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DEVELOPMENT AND VALIDATION OF STABILITY INDICATING ASSAY METHOD FOR QUANTIFICATION OF FLUCONAZOLE IN FLUCONAZOLE TABLET BY SPECTROSCOPY METHOD

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ABSTRACT

For the purpose of quantifying fluconazole in pharmaceutical solid dosage forms such as capsules, uncoated, and dispersible tablets, an RP-HPLC and a UV spectrophotometric assay method were designed and validated. A column was used to do the chromatography. This is a 5µm column with dimensions of 250 mm by 4.6 mm i.d. with a mobile phase of methanol: water (60:40 v/v) at a detector wavelength of 260 nm. It is 40°C in the Column Oven. Using 0.1 M HCl as the solvent, the UV technique was run at 260 nm. For HPLC and UV techniques, the linearity was measured at five different levels, ranging from 50 to 200 µg/ml and 50% to 150% of the test concentration, respectively. With a recovery of 98 to 102%, the HPLC technique proved to be exact and accurate for all dose forms examined. Relative Standard Deviation shouldn't be allowed to get above 2.0%. For the measurement of fluconazole only in tablet dosage form, the UV technique correlated well with HPLC. A broad-spectrum antifungal derived from synthetic triazole, it is effective against a variety of topical and systemic fungal infections orally and orally. It is recommended to treat and prevent the spread of oropharyngeal, oesophageal, or vulvovaginal candidiasis and deep visceral candidiasis. Fluconazole is an extremely picky C-14 sterol suppressor of fungal (Xdemethylated) cytochrome P450. According to the ICH, the developed technique was approved for specificity, linearity, precision, accuracy, reliability, limit of detection, and limit of quantification.

KEYWORDS: Fluconazole, UV Spectrophotometer, RP-HPLC, Assay method, Method development, Method development, validation.

1. INTRODUCTION

When it comes to bulk drug quality assurance and control, pharmaceutical analysis is essential. The processes of Chemistry of analysis include component identification, separation, and quantification within a sample matrix. The development of analytical techniques is crucial to the discovery, creation, and production of medications. RP-HPLC is

perhaps the most sensitive and widely used analytical technique. It is distinct in that it can handle combinations of several components with ease. To get the best separation possible while establishing RP-HPLC analytical procedures for pharmaceuticals, one must have a solid knowledge of chromatographic separation in practice, including how it changes depending on the sample and the type of investigation. Due to its wide range of applications, phase-reversal chromatography is currently the more widely employed division technology in HPLC. The fungus lanosterol 14-α demethylase is actively inhibited by fluconazole, a strong cytochrome P450 inhibitor. It is widely recommended for the therapy and prevention of infections with deep and widespread organ candidiasis. [1,2]

Fluconazole is an antifungal drug belonging to the triazole class, commonly utilized for treating and preventing both superficial and systemic fungal infections. It is typically found in bulk powder form, appearing as a white crystalline substance with very limited solubility in water but soluble in alcohol. Fluconazole boasts advantageous pharmacological qualities like a relatively longer half-life [3,4] and the adaptability of administration via oral or parenteral routes. Similar to other azole-class antifungals [3,4]. A key part of the fungal cytoplasmic membrane, ergosterol, is created when lanosterol is not converted properly for its integrity and function. Therefore, this drug inhibits cell membrane formation by inhibiting ergosterol synthesis. [5,6]

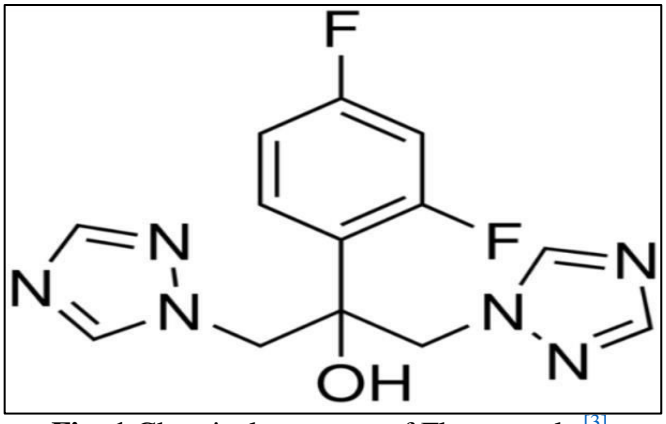


Fig. 1 Chemical structure of Fluconazole [3]

This study reports on quantifying fluconazole in solid dose forms quantitatively using an HPLC approach that has been verified. Additionally, a validated UV spectrophotometric method for quantifying fluconazole in tablet dosage forms is reported in this study. The suggested HPLC approach may be effectively used for regular quality control since it satisfies the specifications of analytical parameters that must be used in the study's content uniformity tests for pharmaceutical products that have been completed. However, only tablet dosage forms were found to be satisfactorily produced using the suggested UV technique. [7,8]

Side effects associated with fluconazole treatment include skin rash, headache, nausea, vomiting, dizziness, pain in the abdomen, and diarrhoea. Plasma-like concentrations of fluconazole are also excreted in breast milk. [9,10] It is not advised for breastfeeding moms to use fluconazole for this reason. Some people are allergic to azoles. Some azole drugs can interfere with oestrogen production during pregnancy and affect pregnancy outcomes.[11]

This study's objective was to determine and validate a straightforward, accurate, and sensitive spectroscopic technique for the measurement of fluconazole in tablet formulations.[11]

2. MATERIAL AND METHOD:

2.1. Chemicals and Reagents:

Vidisha Analytical presented the Pure Fluconazole medication as a gift. Potassium dihydrogen orthophosphate analytical reagent was purchased from Merck and Siddhi Lab. HPLC Water and methanol were of HPLC grade.

2.2 Instrumentation:
An HPLC-1260 infinity II (Agilent) was used for the method development and validation. HPLC binary gradient system, Detector (DEAX02386), Double beam UV visible spectroscopy (Jasco), Weighing Balance (CY224C) from Aczet for sample weighing, Bio-technic Ultra Sonicator (13.5L) and pH meter from Lab Man used for sample preparation.

2.3 Selection of Solvents:
We decided to dissolve fluconazole in methanol as the solvent.

2.4 Getting ready to make basic stock solutions
20 mg of fluconazole were precisely weighed, deposited into a 20 ml volumetric flask along with 15 ml of methanol, and sonicated to completely dissolve the standard. The methanol was then diluted to the appropriate level (1000 PPM) to create a stock solution. Added more methanol to dilute 1mL to 10 mL (100 PPM).

2.5 Choice of the analytical wavelength
From 400 nm to 200 nm, the fluconazole standard solution (100 PPM) and methanol were scanned. The medication's absorption maxima were established. The results indicated that fluconazole's highest absorbance was at 260 nm.

3. UV- SPECTROSCOPIC METHOD FOR FLUCONAZOLE:
3.1 System suitability test for UV analysis (Fluconazole standard solution):
About 20 mg of fluconazole were weighed and then placed in a 20 mL volumetric flask. 15 mL of sonicated methanol was then added to the flask to get the volume up to the required level. 2 ml of the standard stock solution was pipetted out, moved to a 20 ml volumetric flask, and A methanol adjustment was made to the volume.

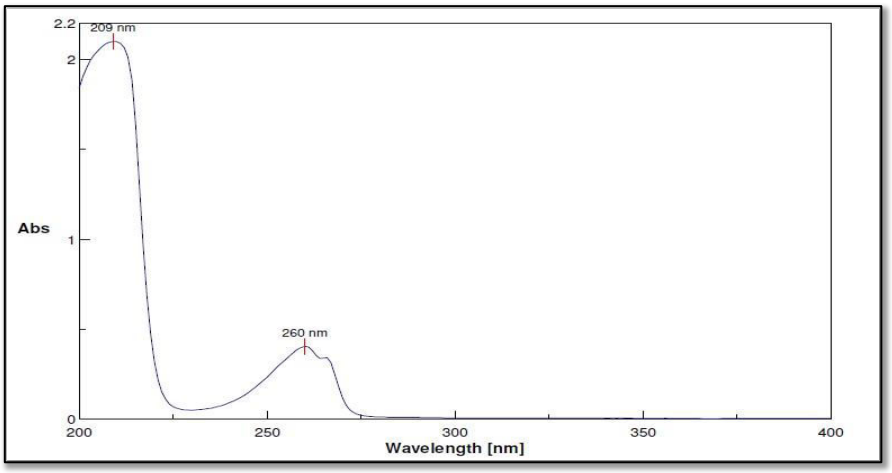


Fig. 2 UV spectrum of Fluconazole

Table 1 The system suitability test's outcomes of Fluconazole for UV

No.	Standard Solution	Absorbance
1	Standard 1	0.4063
2	Standard 2	0.4058
3	Standard 3	0.4067
4	Standard 4	0.4061
5	Standard 5	0.4065
Mean		**0.4063**
STD Dev		**0.00035**
% RSD		**0.09**

System Suitability Acceptance Criteria for UV:
RSD should not be more than 2.0 % for five replicates of absorbance of standard solution.

4. RESULTS AND DISCUSSION:

4.1 VALIDATION OF UV METHOD FOR FLUCONAZOLE

4.1.1 Filtration Study:

The filter's compatibility with the sample, deposition on the filter bed, and interference from extraneous components are all examined during the filtration study of an analytical technique. Carried out on a test specimen.

Table 2 Filter Study Outcomes

Description	Area	% Absolute difference
Unfiltered	0.4012	NA
0.45 µ PVDF filter	0.3969	1.07
0.45 µ Nylon filter	0.3981	0.77

• **Criteria for acceptance:** % Absolute difference between NMT 2.0 filtered samples and unfiltered ones.

4.1.2 Solution Stability:

Both the Test Sample and the Standard underwent a stability assessment. Normal laboratory settings were followed for conducting a stability investigation. The solution was first examined after 12 hours and then again after 24 hours under standard laboratory lighting.

Table 3 The stability of the solution's results

Sample solution			**Standard solution**		
Time point	**Area**	**% Absolute difference**	**Time point**	**Area**	**% Absolute difference**
Initial	0.3991	NA	Initial	0.4036	NA
12 Hours	0.3962	0.73	12 Hours	0.4012	0.59
24 Hours	0.3947	1.10	24 Hours	0.3997	0.97

Criteria for acceptance: % Absolute variation between the initial solution and the stability solution NMT 2.0.

4.1.3 Linearity on UV Spectrophotometer:
Capacity to extract test findings that correspond to analyte concentrations in samples falling within a specified range.

Table 4 Results of UV Linearity for Fluconazole

Level	**Conc (µg/mL)**	**Absorbance**	**Mean**	**% RSD**
50%	50.00	0.1987	0.1986	0.077
		0.1984		
		0.1986		
75%	75.00	0.3045	0.3043	0.066
		0.3041		
		0.3043		
100%	100.00	0.4065	0.4064	0.075
		0.4061		
		0.4067		
125%	125.00	0.5042	0.5043	0.080
		0.5047		
		0.5039		
150%	150.00	0.6129	0.6127	0.034
		0.6126		
		0.6125		

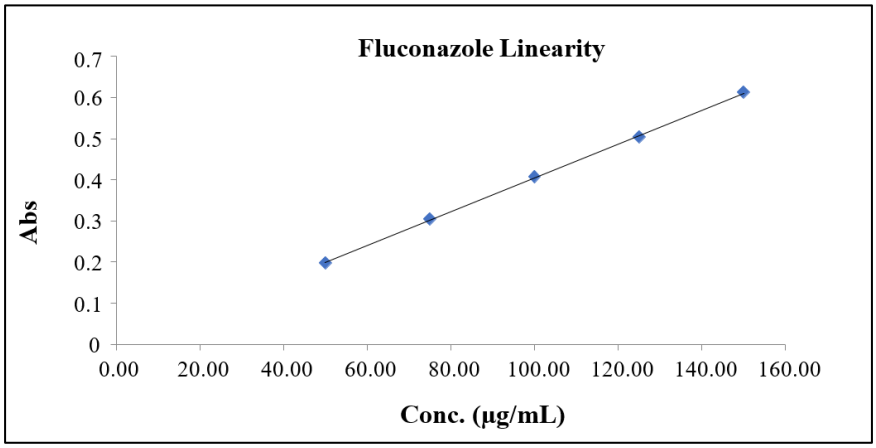


Fig. 3 Rectification curve of Fluconazole on UV

Table 5 UV-linearity of Fluconazole

No.	Parameter	Result value	Acceptance criteria
1.	Beer's linearity range	50.0-150.0 µg/mL	NA
2.	Correlation coefficient (R2)	0.99989	NLT 0.98
3.	Intercept	-0.00602	To be report
4.	Slope	0.00411	To be report
5.	% RSD for the area at each level	NA	NMT 2.0

- **Conclusion:**

It was determined by the calibration curve that Fluconazole has a linear response within the 50.0–150.0 µg/ml limit. Well inside the bounds of the regression value was discovered.

4.1.4. LOD and LOQ:

σ = 0.00242

s = 0.00411

Detection limit (LOD):

LOD = 3.3 σ / S

LOD = 3.3 x 0.00242 / 0.00411

LOD = 1.94 µg/mL

Quantitation limit (LOQ):

LOQ = 10 σ / S

LOQ = 10 x 0.00242 / 0.00411

LOQ = 5.89 µg/mL

4.1.5. ACCURACY:

The degree to which test results produced using that approach resemble the actual value. Analyzing samples that have been added to known concentrations of analyte allows one to assess an analytical method's accuracy.

Table 6 Accuracy of Fluconazole

Level (%)	Absorbance	Recovered conc. (µg/mL)	Added conc. (µg/mL)	% Recovery	Mean Recovery	% RSD
50	0.2032	50.01	50.20	99.62	99.74	1.263
	0.2018	49.67	50.40	98.55		
	0.2053	50.53	50.00	101.06		
100	0.4088	100.62	100.40	100.22	99.51	0.654
	0.4046	99.58	100.20	99.38		
	0.4028	99.14	100.20	98.94		
150	0.6079	149.62	150.20	99.61	99.38	0.472
	0.6024	148.26	150.00	98.84		
	0.6092	149.94	150.40	99.69		

The overall recovery was 99.55 %. The % RSD was 0.767 for the overall recovery.

- **Acceptance criteria:**
  98.0 to 102.0 percent recovery for the total and for each level. Overall recovery and percentage RSD for each level: NMT 2.0.

4.1.6 PRECISION

The degree to which different test results from multiple samplings of a homogenous sample agree when the procedure is repeated. Relative standard deviation or standard deviation can be used to express the precision of an analytical procedure. On the test sample, accuracy was checked.

Table 7 Result of Intra- day and Inter- Day Precision for Fluconazole test sample assay

	Sample	**Test Sample (mg)**	**Absorbance**	**% Assay**
Repeatability	Sample 1	110.9	0.4027	100.51
	Sample 2	111.6	0.3974	98.57
	Sample 3	111.2	0.3985	99.20
	Sample 4	112.1	0.4002	98.82
	Sample 5	111.8	0.4016	99.43
	Sample 6	111.5	0.3934	97.66
	Mean			**99.03**
	STD DEV			**0.9499**
	% RSD			**0.959**
Intermediate precision (Inter-Day)	Sample 1	112.1	0.3992	98.57
	Sample 2	111.4	0.3976	98.80
	Sample 3	111.9	0.4020	99.44
	Sample 4	112.3	0.3947	97.29
	Sample 5	110.8	0.3924	98.03
	Sample 6	111.6	0.3906	96.88
	Mean			**98.17**
	STD DEV			**0.9621**
	% RSD			**0.980**
Repeatability Plus Inter-day	**Mean**			**98.600**
	STD DEV			**1.0169**
	% RSD			**1.031**

- **Acceptance standards:**

The assay yields values for each individual sample as well as the mean assay values for intermediate precision (6 samples) and intermediate precision sample (12 samples) 90–110 %.

% RSD: For six precision study samples, six intermediate precision study samples, and 12 precision plus intermediate precision samples NMT 2.0.

4.1.6. ROBUSTNESS:

An analytical technique's robustness gives an indication of its dependability under typical operating conditions by measuring its ability to withstand slight but intentional changes in method parameters.

- **Changes in wavelength by -3 NM:**

Note: First two samples of Precision study analyzed at this wavelength and calculated its assay value. Abs difference calculated for assay value w.r.t. Precision assay value (Mean value).

Table 8a Results of Test samples by change in – 3 nm wavelength (257 nm)

Sample	**Absorbance**	**% Assay**	**Abs difference w.r.t. Precision assay value**
Sample 1	0.3639	99.15	0.43
Sample 2	0.3621	98.04	
Mean		**98.60**	
STD DEV		**0.7844**	
% RSD		**0.796**	

- **Change in wavelength by +3 NM:**
  Note: First two samples of Precision study analyzed at this wavelength and calculated its assay value. Abs difference calculated for assay value w.r.t. Precision assay value (Mean value).

Table 8b Results of Test samples by change in +3 nm wavelength (263 NM)

Sample	**Absorbance**	**% Assay**	**Abs difference w.r.t. Precision assay value**
Sample 1	0.3463	98.29	0.31
Sample 2	0.3515	99.14	
Mean		**98.72**	
STD DEV		**0.6011**	
% RSD		**0.609**	

4.2. METHOD DEVELOPMENT BY RP – HPLC:

Table 9 Method Development Initial Study

Column Used	Mobile Phase Ratio	Detection Wavelength	Flow Rate	Injection Volume	Run Time	Result	Conclusion
Phenomenex C18 (250 mm X 4.6mm ID, 5 µm)	Methanol : Water (70:30)	260 nm	1.0 ml/min	20µl	7 min	Not satisfactory	Method unaccepted
Phenomenex C18 (250 mm X 4.6mm ID, 5 µm)	Methanol : Water (60:40)	260 nm	1.0 ml/min	20µl	7 min	Fluconazole not eluted until 20 minutes	Method unaccepted
Phenomenex C18 (250 mm X 4.6mm ID, 5 µm)	Methanol : Water (60:40)	260 nm	1.0 ml/min	20µl	7 min	Fluconazole eluted at 7 minutes with unacceptable chromatography	Method Developed

4.2.2. Optimization of HPLC method:

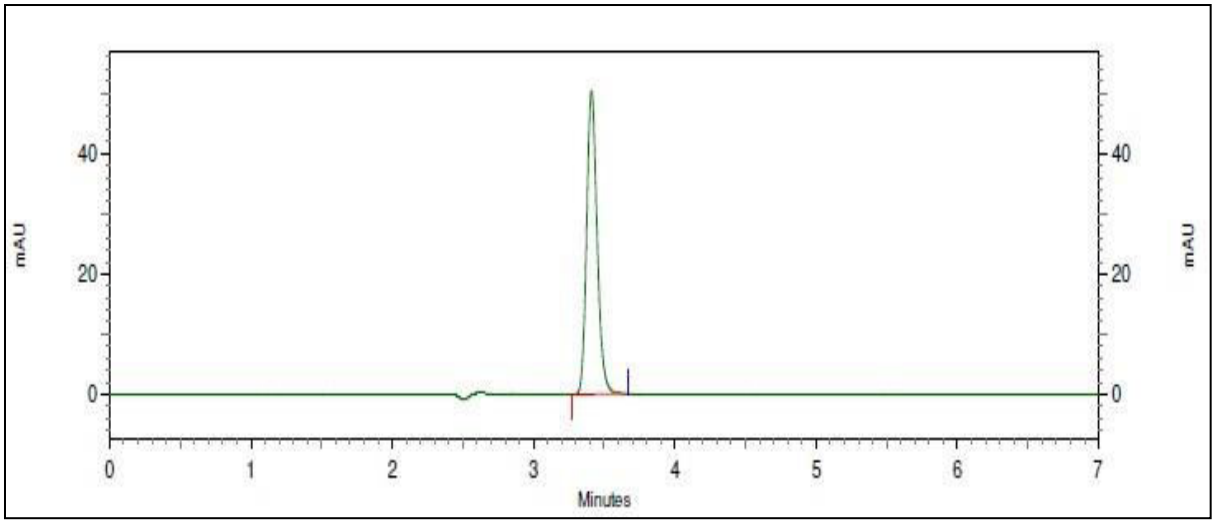


Fig. 4 Typical chromatogram Optimization of RP-HPLC Method Chromatogram

4.2.3. Filtration Study:

The analytical process of filtration study verifies that the filter is compatible with the sample, that it does not clog the filter, and that no foreign materials are deposited on the filter bed. on a test sample of tablets.

Table 10 Output of Filtration study on HPLC

Sample description	**Area**	**% Absolute difference**
Unfiltered	4351203	NA
0.45 µ PVDF filter	4306791	1.02
0.45 µ Nylon filter	4317042	0.79

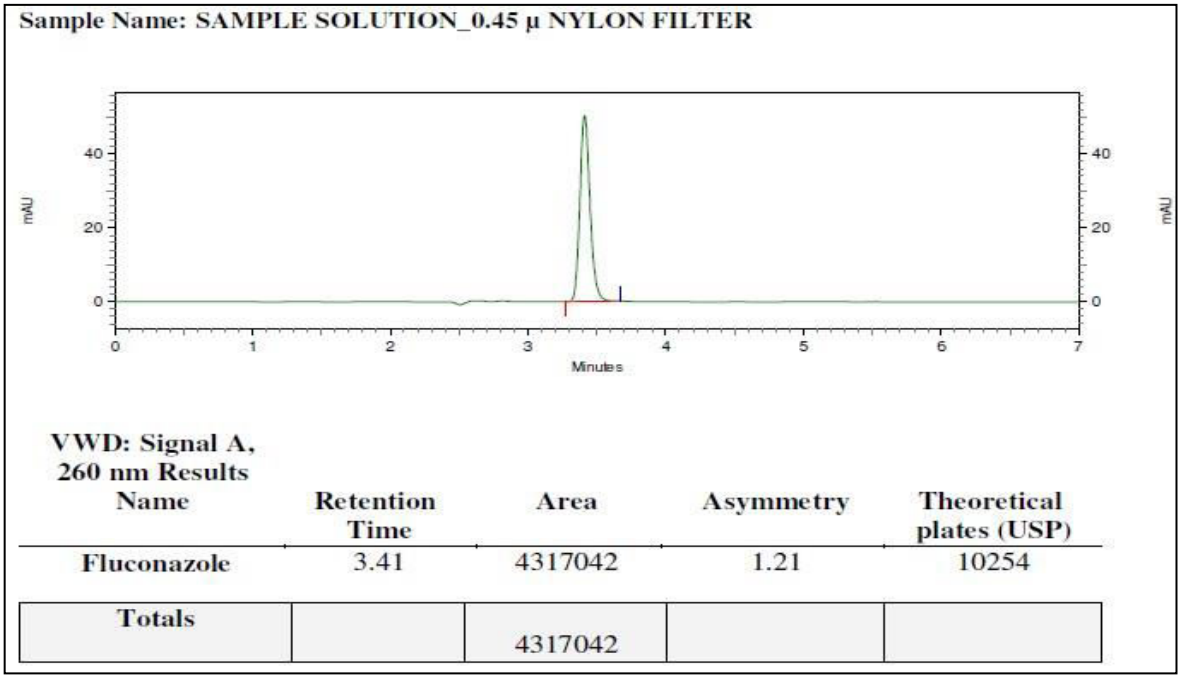


Fig. 5 An example of a chromatogram using a 0.45µ nylon filter is shown

Acceptance criterion: % Absolute variation between filtered samples NMT 2.0. unfiltered specimen.

4.2.4. Solution Stability:

A stability analysis was carried out on both the test and standard samples. The stability research was carried out in a typical laboratory setting. The solution was first examined after six, twelve, and twenty-four hours under standard laboratory lighting.

Sample solution			Standard solution		
Time point	**Area**	**% Absolute difference**	**Time point**	**Area**	**% Absolute difference**
Initial	4348704	NA	**Initial**	4379046	NA
12 Hours	4309921	0.89	**12 Hours**	4342104	0.84
24 Hours	4295516	1.22	**24 Hours**	4331792	1.08

Table 11 Results of Solution stability

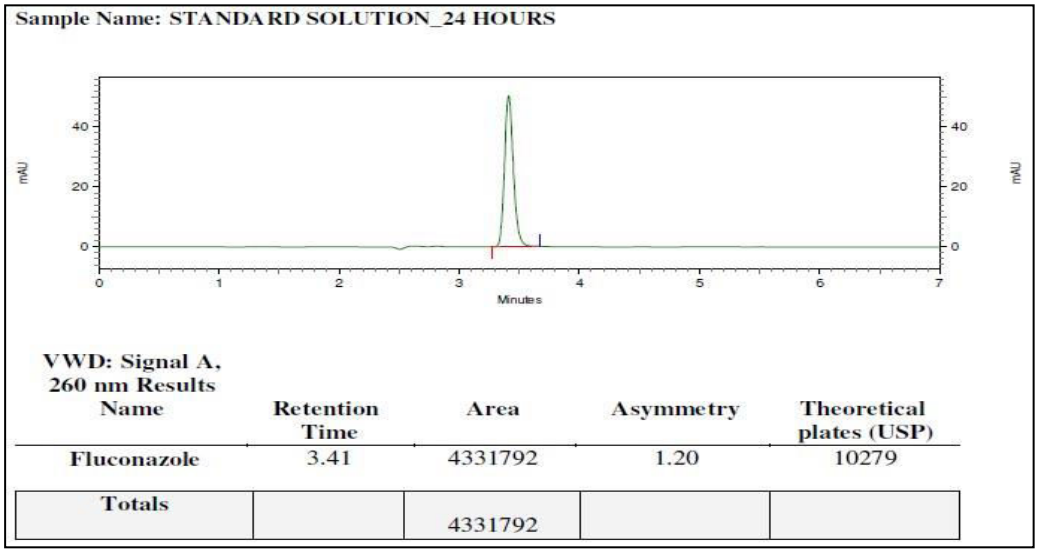


Fig. 6 Standard solution chromatogram 24 hours later

4.2.5. Specificity:

The capacity to clearly identify the analyte in the existence of components that may be anticipated to be present is called specificity. Peak purity is checked by injecting a blank, standard solution.

Table 12 Results of Specificity on HPLC

Description	**Observation**
Blank	No disruption at the fluconazole R.T. because of the blank
Placebo	No placebo-induced disruption at the fluconazole R.T.
Standard solution	maximal purity was 0.994
Test Solution	maximal purity was 0.992

4.2.6. Linearity and Range

Table 13 Linearity Data for Fluconazole on RP-HPLC

Level	Conc. (µg/mL)	Area	Mean	% RSD
50%	50.00	2170944	2170294	0.215
		2165336		
		2174602		
75%	75.00	3261821	3257647	0.131
		3257856		
		3253264		
100%	100.00	4354035	4346162	0.204
		4336591		
		4347859		
125%	125.00	5465635	5458045	0.137
		5457808		
		5450691		
150%	150.00	6560781	6560555	0.192
		6547859		
		6573025		

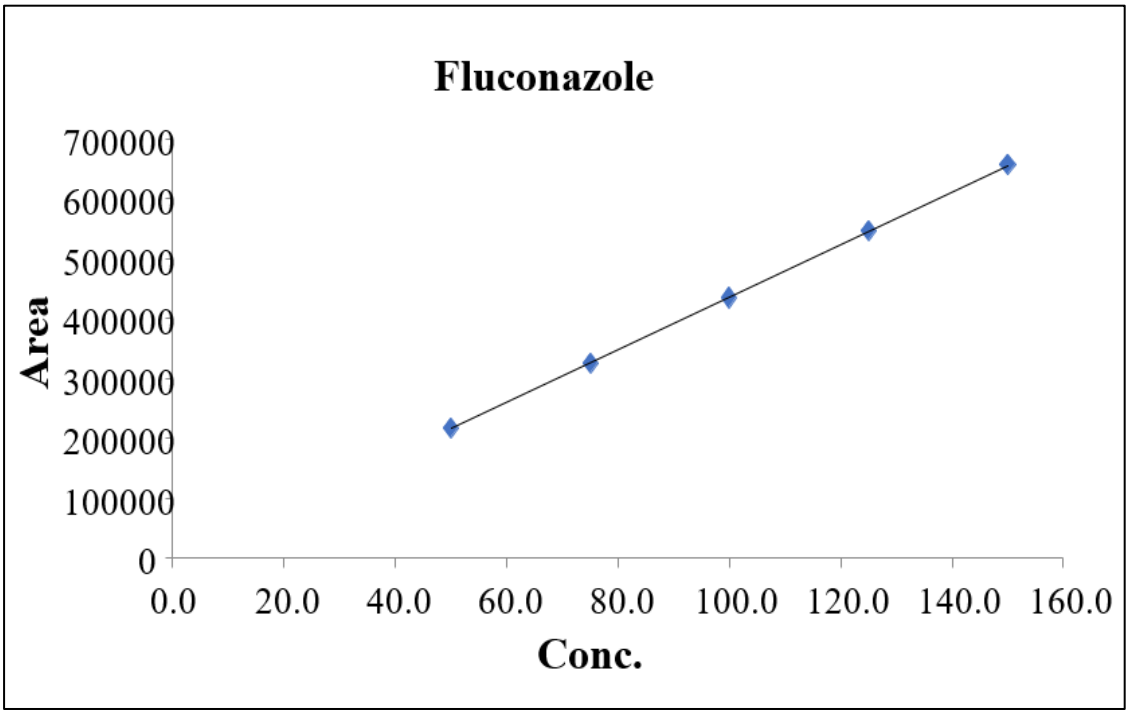


Fig. 7 Calibration curve of Fluconazole on RP-HPLC

4.2.7. ACCURACY (RECOVERY):

The degree to which test results produced using that technique resemble the real number. Analysed samples with known analyte concentrations added are subjected to the procedure to ascertain its accuracy.

Table 14 Accuracy results and statistical information of Fluconazole

Level (%)	Area	Recovered conc (µg/mL)	Added conc (µg/mL)	% Recovery	Mean Recovery	% RSD
50	2186825	50.22	50.60	99.25	99.54	0.8952
	2197582	50.47	50.20	100.54		
	2169140	49.81	50.40	98.83		
100	4385204	100.71	100.40	100.31	99.62	0.7593
	4302604	98.81	100.00	98.81		
	4351420	99.93	100.20	99.73		
150	6612145	151.85	150.20	101.10	100.48	0.7180
	6520475	149.74	150.20	99.69		
	6592103	151.39	150.40	100.66		

Total Recuperation: 99.88, % RSD during the entire recuperation: 0.824.

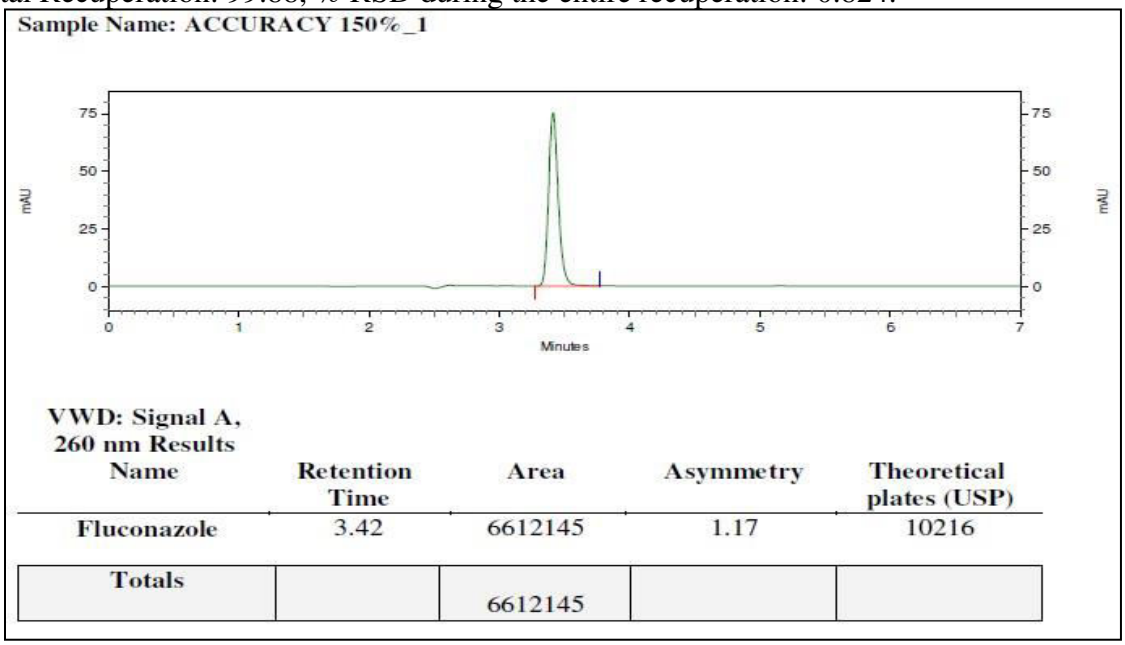


Fig. 8 Accuracy 150% on a typical chromatogram

Acceptance criteria:

Recoveries at each level and overall range from 98.0 to 102%. % RSD for each stage and total recovery NMT 2.0.

4.2.8. PRECISION

Table 15 Result of Intra- day and Inter- Day Precision for Fluconazole test sample assay

	Sample	**Test Sample (mg)**	**Area**	**% Assay**
Repeatability	Sample 1	111.4	4331251	100.42
	Sample 2	110.9	4236920	98.68
	Sample 3	111.8	4323691	99.89
	Sample 4	112.3	4310682	99.14
	Sample 5	111.6	4266978	98.75
	Sample 6	111.9	4203854	97.03
		Mean		**98.99**
		STD DEV		**1.172395**
		% RSD		**1.184**
Intermediate precision (Inter-Day)	Sample 1	112.1	4336920	99.92
	Sample 2	112.5	4205812	96.56
	Sample 3	111.6	4319604	99.97
	Sample 4	111.9	4239561	97.86
	Sample 5	112.2	4336109	99.82
	Sample 6	111.4	4316312	100.07
		Mean		**99.03**
		STD DEV		**1.473169**
		% RSD		**1.488**
Repeatability Plus Inter-day		**Mean**		**99.009**
		STD DEV		**1.26960**
		% RSD		**1.282**

- **Acceptance criteria:**

Same as mentioned in above precision part.

4.2.9. ROBUSTNESS:

Its ability to withstand little but intentional changes in procedure parameters is measured, and this gives an idea of how reliable it is in typical operating conditions.

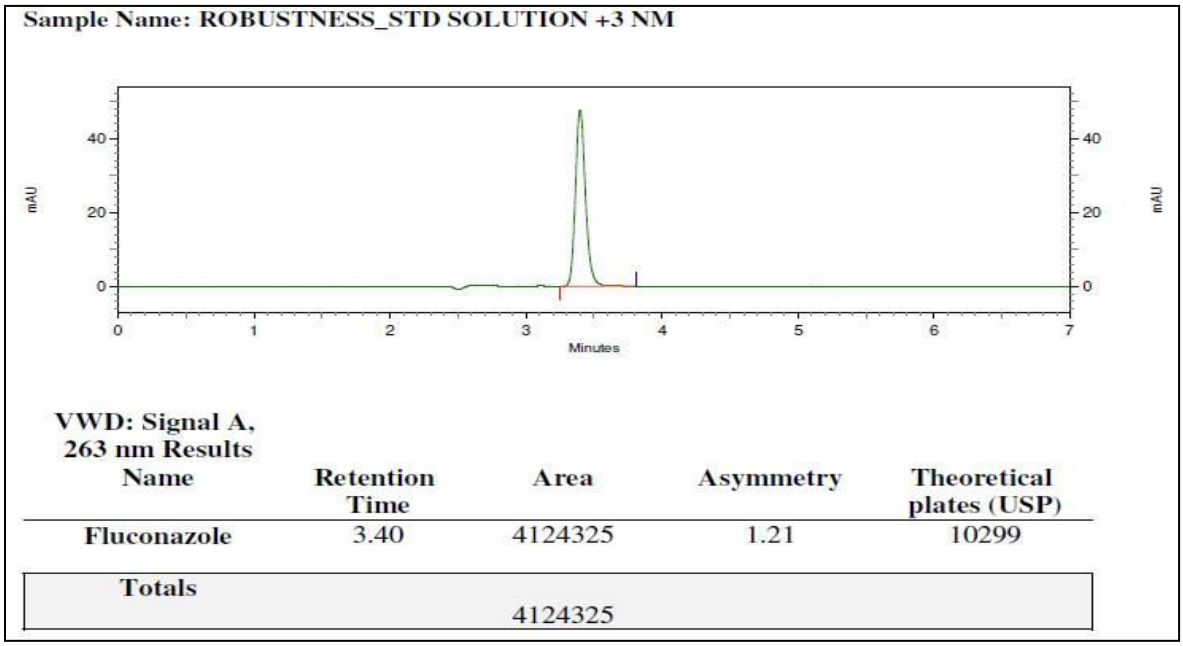
The following modifications were made under robustness: wavelength, flow rate, and column oven temperature adjustments.

Table 16 Result of Robustness study

Change in Parameter	R.T.	Standard area	Asymmetry	Theoretical plates
Wavelength by +3 NM (263 NM)	3.40	4124325	1.21	10299
Wavelength by -3 NM (257 NM)	3.40	4054180	1.21	10192
Ratio of flow by +10% (1.1 mL/min)	3.10	4002602	1.16	9322
Ratio of flow by -10% (0.9 mL/min)	3.78	4822464	1.19	11275
Temperature of columns oven by +2ºC (42 ºC)	3.40	4198512	1.22	10378
Temperature of columns oven by -2ºC (38 ºC)	3.41	4216920	1.19	10224

- **Chromatograms:**

A. Change in Wavelength by +3 NM:

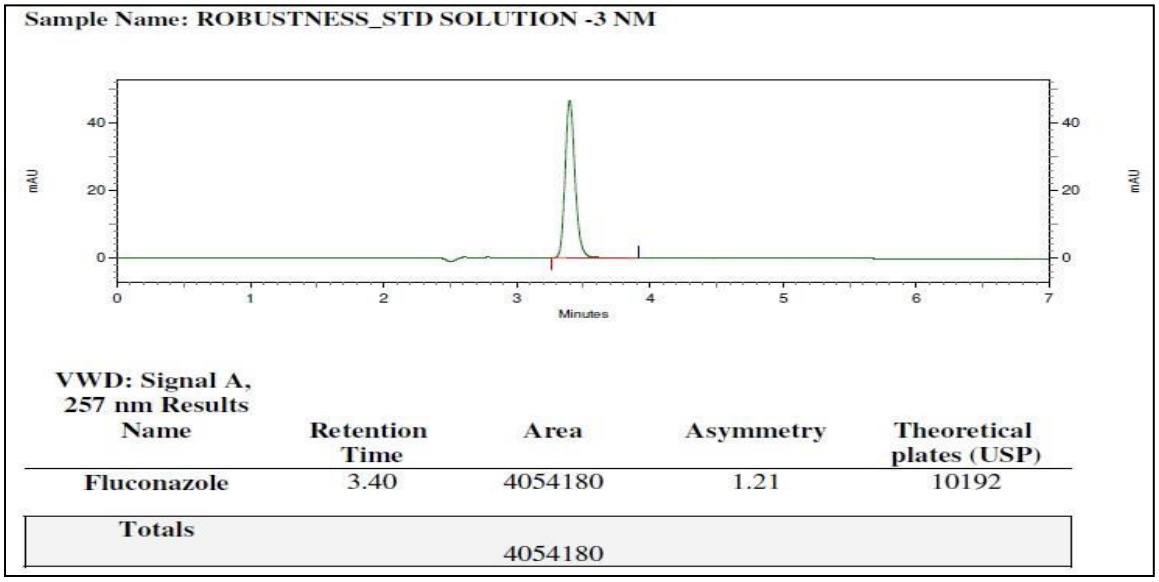


VWD: Signal A, 263 nm Results

Name	Retention Time	Area	Asymmetry	Theoretical plates (USP)
Fluconazole	3.40	4124325	1.21	10299
Totals		4124325		

Fig. 9a Typical chromatogram of Standard +3 NM

B. Wavelength shift of -3 NM:



VWD: Signal A, 257 nm Results

Name	Retention Time	Area	Asymmetry	Theoretical plates (USP)
Fluconazole	3.40	4054180	1.21	10192
Totals		4054180		

Fig. 9b Typical chromatogram of Standard -3 NM

5. CONCLUSION:

In accordance with ICH guidelines, a quick and precise isocratic RP-HPLC technique was developed and authorised to guarantee a Fluconazole measurement structure. There were five degrees of working centralization- 80, 90, 100, 110, and 120% - in the Fluconazole structure. The developed strategy's accuracy is demonstrated by the RSD rate, which is less than 2%. The exact capacity time and great awareness of this procedure are its advantages. The effect of the natural synthesis on the portable stage stream rate and framework similarity boundaries was considered to acquire the optimal chromatographic conditions. The outcomes demonstrate the effectiveness of the presented method in relation to linearity, accuracy, precision, robustness, LOD, and LOQ. As a result, pharmaceutical companies can employ this technique for the methodical screening of fluconazole medications. Thus, routine analysis of Fluconazole was effectively performed using this approach.

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CONFLICT OF INTEREST:

The writers say they have no competing interests.

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