area**	**Tailing factor**
	2008698	1.011		2026578	1.096		2054876	1.056
	2003945	1.013		2027564	1.078		2056842	1.075
	2005682	1.011		2022796	1.053		2053896	1.089
Avg	2003575	1.010		2024505	1.095		2054784	1.036
SD	2005801	1.011	**Avg**	2025499	1.076	**Avg**	2056147	1.065
%RSD	2334.70	0.003	**SD**	2137.66	0.0208	**SD**	1904.91	0.0187

Table no 19: ITRACONAZOLE ROBUSTNESS DATA

Flow 0.8 ml	Std Area	Tailing factor	Flow 1.0 ml	Std Area	Tailing factor	Flow 1.2 ml	Std Area	Tailing factor
	318456	1.145		322654	1.110		326844	1.123
	318860	1.153		322865	1.134		326598	1.135
	318078	1.142		322554	1.120		326870	1.145
Avg	318974	1.129		322680	1.128		326395	1.136
SD	318594	1.141	**Avg**	322643	1.129	**Avg**	326632	1.134
%RSD	320.268	0.008	**SD**	189.241	0.013	**SD**	190.176	0.007

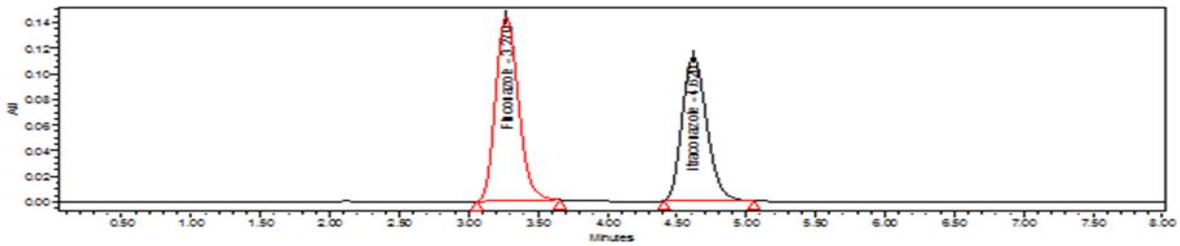


Figure no 30: Chromatograms for a 0.8 ml/min flow rate standard

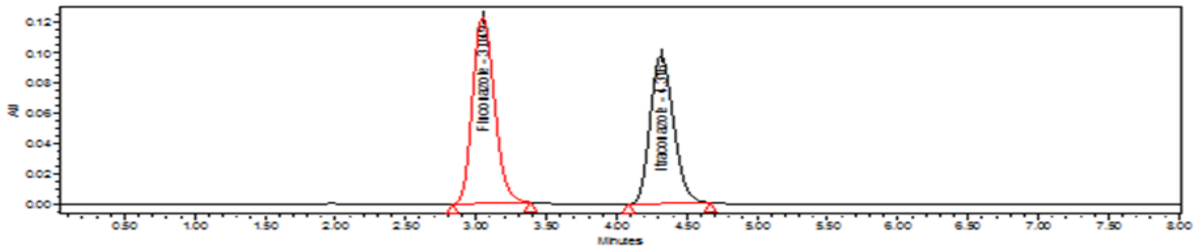


Figure no 31: Chromatograms for a 1ml/min flow rate standard

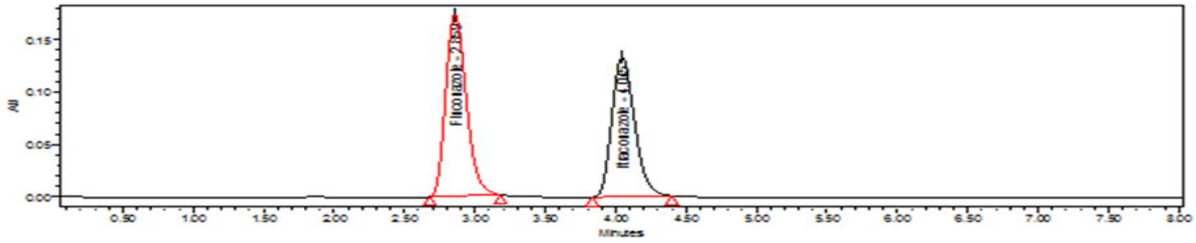


Figure no 32: Chromatogram for a 1.5ml/min flow rate standard

CONCLUSION:

This was the first time Fluconazole and Itraconazole were developed and validated in pure form for pharmaceutical formulations. The proposed approach proved to be a simple, precise, accurate, and appropriate technique for quantifying anti-fungal agents. The optimized selected wavelength was 257 nm using filtered and mixed Degassed Methanol: Acetonitrile (70:30) as a mobile phase. Fluconazole retention period is 3.049 minutes while Itraconazole retention times is 4.316 minutes. The values of LOD, LOQ obtained for the fluconazole and itraconazole by using the relative equations were 5.06 and 1.69 respectively.

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