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Development and Validation of an HPTLC Method for Simultaneous Estimation of Azithromycin, Fluconazole, and Tinidazole in Combined Dosage Form

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Abstract: A high-performance thin-layer chromatographic (HPTLC) method was developed and validated for the simultaneous estimation of azithromycin (AZM), fluconazole (FLZ), and tinidazole (TZ) in pharmaceutical formulations. Various mobile phase combinations were evaluated, and the optimized system—1,4-dioxane: methanol: toluene (2:3:5, v/v/v)—produced sharp, compact, and well-resolved spots with Rf values of 0.202, 0.382, and 0.522 for AZM, FLZ, and TZ, respectively. Densitometric detection was performed at 210 nm. The method exhibited excellent linearity ($r^2 \approx 0.999$) within the concentration ranges of 1,000–5,000 ng/band (AZM), 500–5,000 ng/band (FLZ), and 100–500 ng/band (TZ). Accuracy was confirmed by recovery rates between 98% and 102%, and precision was supported by %RSD values below 2% for intra-day, inter-day, and repeatability assessments. The method also demonstrated acceptable sensitivity with low limit of detection (LOD), and limit of quantification (LOQ).values, and system suitability parameters met all validation criteria. This validated HPTLC method is simple, accurate, precise, and robust, making it suitable for routine quality control of AZM, FLZ, and TZ in combined pharmaceutical dosage forms.

Keywords: HPTLC, Azithromycin, Fluconazole, Tinidazole, AF-Kit, Method Validation, ICH Guidelines

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Introduction

Azithromycin, Fluconazole, and Tinidazole are extensively prescribed drugs used in the treatment of mixed bacterial and fungal infections, particularly in cases of gynecological, gastrointestinal, and respiratory tract infections. The combination of these agents in a single dosage form enhances therapeutic efficacy and patient compliance by targeting a broader spectrum of pathogens (Sweetman, 2009; Brayfield, 2014; Lewis, 2015). However, the simultaneous

presence of these compounds poses a significant challenge in pharmaceutical analysis due to their differing chemical structures and physicochemical properties. Therefore, there is a strong demand for a validated, precise, and rapid analytical technique capable of accurately quantifying all three drugs in a single run, especially in complex matrices such as combined tablet formulations (Muller and Hildebrand, 1989; Lewis, 2015; Basak *et al.*, 2015).

High-performance thin-layer chromatography (HPTLC) has emerged as a highly effective analytical tool for the simultaneous estimation of multiple drugs in pharmaceutical preparations. Compared to conventional chromatographic techniques, HPTLC offers several advantages including low operational cost, high throughput, minimal solvent consumption, and the ability to analyze multiple samples simultaneously under identical conditions. In the case of Azithromycin, Fluconazole, and Tinidazole, HPTLC enables their clear resolution and quantification with good sensitivity and reproducibility (Sethi, 2001; Sherma and Fried, 2003). A validated HPTLC method typically involves optimized mobile phase composition, suitable stationary phase selection, and rigorous method validation according to ICH guidelines, covering parameters such as linearity, accuracy, precision, limit of detection (LOD), and limit of quantification (LOQ). The development of such a method facilitates quality control and regulatory compliance in the pharmaceutical industry, ensuring the safety and efficacy of combined dosage forms containing these critical antimicrobial agents (Sridhar *et al.*, 2002). In the present study a high-performance thin-layer chromatographic (HPTLC) method was developed and validated for the simultaneous estimation of azithromycin (AZM), fluconazole (FLZ), and tinidazole (TZ) in pharmaceutical formulations.

Materials and Methods

Instrumentation

High-Performance Thin-Layer Chromatography (HPTLC) analysis was carried out using a Camag HPTLC system comprising a Linomat V sample applicator, Twin Trough Chamber, TLC Scanner 3, and a digital documentation system (Camag, Switzerland). Sample application was performed using a 100 µl Hamilton syringe. Weighing was done on a Shimadzu Libror AEG-220 analytical balance. An ultrasonic bath was used for solution degassing. Data acquisition and analysis were performed using WinCATS software (version 1.4.2, Camag), while Microsoft Excel was employed for statistical analysis.

Chemicals and Reagents

Standard drug samples of azithromycin, fluconazole, and tinidazole were kindly provided by Cipla Laboratories (Malapur) and Aurobindo Pharmaceuticals (Hyderabad). A marketed formulation (AF-kit, Madras Pharmaceuticals, Chennai) was procured locally. HPTLC-grade solvents—1,4-dioxane, toluene, and methanol—were obtained from Merck Life Sciences, Mumbai. All other analytical-grade chemicals were sourced from SD Fine Chemicals, Mumbai. Milli-Q water, filtered through a 0.45 µm nylon membrane, was used in all analytical procedures.

Analytical Methods

Preparation of Mobile Phase

The mobile phase consisted of a mixture of 1,4-dioxane, methanol, and toluene in the ratio 2:3:5 (v/v/v). The solution was filtered through a 0.45 µm nylon filter and degassed using an ultrasonic bath for 15 min before use.

Chromatographic Conditions

Chromatographic separation was performed on silica gel 60 F254 TLC plates (10 × 10 cm, 250 µm thickness, E. Merck, Germany). Sample bands of 8 mm were applied using a 100 µl Hamilton syringe fitted on a Linomat V applicator. Plates were developed in a twin-trough chamber and scanned at 210 nm using a Camag TLC Scanner 3 under absorbance mode with a deuterium light source. The slit dimensions were set to 2 mm × 0.1 mm, with a scan rate of 4 mm/s and a monochromator bandwidth of 30 mm. Data acquisition was

conducted using WinCATS software.

Preparation of Standard Solution

Stock solutions of the analytes were prepared by dissolving 100 mg of azithromycin in 10 ml of methanol to obtain a concentration of 10,000 ppm, 15 mg of fluconazole in 10 ml of methanol to yield 1,500 ppm, and 75 mg of tinidazole in 10 ml of methanol to achieve a concentration of 7,500 ppm. A mixed working solution was subsequently prepared by transferring 1 ml of each individual stock solution into a 10 ml volumetric flask and diluting to volume with methanol. This resulted in final concentrations of 1,000 ppm for azithromycin, 150 ppm for fluconazole, and 750 ppm for tinidazole. All prepared solutions were stored in amber-colored bottles to protect them from photodegradation and ensure their stability during the course of the study.

Preparation of Sample Solution

Tablet powders equivalent to 100 mg azithromycin, 15 mg fluconazole, and 75 mg tinidazole were extracted with methanol, sonicated for 10 min, and filtered. Equal volumes (1 ml each) of the extracts were combined in a 10 ml volumetric flask and diluted with methanol to prepare the final working solution of the same strength as the standard.

Selection and Detection Wavelength

Standard stock solutions (10 µg/ml each) of azithromycin, fluconazole, and tinidazole were scanned over the UV range (200–400 nm) to determine the optimal detection wavelength. A common wavelength of 210 nm was selected based on the overlay UV spectrum for densitometric analysis.

Method Validation

Method validation was performed according to ICH Q2(R1) guidelines, assessing key parameters: system suitability, linearity, accuracy, precision, robustness, specificity, limit of detection (LOD), and limit of quantification (LOQ) (Sethi, 2001; ICH, 2005; Snyder *et al.*, 2010).

System Suitability

System suitability was evaluated by performing five replicate injections of the standard mixture under the optimized chromatographic conditions. The peak areas for each analyte—azithromycin, fluconazole, and tinidazole—were recorded, and the relative standard deviation (%RSD) was calculated to assess the precision of the method. The system was considered suitable for analysis if the %RSD for each analyte was less than or equal to 2%, in accordance with the established acceptance criterion (Sherma and Fried, 2003; Bhatt and Vora, 2007).

Linearity

Linearity was assessed over a concentration range corresponding to 50–150% of the working standard concentrations, specifically 500–1,500 ppm for azithromycin, 75–225 ppm for fluconazole, and 375–1,125 ppm for tinidazole. Calibration curves were constructed for each analyte by plotting peak area against concentration, and linear regression analysis was conducted to determine the correlation coefficient (r^2). The method was considered linear within the tested range if the correlation coefficient for each analyte was greater than or equal to 0.99, in accordance with the established acceptance criterion (Dhalwal *et al.*, 2005; Gurumurthy *et al.*, 2009; Patel *et al.*, 2010).

Accuracy

Accuracy of the method was evaluated through recovery studies conducted at three concentration levels: 75%, 100%, and 125% of the working standards. Each level was analyzed in triplicate by spiking known amounts of the analytes into the matrix, and the percentage recovery was calculated by comparing the measured concentrations to the actual amounts added. The method was considered accurate if the recovery values for each analyte fell within the acceptance range of 98% to 102% (Rele and Tiwatane, 2011; Singhal and Kansara, 2012).

Precision

Precision of the method was evaluated by assessing both intra-day and inter-day repeatability. This involved performing six replicate analyses of the test mixture on the same day (intra-day) and on different days (inter-day). The relative standard deviation (%RSD) of the peak areas was calculated for each analyte to determine the consistency of the method. The method was considered precise if the %RSD was less than or equal to 2%, in accordance with the predefined acceptance criterion (Wankhede *et al.*, 2005; Jain *et al.*, 2009).

Robustness

Robustness of the method was evaluated by deliberately varying key chromatographic parameters to assess the reliability of the method under slight changes. The flow rate was adjusted by ±10 µl/min, ranging from 490 to 510 µl/min, and the mobile phase composition was varied between 48:52 and 52:48 (v/v). The impact of these variations on the peak area and the relative standard deviation (%RSD) of the analytes was recorded. The method was considered robust if the %RSD remained within the acceptance limit of ≤ 2% (Shah *et* al., 2006; Jadhav *et al.*, 2011).

Specificity

Specificity was evaluated by comparing chromatograms of standard, sample, and blank. No interfering peaks were observed at the retention times of the target analytes, confirming the specificity of method (Ravishankar *et al.*, 2015).

Limit of Detection (LOD)

The limit of detection (LOD) was determined using the standard formula: LOD = 3.3 × (σ / S), where σ represents the standard deviation of the response and S denotes the slope of the calibration curve. This calculation provides an estimate of the lowest concentration of the analyte that can be reliably detected, but not necessarily quantified, under the specified experimental conditions.

Limit of Quantification (LOQ)

The limit of quantification (LOQ) was calculated using the formula: LOQ = 10 × (σ / S), where σ is the standard deviation of the response and S is the slope of the calibration curve. This value represents the lowest concentration of the analyte that can be quantitatively determined with acceptable precision and accuracy under the given experimental conditions (Sridhar *et al.*, 2002; Sharma *et al.*, 2011).

Results and Discussion

Optimization of Chromatographic Conditions

Several trials were conducted to optimize the mobile phase for the simultaneous estimation of azithromycin (AZM), fluconazole (FLZ), and tinidazole (TZ). The objective was to achieve sharp, compact, and well-resolved peaks. Various combinations of solvents were tested, and their outcomes are detailed in Table 1. Most trials (Trials 1–7) resulted in poor resolution, high or very low Rf values, or diffused spots, making them unsuitable. The best result was achieved with 1,4-dioxane: methanol: toluene (2:3:5, v/v/v), which gave good resolution and compact spots. Thus, this mobile phase system was selected for further method development and validation.

Detection of Wavelength and Spectral Analysis

The overlay spectra of AZM, FLZ, and TZ revealed that all three drugs exhibited significant absorbance at 210 nm, which was selected as the detection wavelength for densitometric scanning (Fig. 1).

Selectivity

Selectivity was confirmed by comparing the Rf values of standard drugs with those in the sample matrix. There was no interference from excipients at the Rf values of AZM (0.202), FLZ (0.382), and TZ (0.522), indicating that the method was selective. Peak purity values greater than 0.999 for all analytes further supported the method's selectivity.

Linearity

The method demonstrated excellent linearity across the specified concentration ranges for each analyte: 1,000–5,000 ng/band for azithromycin

Table 1: Observation and remarks of Mobile phase optimization

S. No.	Trials	Observation	Remarks
1	n-butyl acetate : glacial acetic acid : water (12 : 3 : 5, v/v/v) (10:5:5, v/v/v/v)	Improper resolution, diffused spot	Not satisfactory
2	Methanol : Ethyl acetate : 1,4 dioxone : Toluene (3: 5: 2:1 v/v/v/v) (5: 3: 2:1, v/v/v/v) (4: 4: 2:1, v/v/v/v)	Improper resolution, diffused spot	Not satisfactory
3	Toluene: ethyl acetate: glacial acetic acid (4: 2: 3.5, v/v/v/) (4: 2: 4.5, v/v/v)	Very high Rf values, diffused spots	Not satisfactory
4	Acetone: chloroform: n-butanol: glacial acetic acid (6: 4: 4: 4, v/v/v/v) (6: 4: 4: 3.5, v/v/v/v)	Very high Rf values, diffused spots	Not satisfactory
5	Acetone: chloroform: n-butanol: glacial acetic acid: water (6: 4: 4: 4: 3.5, v/v/v/v/v) 5: 5: 4: 4: 3.5, v/v/v/v/v)	Very high Rf values, diffused spots	Not satisfactory
6	Toluene: Chloroform: Methanol (5:4:1, v/v/v)	Very low difference Rf values, diffused spots,	Not satisfactory
7	Toluene: Chloroform: Methanol (5:3:2 v/v/v)	Very low difference Rf values, diffused spots,	Not satisfactory
8	Toluene: Chloroform: Methanol (5:2:3 v/v/v)	Very low difference Rf values, diffused spots,	Not satisfactory
8	**1,4-dioxane, methanol, toluene 2:3:5 (V/V/V)**	**Good resolution, compact spots.**	**Satisfactory**

(AZM), 500–5,000 ng/band for fluconazole (FLZ), and 100–500 ng/band for tinidazole (TZ). Calibration curves were constructed by plotting peak area against concentration, yielding high correlation coefficients ($r^2 \approx 0.999$), which confirmed strong linear relationships. The regression equations obtained were: AZM: $y = 4.506x + 2189.4$, FLZ: $y = 9.7948x + 3078.4$, and TZ: $y = 4.8778x + 340.33$. While the high r^2 values indicated good correlation, linearity was further validated through appropriate statistical methods in accordance with FDA guidelines, ensuring the reliability and accuracy of the method

Accuracy and Recovery Study

Accuracy of the method was assessed through recovery studies conducted at three concentration levels—80%, 100%, and 120%—for each analyte. The recovery results fell within the acceptable range of 98–102%, confirming the method's accuracy. The mean recovery values obtained were 99.27% for azithromycin (AZM), 99.85% for fluconazole (FLZ), and 99.98% for tinidazole (TZ). Additionally, the %RSD values for all analytes

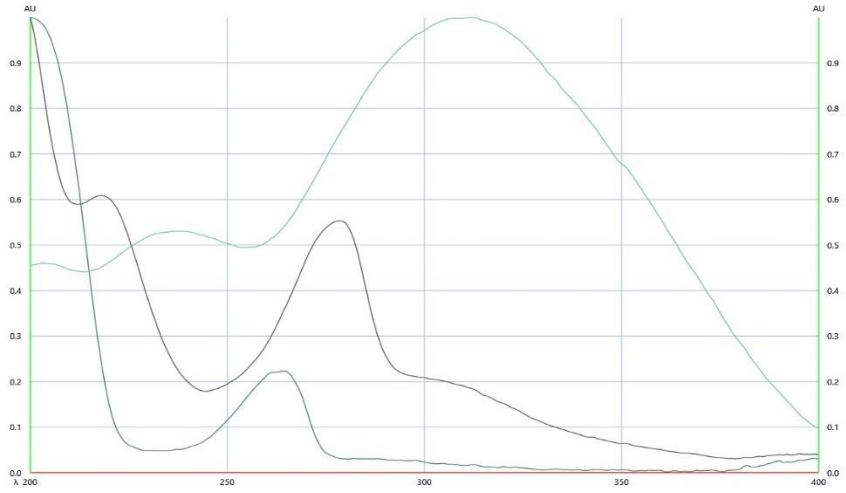

Fig. 1: Overlain spectrum of mixed standard solution containing AZM, FLZ and TZ.

Table 2: LOD and LOQ of AZM, FLZ and TZ

Drug	LOD (µg/band)	LOQ (µg/band)
AZM	0.20	0.146
FLZ	0.0524	0.150
TZ	0.826	0.168

Table 3: Summarizes the repeatability values for AZM, FLZ, and TZ

Parameter	AZM	FLZ	TZ
% RSD	<2%	<2%	<2%

were well below 2%, indicating excellent precision and reliability of the method.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

Based on ICH Q2(R1) guidelines using the formulae LOD = 3.3σ/S and LOQ = 10σ/S, the sensitivity of the method was calculated and depicted in Table 2. The results indicate that the method is sensitive enough for quantitative analysis of all three drugs.

Precision

System precision and method precision were assessed through intra-day and inter-day studies. For all analytes, the %RSD values obtained from both intra-day and inter-day analyses were below 2% (Table 3), confirming the method's precision and reproducibility. Furthermore, repeatability was evaluated using six replicate applications of the standard solution, and the results demonstrated minimal variation in peak areas, further supporting the robustness and reliability of the method.

System Suitability

The system suitability parameters were within acceptable limits. All drugs showed high peak purity (>0.999) (Table 4), distinct Rf values, and

Table 4: System suitability of AZM, FLZ and TZ

Parameter	AZM	FLZ	TZ
RF	0.202	0.382	0.522
Peak Purity	0.9996	0.9998	0.9995

consistent peak shape, confirming the suitability of the system for routine analysis.

Conclusion

In the present study the developed HPTLC method for the simultaneous estimation of azithromycin, fluconazole, and tinidazole was found to be simple, precise, accurate, and robust. The method demonstrated excellent linearity with high correlation coefficients ($r^2 \approx 0.999$) across the specified concentration ranges for all three analytes. Accuracy studies showed recovery values within the acceptable range of 98–102%, with %RSD well below 2%, confirming the method's reliability. Precision studies, including intra-day, inter-day, and repeatability analyses, further validated the reproducibility of the method. Robustness was confirmed by small deliberate variations in chromatographic conditions, and both LOD and LOQ values indicated the method's sensitivity. Overall, the method is suitable for routine quality control and simultaneous determination of azithromycin, fluconazole, and tinidazole in pharmaceutical formulations.

Ethical Statement

No animal or microbes have been used or sacrificed for this study, hence Ethical Approval not required.

Author Contributions

Each author has collaborated equally in designing the research, conducting thorough analysis, drafting the manuscript, and refining its content. All authors have read and agreed to the published version of the manuscript.

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Conflict of Interest

The authors declare no conflicts of interest.

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