RESEARCH ARTICLE
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Development and Validation of a Rapid Reverse-Phase High-Performance Liquid Chromatography Method for Quantification of Fluconazole in Pharmaceutical Preparations

ABSTRACT

Background: Fluconazole is a triazole antifungal agent widely employed in the treatment of systemic and superficial fungal infections. Accurate, precise, and reliable quantification of the active pharmaceutical ingredient in dosage forms is of critical importance for both quality control and therapeutic efficacy.

Methods: In this study, a reverse-phase high-performance liquid chromatography (RP-HPLC) method was developed and validated for the quantitative determination of fluconazole in pharmaceutical preparations. Chromatographic separation was performed using a Zorbax C18 column (100 mm × 4.6 mm, 3.5 µm). The mobile phase consisted of a methanol–water mixture (70 : 30, v/v) in an isocratic system at a flow rate of 1.0 mL/min. The injection volume was set at 5 µL, the detection wavelength at 210 nm, and the column temperature at 30°C. The system suitability parameters of the method were found to be within the acceptable limits.

Results: The retention time for fluconazole was determined as 1.13 ± 0.03 minutes. The method demonstrated linearity within the concentration range of 3.0×10^{-7} M to 9.0×10^{-6} M, with a correlation coefficient (r^2) of 0.9999. The limit of detection (LOD) and limit of quantification (LOQ) were calculated as 6.0×10^{-8} M and 1.8×10^{-7} M, respectively. Accuracy and precision values were within acceptable ranges for both intra-day and inter-day analyses. Recovery values ranged from 96.2% to 101.3%, while the relative standard deviation values remained below 2%.

Conclusion: The developed RP-HPLC method is a simple, accurate, precise, and robust approach that can be employed for the routine quality control of fluconazole in pharmaceutical preparations. Moreover, the high sensitivity and reproducibility of the method indicate its potential applicability for the determination of fluconazole in biological samples in future studies.

Keywords: Fluconazole, method validation, pharmaceutical analysis, quality control, RP-HPLC

What is already known on this topic?

- Fluconazole is a commonly used triazole antifungal drug, and its accurate quantification is essential for ensuring product quality and therapeutic efficacy.
- Existing HPLC methods for its analysis often require complex conditions or longer run times, which can limit their practicality for routine analysis.
- Therefore, there is a need for a simpler, faster, and more reliable analytical approach for fluconazole determination in pharmaceutical preparations.

What this study adds on this topic?

- This study presents a simple, rapid, and highly precise RP-HPLC method for fluconazole quantification using a methanol–water mobile phase with a short retention time.
- The validated method demonstrated excellent linearity, accuracy, and reproducibility.
- The method makes it suitable for routine quality control and potential application to biological samples.

INTRODUCTION

Fluconazole, a member of the triazole class of antifungal agents, is widely used in the treatment of systemic and superficial fungal infections. It is particularly notable for its high efficacy against infections caused by *Candida* and *Cryptococcus* species and is also administered as prophylaxis in immunocompromised patients.[1] Fluconazole exerts its pharmacodynamic effect by inhibiting ergosterol biosynthesis in fungal cells, leading to disruption of membrane integrity and subsequent cell death. Because ergosterol is an essential component of the fungal membrane, its inhibition impairs critical cellular functions and produces a fungicidal effect.[2]

Fluconazole possesses high oral bioavailability (approximately 90%) and exhibits low plasma protein binding, facilitating widespread tissue distribution, including penetration into the brain, lungs, kidneys, liver, and urine. Its elimination occurs via both renal and hepatic pathways, with a half-life of approximately 30 hours, enabling maintenance of therapeutic concentrations through once-daily dosing. These pharmacokinetic properties underpin its safe and

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effective application in the treatment of both acute and chronic fungal infections.[3]

The chemical structure of fluconazole is 2-(2,4-difluorophenyl)-1,3-bis(1H-1,2,4-triazol-1-yl) propan-2-ol (Figure 1). The polar nature of the molecule and its stability at neutral pH enhance its solubility in both aqueous and organic solvents, allowing effective dosing across a wide pH range in pharmaceutical formulations. The molecule predominantly exists in a non-ionized form and therefore exhibits favorable absorption and distribution in biological systems. Moreover, fluconazole is thermally stable and relatively resistant to light, supporting its suitability for long-term storage and for use in various dosage forms.[4]

Accurate and precise quantitative determination of active compounds in pharmaceutical and biological samples is of critical importance for both quality control and pharmacokinetic-pharmacodynamic studies. In this context, chromatographic techniques, particularly high-performance liquid chromatography (HPLC), are among the most widely preferred methods in drug analysis. By providing high specificity, sensitivity, and precision, HPLC offers more reliable quantitative analysis compared to spectral or microbiological approaches. Furthermore, the ability to employ HPLC with different column types, diverse mobile phase compositions, and various detectors makes it a flexible and comprehensive tool suitable for the analysis of both pharmaceutical formulations and biological matrices.[5]

Several analytical methods have previously been reported for the determination of fluconazole, including spectrophotometric techniques[6] and HPLC methods with relatively long run times or complex gradient elution programs.[7-9] While these approaches are useful, they often suffer from limitations such as lower sensitivity, insufficient specificity, or lengthy analysis times, making them less suitable for routine quality control.

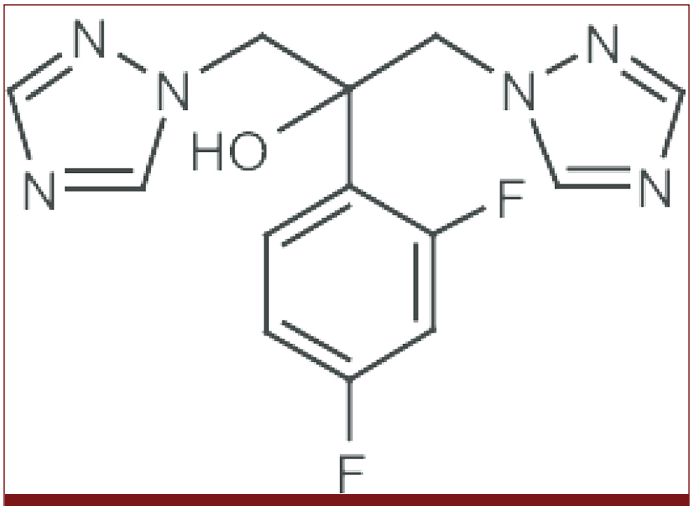

Figure 1. Chemical structure of fluconazole.

The validation phase is of paramount importance in the method development process. Validation provides evidence of a method's reliability through parameters such as accuracy, precision, linearity, limit of detection (LOD), limit of quantification (LOQ), robustness, and specificity.[10,11] The guidelines of the International Council for Harmonisation (ICH)[12] serve as a fundamental reference for standardizing these parameters and ensuring that methods are validated according to internationally accepted criteria. System suitability tests—such as retention time reproducibility, theoretical plates, and tailing factor—are also critical indicators demonstrating that the method operates reliably and reproducibly.[13,14]

In this study, a simple, sensitive, robust, and reproducible reverse-phase HPLC (RP-HPLC) method was developed and validated for the quantitative determination of fluconazole in pharmaceutical preparations.

Compared to previously reported methods, the proposed approach shortens the analysis time while maintaining high sensitivity and specificity, thereby offering a reliable platform for routine quality control. Furthermore, the developed method is considered applicable for the determination of fluconazole in biological samples in future studies, potentially contributing to both clinical research and therapeutic drug monitoring.

MATERIAL AND METHODS

Reagents and Solutions

Fluconazole standard and all other analytical-grade chemicals were obtained from Sigma-Aldrich. Methanol was used as the solvent. Standard and working solutions prepared during the study were stored in amber glass vials at −20°C.

Instrumentation and Chromatographic Conditions

Analyses were performed using an Agilent 1260 Infinity Series HPLC-DAD system (Agilent Technologies, USA). Separation was achieved on a Zorbax C18 column (100 mm × 4.6 mm, 3.5 µm). The mobile phase consisted of a methanol–water mixture (70 : 30, v/v) and was applied in an isocratic mode at a flow rate of 1.0 mL/min. The injection volume was 5 µL, the column temperature was maintained at 30°C, and the detector wavelength was set at 210 nm. Prior to each run, the column was equilibrated for 20 minutes.

Preparation of Standard and Working Solutions

A fluconazole stock solution was prepared at a concentration of 1 mg/mL in methanol. All diluted solutions were stored in amber glass vials at −20°C. Prior to analysis, an appropriate volume of the solution was taken and prepared for injection.

Preparation of Pharmaceutical Preparations

The powder from fluconazole-containing capsules was collected and dissolved in an appropriate solvent to obtain

a homogeneous mixture. The mixture was filtered through a 0.45 μm PTFE membrane filter to render it suitable for HPLC analysis. If necessary, the samples were appropriately diluted prior to injection.

Method Validation

The method was evaluated in accordance with the ICH Q2(R2) guidelines[15] with respect to linearity, accuracy, precision, sensitivity, specificity, robustness, and stability.

Linearity: Calibration curves were constructed using at least 6 different standard concentrations. Each concentration was analyzed in triplicate, and the peak areas were plotted against concentrations to determine the regression equations.

Accuracy and Precision: Accuracy and precision were assessed through intra-day and inter-day analyses with 6 replicates. Accuracy was determined at different selected concentration levels, while precision was calculated at a single concentration.

Sensitivity: The LOD and LOQ were determined using the signal-to-noise (S/N) ratio (LOD = 3 S/N, LOQ = 10 S/N).

Specificity, Robustness, and Stability: Specificity was evaluated using control samples in the tablet matrix. Robustness was tested by making minor deliberate changes in the method across 6 analyses. Stability was examined by analyzing the prepared solutions under short-term (+4°C, 24 hours) and long-term (−18°C, 1 month) storage conditions.

RESULTS

Method Development and Optimization

An HPLC method was developed and optimized for the quantitative determination of fluconazole in pharmaceutical preparations. During the optimization of chromatographic conditions, parameters such as wavelength, organic solvent ratio, flow rate, and column temperature were systematically evaluated.

Wavelength Optimization: Analyses were performed at different wavelengths to determine the optimal detection wavelength for fluconazole, and 210 nm was found to be most suitable. At this wavelength, a high peak area and favorable signal-to-noise ratio were achieved.

Mobile Phase Optimization: Methanol–water mixtures in different ratios (60 : 40, 70 : 30, 80 : 20 v/v) were tested to evaluate their effects on peak morphology and retention time. The 70 : 30 (methanol : water) ratio was selected as optimal, providing short retention time and symmetrical peak shapes.

Flow Rate Optimization: Analyses were conducted at flow rates of 0.8, 1.0, and 1.2 mL/min, and retention times and peak shapes were assessed. Considering total analysis time and peak quality, 1.0 mL/min was determined to be optimal.

Temperature Optimization: The column temperature was tested at 25, 30, and 35°C. A temperature of 30°C provided the best peak symmetry and reproducible retention time and was therefore selected.

Based on these evaluations, the optimal chromatographic conditions were determined as follows: detection wavelength 210 nm, mobile phase methanol:water (70 : 30, v/v), flow rate 1.0 mL/min, column temperature 30°C, and injection volume 5 μL. The retention time was 1.13 ± 0.03 minutes. The chromatogram under optimum conditions is shown in Figure 2. System suitability parameters are presented in Table 1, with all criteria falling within acceptable limits.

Method Validation

The method was developed according to ICH Q2(R2) guidelines. Validation parameters were evaluated using

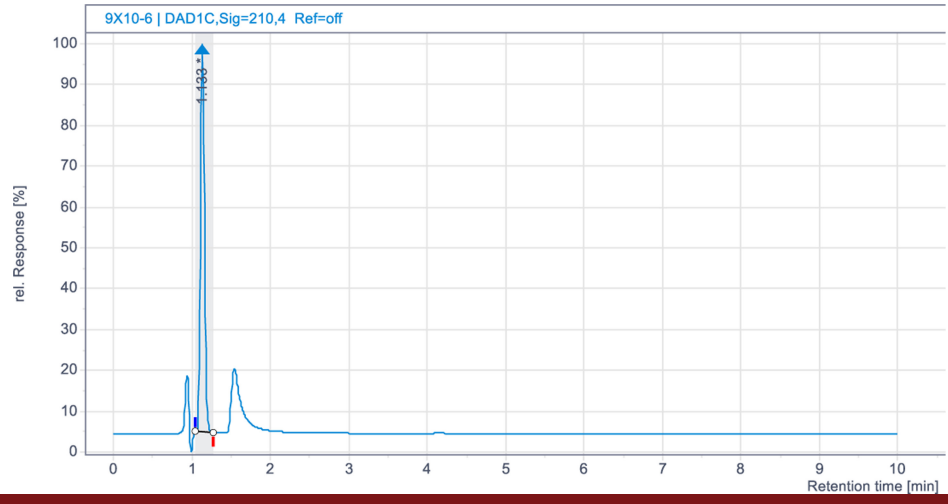

Figure 2. Chromatogram of fluconazole under optimal conditions.

Table 1. System Suitability Data for Flucanazole

Parameters/Methods	Flucanazole
t_R (minutes)	1.13 ± 0.03
Asymmetric factor (*T*)	1.05
Theoretical plate number (*N*)	412189

pharmaceutical preparations, and results were statistically analyzed.

Linearity: The linear range of the method was first determined by evaluating the response of fluconazole at various concentrations (3.0×10^{-8} M-1.8×10^{-5} M). A linear relationship between concentration and peak area was observed within the range of 3.0×10^{-7}-9.0×10^{-6} M, and a calibration curve was constructed using solutions within this established linear range. The calibration curve exhibited a highly correlated regression line ($r = 0.9999$; $y = 2.6 \times 10^{7}x - 0.044$), and the corresponding linearity graph is shown in Figure 3. The correlation coefficient (r^2) confirmed excellent linearity for the selected range (Table 2).

Accuracy: Intra-day and inter-day analyses were performed using preparations at different concentration levels (6.0×10^{-7} M, 3.0×10^{-6} M, 6.0×10^{-6} M). Recovery values ranged from 96.2% to 101.3%, confirming the method's accuracy (Table 3).

Precision: For precision studies, a fluconazole solution at 3.0×10^{-6} M was used. Intra-day and inter-day relative standard deviation (RSD%) values were below 2%, verifying the reproducibility of the method (Table 4).

Specificity: Blank analyses conducted on the tablet matrix showed no interference at the fluconazole peak, confirming method specificity.

Sensitivity: LOD and LOQ were determined as 6.0×10^{-8} M and 1.8×10^{-7} M, respectively.

Stability: Prepared solutions were stored at +4°C for 24 hours and −18°C for 1 month. Relative standard deviation values remained below 2%, indicating the method's stability.

Robustness: The method was tested under minor deliberate variations (flow rate ±0.1 mL/min, temperature ±2°C, mobile phase ratio ±2%), with RSD% remaining below 2%, confirming that the method is robust and reliable.

Application of the Method

The determination of fluconazole in pharmaceutical preparations was carried out using Flucan® capsules containing 150 mg of fluconazole. Solutions prepared from the capsules within the calibration range were analyzed 6 times under the optimized conditions. The chromatogram of the analysis is presented in Figure 4.

In the analysis of pharmaceutical preparations, chromatograms demonstrating the peak characteristics of the standard compounds were obtained. Statistical evaluations related to the quantitative determination of fluconazole in pharmaceutical preparations are presented in Table 5. The percent recovery values at different concentration levels of the drug were found to be approximately 100%. These results demonstrate that the developed method can be effectively applied for the quantitative determination of fluconazole in pharmaceutical preparations.

DISCUSSION

The developed RP-HPLC method has been validated as a reliable, sensitive, and reproducible approach for the quantitative determination of fluconazole in pharmaceutical

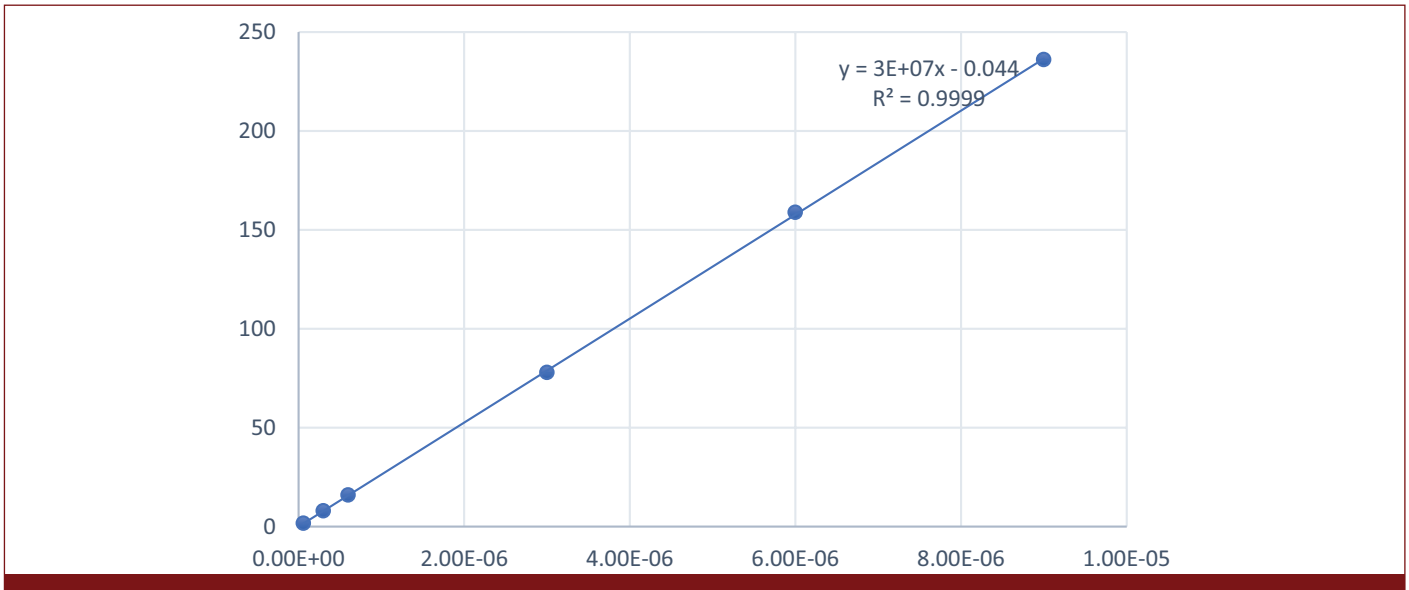

Figure 3. The graph of linearity.

Table 2. Linearity, Calibration, and Sensitivity Data for Flucanazole

Linearity	
Linearity range	3.0×10^{-7}-9.0×10^{-6} M
Calibration	
Slope	26283180
Intercept	−0.04
Correlation coefficient (*r*)	0.9999
Sensitivity	
LOD	6.0×10^{-8} M
LOQ	1.8×10^{-7} M

Table 3. Accuracy Data for Flucanazole

	Added (M)	Recovery (%)
Day I (n = 6)	6.0×10^{-7} M	96.87
	3.0×10^{-6} M	101.30
	6.0×10^{-6} M	99.75
Day II (n = 6)	6.0×10^{-7} M	97.56
	3.0×10^{-6} M	100.99
	6.0×10^{-6} M	99.27
Day III (n = 6)	6.0×10^{-7} M	96.20
	3.0×10^{-6} M	101.02
	6.0×10^{-6} M	99.72
Interdays (n = 18)	6.0×10^{-7} M	96.88
	3.0×10^{-6} M	101.10
	6.0×10^{-6} M	99.58

preparations. During wavelength optimization, a high peak area and favorable signal-to-noise ratio were achieved at 210 nm. Optimization of the mobile phase and flow rate provided short retention times and symmetrical peak shapes, establishing ideal conditions for routine analyses.

Validation results demonstrate that the method complies with ICH Q2(R2) guidelines. The linear range, accuracy, and precision values confirm the method's reliability for the quantitative analysis of pharmaceutical preparations. The LOD and LOQ values indicate the high sensitivity of the method, while stability and robustness tests verify its resistance to minor variations and storage conditions.

When compared with previously reported analytical methods for fluconazole determination, the advantages of the proposed method become evident. Spectrophotometric approaches, while simple, often lack the sensitivity and specificity required for reliable quantification in complex matrices.[16] Microbiological assays provide information about biological activity but are time-consuming and less precise for routine quality control.[17] Reported HPLC

Table 4. Precision Data for Flucanazole

	Intraday			
	Day I (n = 6)	**Day II (n = 6)**	**Day III (n = 6)**	Interdays (n = 18)
Mean	3.292	3.289	3.288	3.290
SD	0.087	0.089	0.089	0.088
RSD%	1.18	1.21	1.23	1.21
CI (95%)	±0.036	±0.038	±0.038	±0.037

RSD%, relative standard deviation; SD, standard deviation.

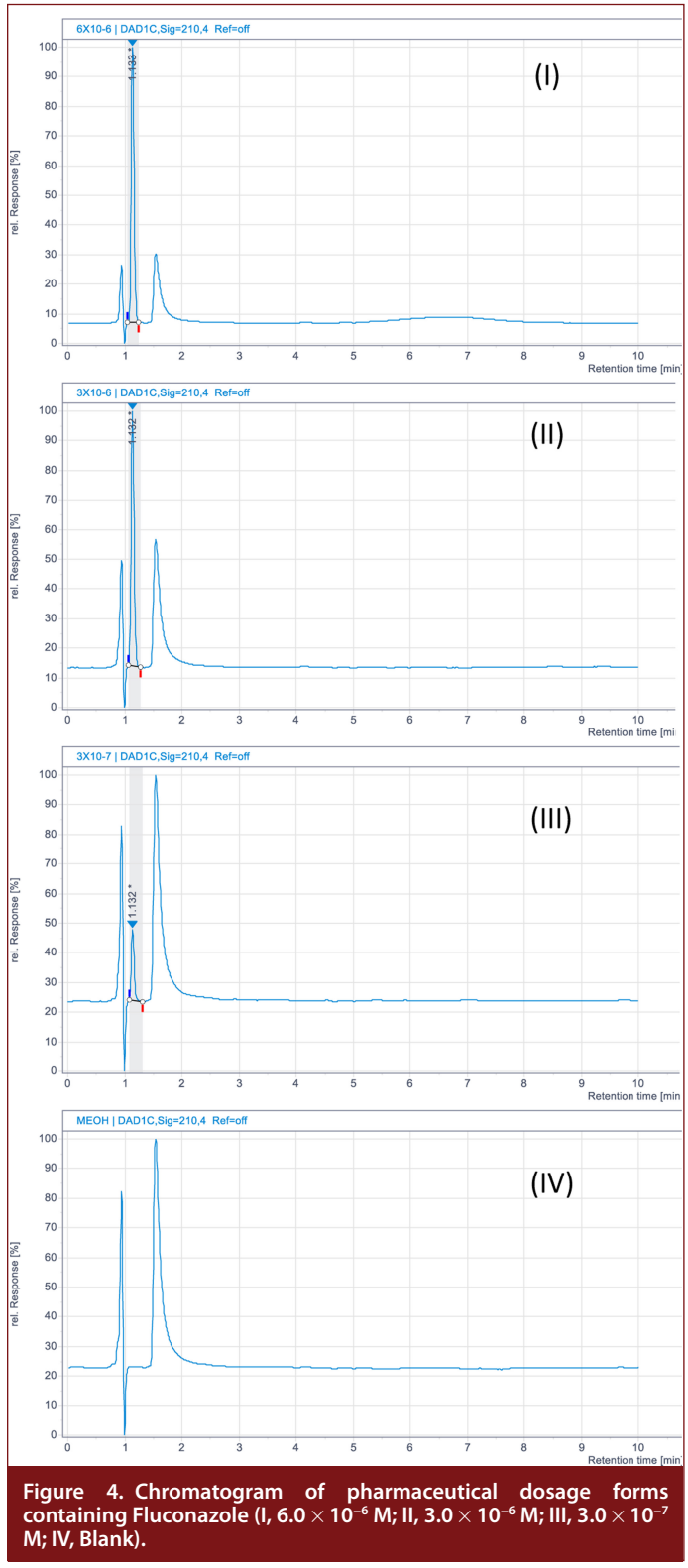

Figure 4. Chromatogram of pharmaceutical dosage forms containing Fluconazole (I, 6.0×10^{-6} M; II, 3.0×10^{-6} M; III, 3.0×10^{-7} M; IV, Blank).

Table 5. Statistical Evaluation of the Assay Data of Pharmaceutical Dosage Forms Containing Fluconazole

Flucanazole (150 mg)	Mean (n = 6)	6.0 × 10^{-6} M	3.0 × 10^{-6} M	3.0 × 10^{-7} M
	SD	0.02	0.03	0.03
	RSD%	0.02	0.03	0.03
	Recovery%	99.82	98.89	100.87

RSD%, relative standard deviation; SD, standard deviation.

methods have employed gradient elution systems or longer run times exceeding 15-20 minutes, which, although effective, may not be efficient for routine pharmaceutical analysis.[18] In contrast, the present method employs a simple isocratic elution system with a total run time under 2 minutes, offering faster analysis without compromising sensitivity or reproducibility. The rapid analysis not only facilitates high-throughput routine pharmaceutical testing but also aligns with green chemistry principles by reducing solvent consumption and energy usage, providing a more sustainable alternative compared with longer conventional HPLC methods reported in the literature.

These findings indicate that the method is suitable for routine quality control analyses. Moreover, due to its adequate sensitivity and reproducibility, the method can be adapted for the determination of fluconazole in biological samples in future studies. This capability makes the method a valuable tool for both clinical pharmacokinetic studies and therapeutic drug monitoring applications.

CONCLUSION

In this study, a sensitive, reliable, and reproducible RP-HPLC method was developed and optimized for the quantitative determination of fluconazole in pharmaceutical preparations. The method was optimized through systematic evaluation of parameters such as wavelength, mobile phase composition, flow rate, and column temperature. Validation results demonstrate that the method complies with ICH Q2(R2) guidelines, providing high accuracy and precision, along with short retention times and well-shaped peaks.

The LOD and LOQ values of the developed method indicate that it can reliably detect fluconazole even at low concentrations in pharmaceutical preparations. Furthermore, the method is robust and stable, showing resistance to minor variations and storage conditions.

This method is suitable for routine quality control analyses and can also be adapted for the determination of fluconazole in biological samples in future studies. Consequently, it represents a valuable tool for clinical pharmacokinetic studies and therapeutic drug monitoring applications.

In conclusion, the developed RP-HPLC method offers an effective and applicable approach for fluconazole analysis, providing both sensitivity and reliability.

Data Availability Statement: The data that support the findings of this study are available on request from the corresponding author.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept – A.G.D.P.; Design – A.G.D.P.; Resources – A.G.D.P.; Materials – A.G.D.P.; Data Collection and/or Processing – A.G.D.P.; Analysis and/or Interpretation – A.G.D.P.; Literature Search – A.G.D.P.; Writing – A.G.D.P.

Declaration of Interests: The authors have no conflicts of interest to declare.

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