

Analytical Method Development, Validation and Optimization of Fluconazole Drug Using RP- HPLC

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ABSTRACT

This study reported the development and validation of a simple, precise, and cost-effective Reverse Phase High-Performance Liquid Chromatography (RP-HPLC) method for the quantitative estimation of Fluconazole. Method development was systematically performed through nine optimization trials by varying the mobile phase composition, flow rate, and column type to achieve a sharp, symmetrical, and well-resolved peak with minimal tailing. The final optimized chromatographic conditions comprised a Kromasil C18 column (250 mm × 4.6 mm, 5 µm) with a mobile phase of Water: Acetonitrile (60:40 v/v) at a flow rate of 1.5 mL/min, which yielded a retention time of 2.231 minutes. The method exhibited excellent specificity, with no interfering peaks observed at the retention time of Fluconazole. Validation was performed in accordance with ICH guidelines, confirming the method's reliability for routine quality control applications. System suitability parameters, including tailing factor, theoretical plates, and %RSD, were found to be within acceptable limits. The method demonstrated excellent linearity across 20–150% of the target concentration ($r^2 = 0.999$), along with high precision and accuracy. The assay of the drug substance was determined to be 99.49%, which was within the acceptable range. Overall, the developed RP-HPLC method proved to be simple, rapid, accurate, precise, robust, sensitive, and specific, making it suitable for the routine estimation of Fluconazole in pharmaceutical formulations.

Keywords: Fluconazole; RP-HPLC; Optimization; Validation; ICH guidelines; Analytical method; System suitability; Precision; Accuracy; Linearity; Specificity

INTRODUCTION

Pharmaceutical analysis plays a crucial role in ensuring the quality, safety, and efficacy of bulk drug substances and their formulations. Among the various analytical techniques available, High-Performance Liquid Chromatography (HPLC) has emerged as one of the most reliable and versatile tools for both qualitative and quantitative analysis in the pharmaceutical industry.[1,2] The data generated through HPLC can be either numerical, representing the exact amount of a compound present in a sample, or qualitative, confirming the presence or absence of specific analytes.[3,4] Owing to its high sensitivity, accuracy, and reproducibility, HPLC is employed at every stage of drug development from the analysis of raw materials to in-process controls and final product testing.[5,6] The specific objective of each analysis depends on the nature of the sample and the stage of

pharmaceutical development. Therefore, a clear understanding of the fundamental principles of chromatography is essential to comprehend the operation and applications of HPLC in pharmaceutical analysis. In this research work, Fluconazole was selected as the model drug because it is a widely used triazole antifungal agent employed in the treatment of various systemic and superficial fungal infections. Despite its extensive clinical use, the available analytical methods for Fluconazole are often reported in combination with other drugs or involve complex mobile phase systems containing buffers or multiple solvents, which make them less suitable for routine analysis. Several analytical methods for the estimation of Fluconazole have been previously reported in the literature; however, most of these methods involve complex mobile phase compositions, buffer systems, or drug combinations, which limit their simplicity and routine applicability.

Relevant conflicts of interest/financial disclosures: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Therefore, the present study aimed to develop a simple, reliable, and eco-friendly RP-HPLC method for the quantitative estimation of Fluconazole as a single drug component. Multiple optimization trials were performed using different ratios of Water and Acetonitrile as the mobile phase to achieve a sharp, symmetrical peak with an acceptable retention time and minimal tailing. The use of a this mobile phase not only reduced the organic solvent consumption but also made the method more environmentally friendly, cost-effective, and suitable for routine quality control analysis. Fluconazole, α -(2,4-difluorophenyl)- α -(1H-1,2,4-triazol-1-ylmethyl)-1H-1,2,4-triazol-1-ethanol an antifungal medication, was discovered and developed by Pfizer. [7,8] Fluconazole is a first-generation triazole antifungal medication. It differs from earlier azole antifungals (such as ketoconazole) in that its structure contains a triazole ring instead of an imidazole ring. [9,10] The presence of a triazole and a difluoro phenyl produces potent antifungal activity but replacement of the usual imidazole group by triazole leads to improved selectivity.[11,12] Fluconazole is much less lipophilic than other azole antifungals and this leads to excellent penetration throughout the body, low protein-binding and water-solubility.[13,14] Fluconazole, was chosen for development on the basis of an optimal combination of antifungal efficacy, pharmacokinetic characteristics, aqueous solubility, and safety profile.[15]

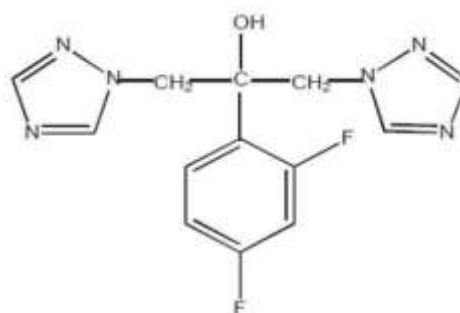


Figure 1: Structure Of fluconazole [16]

Fluconazole is a very selective inhibitor of fungal CYTOCHROME P450 dependent enzyme lanosterol 14- α -demethylase. This enzyme normally works to convert lanosterol to ergosterol, which is necessary for fungal cell wall synthesis. [17]

MATERIALS AND METHOD

Chemicals and reagents

A. API used in Research Work

Fluconazole Drug Trials (Sample and Standard) was provided by R&D Department of Axiom Analytical Services, Rau, Indore which was manufactured by Rusan Pharma Ltd., Pithampur, Indore.

B. Chemical used

Methanol (HPLC grade) and Acetonitrile (HPLC grade) from Advent Chembio Pvt. Ltd. and Water (HPLC grade) from Rankem Laboratory Chemicals.

C. Major Instrument Used

Instrument Name	Model No.	Company Name
HPLC	Shimadzu i-series LC-2050 C	Shimadzu Corporation, Japan
HPLC detector (UV)	Ultraviolet-Visible (UV-Vis) Detector	Shimadzu Corporation, Japan
System controller HPLC	LabSolutions Workstation software	Shimadzu Corporation, Japan
Analytical Balance	ATX224	Shimadzu Corporation, Japan
Ultrasonicator	2.5L	LeelaSonic, India

➤ HPLC method Development and Validation:

Mobile phase optimization: Initially, the mobile phase tried was Water: Acetonitrile and then one trial with Water and Methanol with various combinations of then varying proportions. Finally, the mobile phase was optimized to Water and Acetonitrile in proportion 60: 40 v/v respectively.

Wave length selection: UV spectrum of 10 μ g / ml Fluconazole in diluents (mobile phase composition) was recorded by scanning in the range of 200nm to 400nm. From the UV spectrum wavelength selected as 260nm.

Preparation of Mobile Phase: Accurately measured of 60 ml volume of water and 40ml volumes of

Acetonitrile. were mixed and degassed in an ultrasonic water bath for 10 minutes and then filtered through 0.45 μ filter.

Standard solution preparation: About 10 mg of Fluconazole drug was weighed using weighing balance. The 10mg drug was transferred into clean 100 ml volumetric flask dissolved the drug with H₂O (60) & ACN (40) (diluent) and after dissolving, made up the volume (100ml) of the volumetric flask up to the mark, sonicate gently to dissolve completely. Labelled flask as Fluconazole standard solution of 100ppm.

Sample solution preparation: About 10 mg of pure Fluconazole drug was weighed using analytical balance. The 10mg drug was transferred into clean 100 ml volumetric flask dissolved the drug with H₂O (60) & ACN (40) (diluent) and after dissolving, made up the volume (100ml) of the volumetric flask up to the mark sonicate gently to dissolve completely. Labelled flask as Fluconazole sample solution of 100ppm.

Method development: The method development for Fluconazole by Reverse Phase High-Performance Liquid Chromatography (RP-HPLC) was carried out

with the aim of achieving an efficient, precise, and eco-friendly analytical procedure that provided a sharp, symmetrical peak with reduced tailing factor, shorter retention time, and satisfactory theoretical plates. Various chromatographic conditions were systematically optimized by varying the mobile phase composition, flow rate, wavelength, and column type. Several mobile phase combinations of Water and Acetonitrile (ACN) were evaluated in different ratios ranging from 70:30 to 60:40 (v/v) to obtain optimum separation and peak symmetry. Initial trials did not meet the desired chromatographic performance as the peaks exhibited higher tailing and longer retention times. Subsequently, the mobile phase composition was optimized to Water: Acetonitrile (60:40 v/v), which provided a sharp, symmetrical, and well-resolved peak under the acceptance criteria. The flow rate was adjusted at 1.5ml/min, which provides good peak shape, retention time, tailing factor and run time. Wavelength was chosen at 260nm at this wavelength Fluconazole shows strong absorbance. Injection volume was adjusted at 10 μ l and run time was 10 minutes. Under those conditions the Fluconazole eluted at 2.231 minutes.

❖ Chromatographic Condition for Method Validation:

HPLC System	HPLC Shimadzu i-series LC-2050 C With UV Detector
Column	Kromasil 100-5C18 (250x4.6mm) 5 micron
Mobile Phase	Water: Acetonitrile (60:40)
Flow rate	1.5ml/min
Wavelength	UV-260 nm
Sample volume	10 μ l
Run time	10 min.
Column oven temperature	40°C
Retention time	2.231

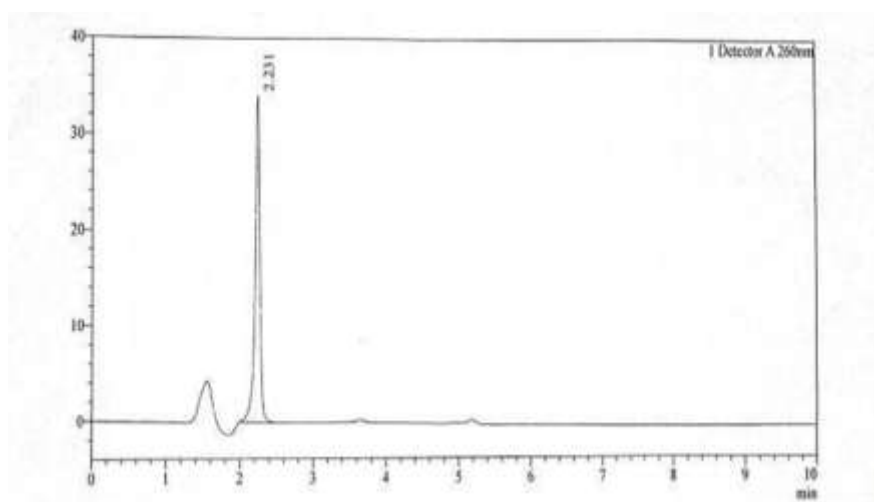


Figure No. 2: Optimized Chromatogram of Fluconazole

- **Method validation:** The method validation was conducted according with International Conference on Harmonization the guidelines. The methods were accuracy, precision, linearity, specificity, and system suitability all were verified. [18]
- **System suitability:** Standard solution of drug was prepared using water and acetonitrile as solvent (ppm). Blank sample and standard solution were injected and the run was performed. Parameters such as retention time, tailing factor, plate count, peak area was determined. The measured results were compared against the pre-defined limits. By inject blank, standard solution and parameters such as retention time, tailing factor, plate count, peak area was found in acceptance criteria, it concluded that system was suitable for further analysis. [19]
 - Tailing factor < 2.0
 - Theoretical plates > 2000
 - Should be consistent ($\pm 2\%$)
- **Specificity:** In order to verify specificity by inject blank, sample solution and standard solution(100ppm) to check whether there is no interference of blank chromatogram in retention time of Fluconazole and that confirmed that the method is specified for fluconazole drug. [20]
- **Linearity:** Along with the blank and standard, six different concentrations of the standard solution

(120–900 $\mu\text{g/mL}$) of the target concentration were injected to ensure linearity. After the calibration curve was established between concentrations and peak regions, regression analysis was performed. A linear technique was demonstrated if the correlation coefficient fell within the permitted range.

Correlation coefficient (R^2) ≥ 0.999

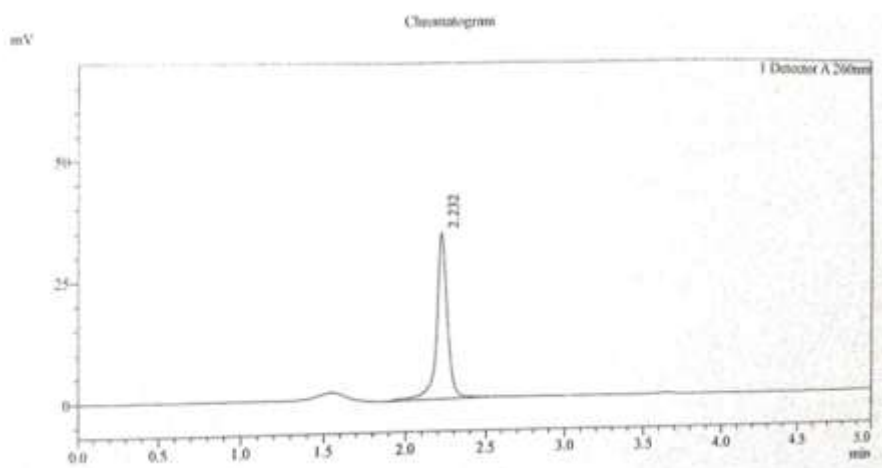
- **Precision:** Method were assessed by preparing and injecting six individual sample solution on identical analyte condition and record peak area or region of all injection and calculate the assay, %RSD if all are within the acceptance criteria than the method was precised. [21]
- **Accuracy:** By injecting blank, standard and sample solution in triplicate time at different concentration of 50%,150% and 100%. Calculate peak area that how much amount of drug or API were recovered at each level. Determined mean recovery % and % RSD for each level and if mean recovery % and % RSD were within acceptance limit than the method was accurate. [22]

RESULTS AND DISCUSSION:

- **System suitability:** Inject blank, standard solution and observed the chromatograph.

Table No 1: System suitability and precision

Standard solution	Retention Time	USP Tailing Factor (< 2)	USP Plate Count (> 2000)	Peak Area of Fluconazole
Standard solution-1	2.230	0.84	4875	165151
Standard solution-2	2.234	0.87	4987	165060
Standard solution-3	2.238	1.01	5855	164707
Standard solution-4	2.237	0.62	4683	165080
Standard solution-5	2.232	0.81	4910	165017
Mean	2.2342	0.83	5062	165003
SD (±)	0.0033			172.4050
% RSD (< 2%)	0.1498			0.1045

**Figure No.3: System suitability chromatogram**

- **Specificity:** Inject blank, standard, sample solution of 100ppm and observed the chromatograph.

Table No. 2: Specificity of Fluconazole

Sr. No.	Injection	Observation (Interference)	Result	Assay (%)
1	Blank (Diluent)	No peak found at analyte RT 2.234min	No interference	99.18%
2	Standard Solution	RT at 2.234min	Passed	
3	Sample Solution	RT at 2.235min	Passed	

- **Linearity:** Inject blank, standard of 6 replicates of different concentration and observed the data.

Table No. 3: Specificity of Fluconazole

(%) Level	Standard Concentration (µg/ml)	Peak Area
20 %	20	33021
50 %	50	825550
80 %	80	132078
100 %	100	165102
120 %	10	198125
150 %	150	247654
Correlation Coefficient (r ²)	0.9999	

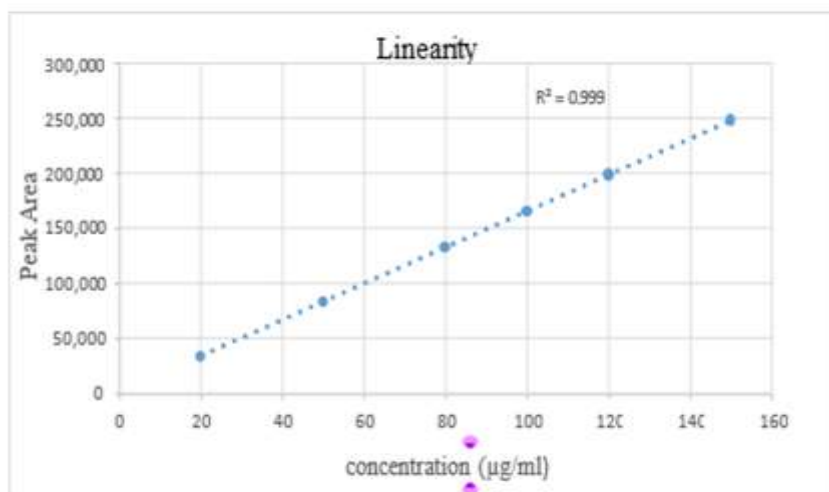


Fig No. 4: Linearity of Fluconazole Graph

- **Precision:** Inject blank, standard, six samples

Table No. 3: Precision of Fluconazole

Sample	Sample weight	Area of Fluconazole	Assay (%)
Precision sample 1	10.2	165252	100.8
Precision sample 2	10.5	165102	99.7
Precision sample 3	10.7	165240	99.2
Precision sample 4	10.4	165100	100.3
Precision sample 5	10.8	165090	99.0
Precision sample 6	10.4	165215	100.5
Mean			99.92
SD (\pm)			0.73
%RSD (<2%)			0.73

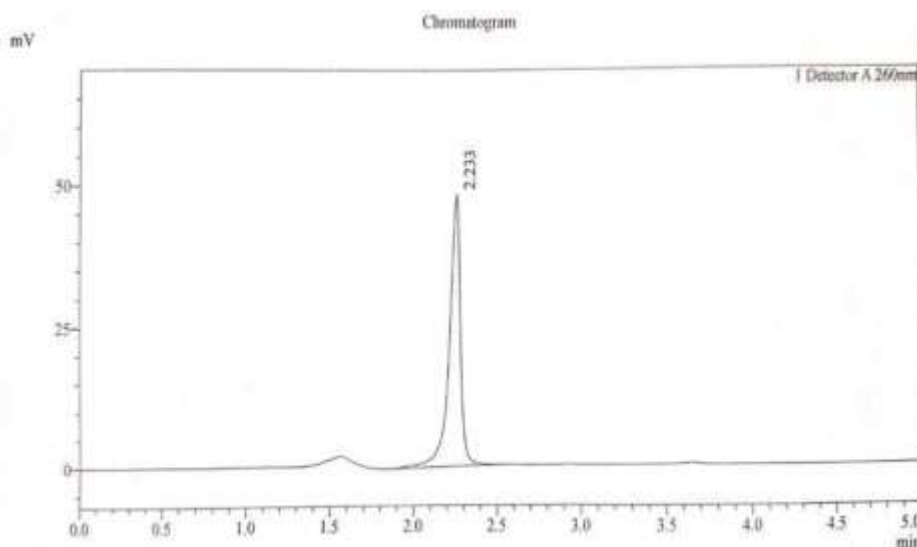
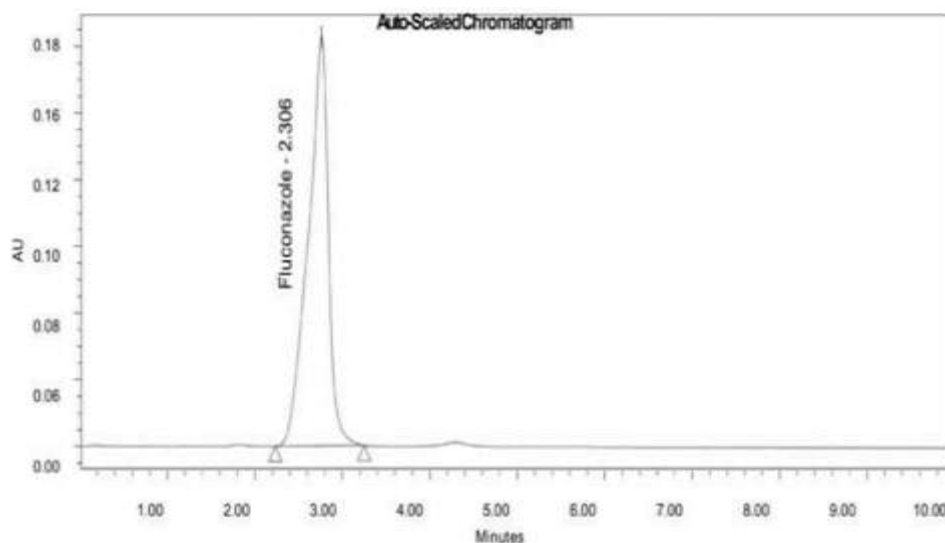
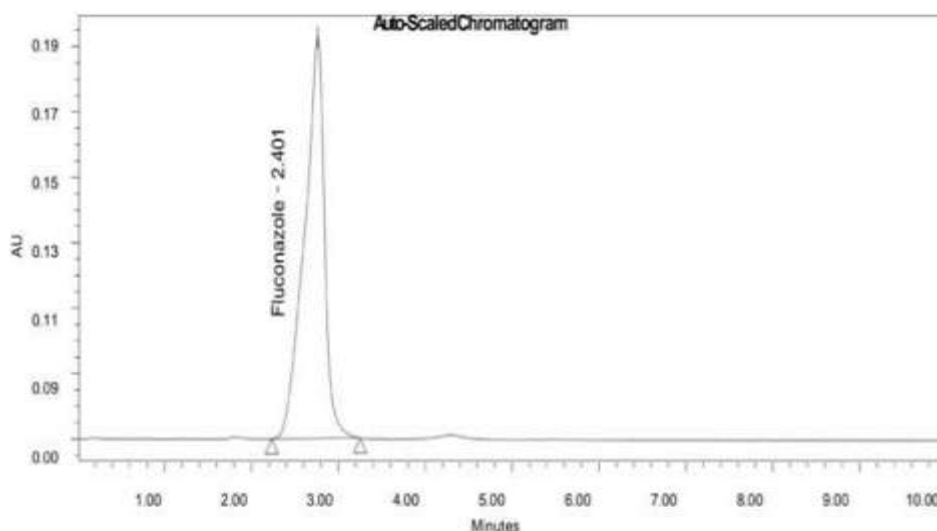


Fig. No. 5: Precision chromatogram

- **Accuracy:** Inject blank, standard, 3 replicate of each 50% sample, 100% sample, 150% sample and observed the chromatograph.

Table No. 4 Accuracy of Fluconazole

Spiked amount (%)	Amount added (mg)	Amount found (mg)	Recovery (%)	Mean recovery (%)	% RSD
50%	5.0	4.9	98.0	99.33	1.16
	5.0	5.0	100.0		
	5.0	5.0	100.0		
100%	10	9.8	98.0	99.17	1.05
	10	9.95	99.5		
	10	10.0	100.0		
150%	15	14.78	98.5	99.17	0.77
	15	14.85	99.0		
	15	15.0	100.0		

**Figure No. 6: Standard Chromatogram 50%****Figure No.7: Standard Chromatogram 150%****CONCLUSION: -**

The developed RP-HPLC method provides a simple, rapid, precise, and cost-effective approach for the quantitative estimation of Fluconazole. Optimization through systematic trials ensured excellent peak

symmetry, resolution, and reproducibility. Validation in accordance with ICH guidelines confirmed that the method meets all analytical performance parameters. With an assay value of 99.49% the method demonstrates high reliability for routine quality control analysis of Fluconazole in bulk and

pharmaceutical formulations. Overall, this validated method can be effectively employed for regular laboratory use due to its simplicity and strong analytical performance its simplicity, reliability, and eco-friendly approach make it applicable for routine quality control analysis in pharmaceutical industries.

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