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Development And Validation Of Stability Indicating Hptlc Method For Simultaneous Estimation Of Azithromycin, Fluconazole And Ornidazole In Combined Dosage Form

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ABSTRACT

For the simultaneous quantitative determination of azithromycin (AZM), fluconazole (FLZ), and ornedizole (OZ) in tablet Combi-kit quantities, a quick, reliable, and high-performance thin-layer chromatography (HPTLC) method was developed and tested. HPTLC silica gel 60 F254 plates comprised the stationary phase, and a mixture of toluene, methanol, and 1,4-dioxane in a volume ratio of 2:2:6 made up the mobile phase. A CAMAG TLC Scanner 3 was used to conduct densitometric scanning at a wavelength of 210 nm. The International Council for Harmonization's standards were used to validate the procedure. The RF values for AZM, FLZ, and OZ were 0.202, 0.382, and 0.522, respectively. In this instance, the statistical tests for assessing the appropriateness of the simple linear regression model that were previously described are used to our models AZM (y = 4.506x + 2189.4), FLZ (y = 4.506x + 2189.4), and OZ (y = 4.8969x + 240.53). LOD= 3s/S and LOQ = 10s/S (2) where s is the standard deviation of y-intercept and S is the slope of the calibration curve. The LOD and LOQ were found to be 0.0467and 0.141 g/zone, respectively. Using these methods, it is possible to routinely analyze these three medications in their pharmaceutical dosage form. For several parameters, the results for both analytical methods were tested and validated in accordance with ICH recommendations.

Keywords: *Azithromycin, Fluconazole, Ornedizole, High Performance Thin Layer Chromatography, Pharmaceutical dosage form, Stability, Validation*

1. INTRODUCTION

Only after a solid scientific rationale for the application has been developed, proven, and supported, and the suggested methodology has been approved by internal business procedures [1], can new measurement technologies be used in industries. Each year, several drugs are released, either as brand-new treatments or as changes to currently available molecules. The time between the introduction of these treatments and their inclusion in pharmacopoeias is due to the possibility that they may have unanticipated toxicities, that patients may develop resistance, or that a better medication may be introduced. Consequently, new analytical methods for such drugs, which may not be found in pharmacopoeias [2], must be created and validated Since validation procedures demonstrate the analytical laboratory's certification and competence [3], it is widely understood that a developed method needs to be validated. Analytical measures are a part of every facet of society, and there are a variety of reasons why they are carried out. It is obviously imperative to determine the correct result and be able to prove that it is correct. Consequently, technique validation is required. The growing use of novel botanical ingredients in meals and dietary supplements, for instance, has sparked a rush of research focused on creating and validating analytical methods for accurately quantifying active compounds [4].

Because drugs include the taking of human life [5], analysis is essential for any good or service. Analytical chemistry is the study of the separation, measurement, and identification of chemical additives in synthetic and herbal materials that are made up of one or more chemicals or elements. The two main types of analytical chemistry are quantitative and qualitative evaluation. The latter identifies the amount of beneficial substance or compound present in the sample, while the former identifies the presence of chemical additions in the material or sample [6]. New medications are introduced to the market every year. Additionally, the substances may be little changes to what we currently have or completely new discoveries. Medicines should be available in a way that guarantees their quality, bioavailability, ideal plasma concentration, timing, and method of delivery. Regulatory approval, drug discovery, research laboratory testing, clinical testing, preclinical testing, and other steps are all part of the protracted process of producing a medication [7]. The initiation of action, safety, appropriate dose, effectiveness, and stability of the product throughout storage are all taken into account.

Before a drug product may be released for usage, a number of regulatory agencies, including the US Food and Drug Administration (USFDA), also require that it be tested for its identification, potency, characteristics, quality, stability, and purity in order to further enhance the adequacy and safety of the medication after acceptance. Process controls and pharmaceutical validation are essential to prevent these issues [8]. There are occasions when the day a drug is first released to the market and the day it is taken into account for inclusion in pharmacopoeias might be different. This is because of the possibility of flaws in the ongoing and widespread usage of those pharmaceuticals, accusations of ongoing toxicity (resulting in their market withdrawal), the development of patient resistance, and the development of superior medical therapies in an attempt to compete. For some medicines, there may be requirements and analytical methods that are beyond the scope of pharmacopoeias. In order to develop new analytical techniques for such medications, it becomes necessary [9]. The research, development, and manufacturing of pharmaceuticals depend heavily on the creation and validation of analytical approaches. The fundamental goal of an analytical measure is to obtain information that is precise, realistic, and consistent. The key to achieving this goal is through validated analytical techniques. Validating analytical methods helps to assess the quality, consistency, and reliability of analytical results, which are crucial to any sound analytical process. The majority of regulations and quality standards that govern labs mandate the validation of analytical methods [10].

Analytical method development

In the absence of established approaches, new methodologies are being developed for the evaluation of innovative products. Innovative procedures are created to decrease the value aside from time for greater precision and strength in order to analyse the existence of either pharmacopoeial or nonpharmacopoeial product. Through test runs, these approaches have been optimised and proven to be reliable. Alternative methods are developed and put into use to replace the current approach in the context of comparing laboratory data with all available benefits and drawbacks.

2. MATERIALS AND METHODS

Preparation of mobile phase

The mixture of 1,4-dioxane, methanol, and toluene in a ratio of 2:2:6 (V/V/V) was then filtered using a 0.45 µ millipore nylon filter. For 15 minutes, the solution was degassed using an ultrasonic cleaner. The mobile phase was the combination that resulted.

Chromatographic conditions

In HPTLC, chromatographic separation of drug was performed with silica gel 60 F254 (10.0 × 10.0 cm with 250 mm layer thickness) from E. Merck, Germany. Samples were applied as 8 mm bands by means of Camag 100 µL, sample syringe (Hamilton, Switzerland) with Linomat 5 applicator (Camag, Switzerland). Densitometric scanning was performed in the absorbance/reflectance mode at 210 nm using Camag TLC scanner 3 with deuterium source, slit dimension settings of length 2 mm, width 0.1 mm, monochromator band width 30 mm, and scan rate of 4 mm s−1. Win CATS software (V 1.4.2, Camag, Switzerland) was used for scanner control and data processing.

Preparation of a standard mixture of azithromycin, fluconazole and ornidazole

100 mg of azithromycin was precisely weighed and dissolved in 10 mL of methanol to produce a stock solution with a concentration of 10,000 ppm (10 mg/mL). In a similar manner, 15 mg of fluconazole was dissolved in 10 mL of methanol to produce a 1,500 ppm (1.5 mg/mL) solution. Furthermore, a stock solution of 7,500 ppm (7.5 mg/mL) was produced by dissolving 75 mg of ornidazole in 10 mL of methanol. One milliliter each of the azithromycin, fluconazole, and ornidazole stock solutions were pipetted into a 10 mL volumetric flask to make the working standard solution. A mixed working standard solution containing azithromycin (1,000 ppm), fluconazole (150 ppm), and ornidazole (750 ppm) was produced by diluting the volume with methanol. Every solution was used during its stability period and freshly made, and it was kept in amber-colored glass bottles to avoid photodegradation.

Preparation of sample mixture of azithromycin, fluconazole and ornidazole

For the preparation of the sample solution, one tablet each containing azithromycin, fluconazole, and ornidazole was

accurately weighed and finely powdered. The powdered content of each tablet was transferred into separate 100 mL volumetric flasks, and approximately 70 mL of methanol was added to each. The flasks were sonicated for 10 minutes to ensure complete dissolution of the active pharmaceutical ingredients and then made up to volume with methanol. The resulting solutions were filtered through Whatman filter paper to remove any insoluble excipients. From each of these stock solutions, 1 mL was pipetted and transferred into a common 10 mL volumetric flask. The final volume was adjusted to the mark with methanol to prepare a combined working solution containing 1,000 ppm of azithromycin, 150 ppm of fluconazole, and 750 ppm of ornidazole.

Selection of detection wavelength

A solvent blank was used to compare each drug concentration in a standard stock solution of azithromycin, fluconazole, and ornidazole at a concentration of 10 µg/ml, which was then scanned with three-dimensional overlay spectrum displays a 10 mm path length in the UV area (200–400 nm) against a solvent blank.

Method Validation

After creating a method, it's crucial to test it to make sure it's appropriate for its intended use. The validity demonstrates the quality of the approaches, especially whether it is sufficient for the intended use. In analytical chemistry labs, method validation is now a crucial issue. The pharmaceutical industry has already implemented it effectively. However, laws have not reached that degree of stringency in other sectors, such as biotechnology, petroleum chemistry, and food. The International Conference on Harmonisation (ICH) has defined validation challenges covered under "analytical procedures" for the domains of bioanalytical techniques, pharmaceutical, and biotechnological procedures. The US Pharmacopeia (USP) has also released guidelines for method validation for analytical techniques used to analyze pharmaceutical products. Nevertheless, the guidelines issued by the ICH and USP are not as comprehensive as those issued by the FDA, and there are no specific validation guidelines for the analytical biotechnology field. Below is a short explanation of the most typical validation criteria.

Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between the series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions.

Preparation of Standard Stock Solution: A primary standard stock solution was prepared by dissolving 100mg, 15mg and 75mg of Azithromycin, Fluconazole and Ornidazole in 70mL of diluent. Secondary stock solution was prepared by pipetting out 20µL of primary standard stock solution and make up the volume up to 10mL with diluent.

Preparation of Sample Solution: Twenty tablets were weighed, their mean weight was determined, and they were crushed in mortar. An amount of powdered mass of Azithromycin equivalent to 100mg, Fluconazole equivalent to 15mg and Ornidazole equivalent to 75mg weighed, dissolved in diluent completely then again diluent was added to make up the volume up to 70mL. 20µL from the sample stock solution was pipetted out and diluted with diluent to make up the volume up to 10mL.

Precision procedure: In method precision, a homogenous sample of a single batch should be analyzed six times. The precision of the method was evaluated as intra-day and inter-day by carrying out six independent assays of test sample Azithromycin, Fluconazole and Ornidazole (tablet) against a qualified Azithromycin, Fluconazole and Ornidazole standard and the %RSD of assay was calculated.

Acceptance Criteria: %RSD should not be more than 2%.

Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. The typical variations are mobile phase composition and flow rate variation.

Preparation of Standard Stock Solution: A primary standard stock solution was prepared by dissolving 100mg, 15mg and 75mg of Azithromycin, Fluconazole and Ornidazolein 70mL of diluent. Secondary stock solution was prepared by pipetting out 20µL of primary standard stock solution and make up the volume up to 10mL with diluent.

Robustness procedure:

Effect of variation in flow rate: The 100% level standard solution was taken from the secondary stock of Azithromycin, Fluconazole and Ornidazole and then injected in triplicate by varying flow rate from 490µL/min to 510µL/min. Then %RSD was calculated.

Effect of variation in mobile phase ratio: The 100% level standard solution was taken from the secondary stock of Azithromycin, Fluconazole and Ornidazole and injected in triplicate by varying mobile phase ratio from 52:48 to 48:52. Then %RSD was calculated.

Acceptance Criteria: %RSD should not be more than 2%.

3. RESULT AND DISCUSSION

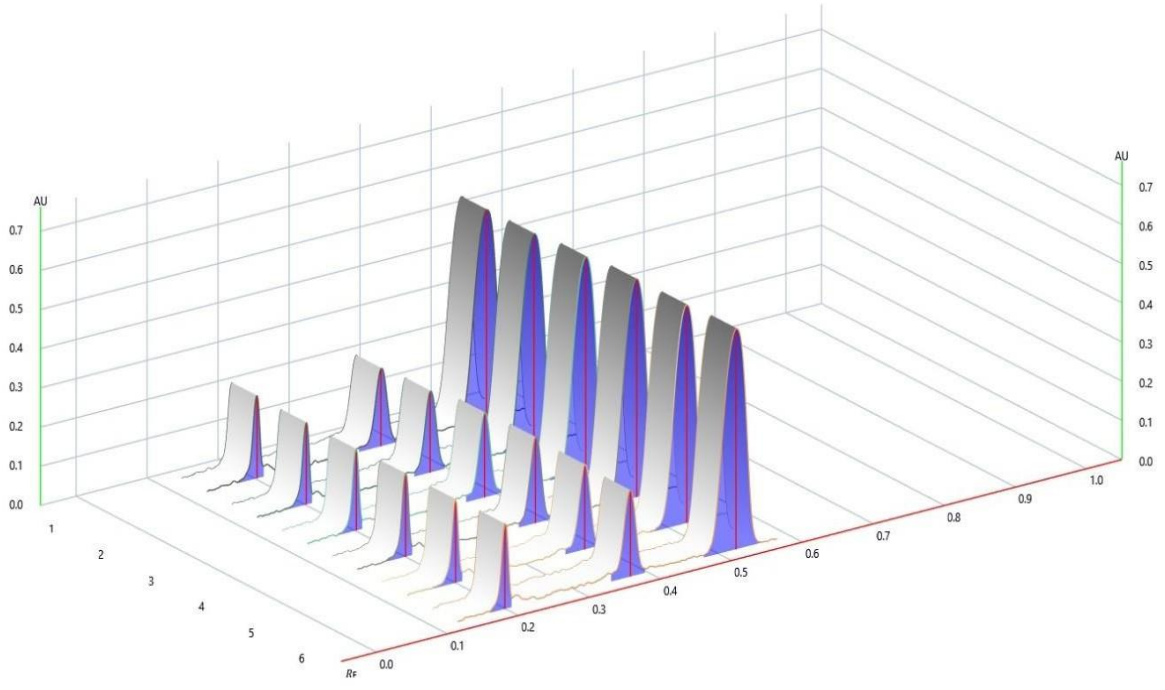

Figure 1. 3D Structure of AZM, FLZ and OZ

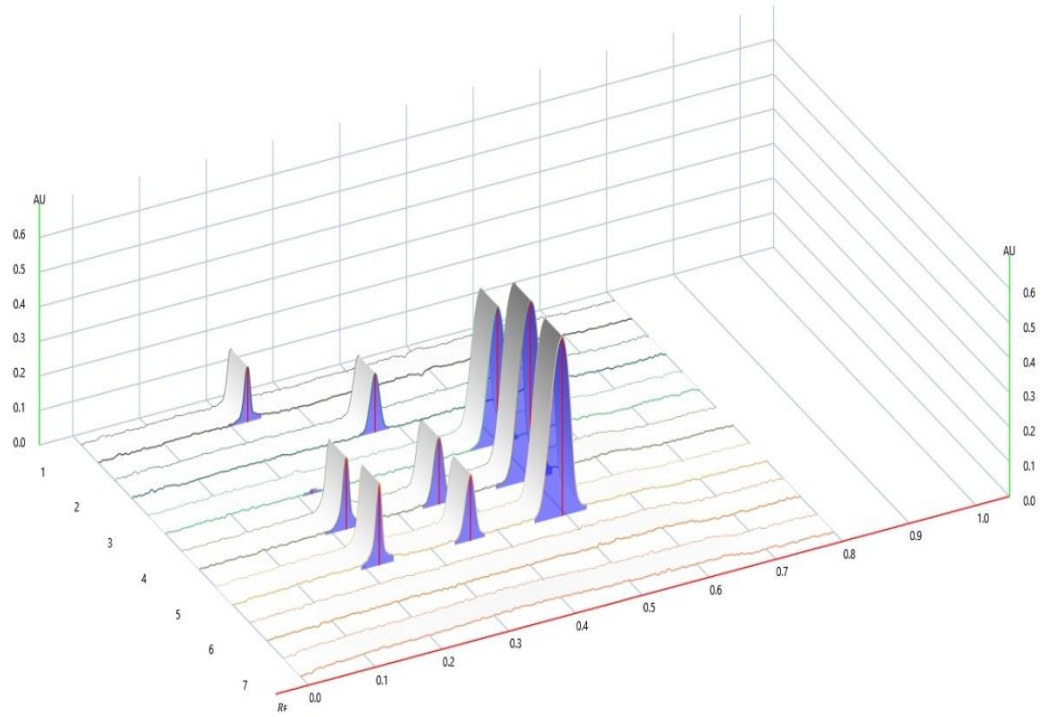

Figure 2. 3D Overlay Specificity spectra of AZM, FLZ and OZ

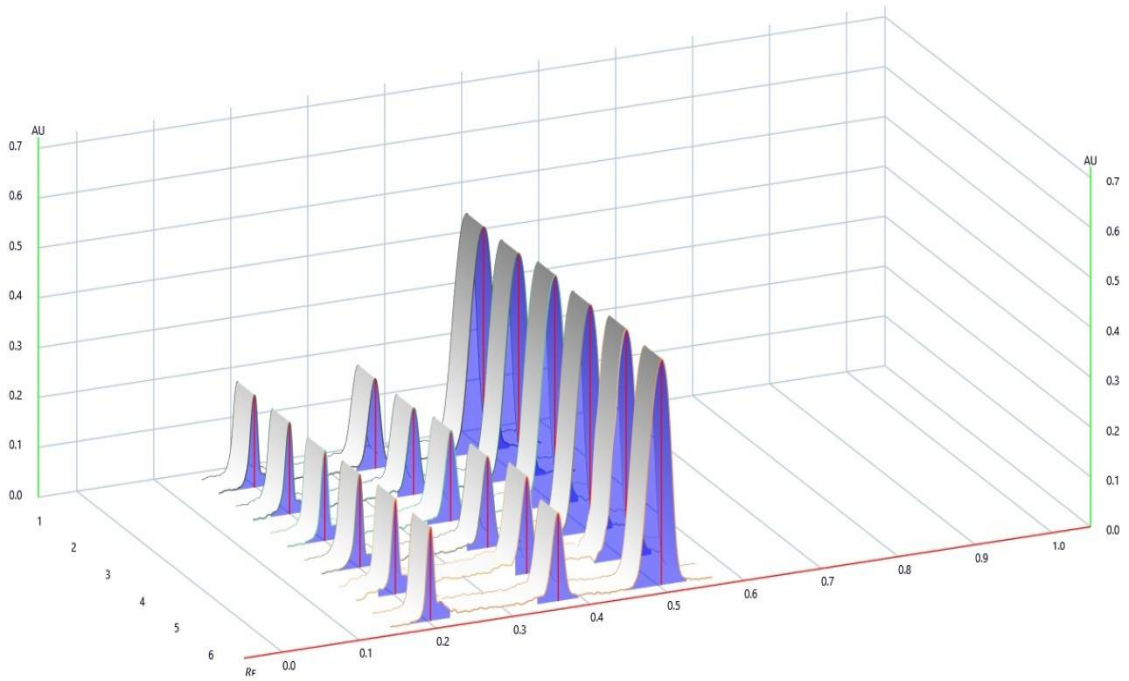

Figure 3. 3D Overlay Precision spectra of AZM, FLZ and OZ

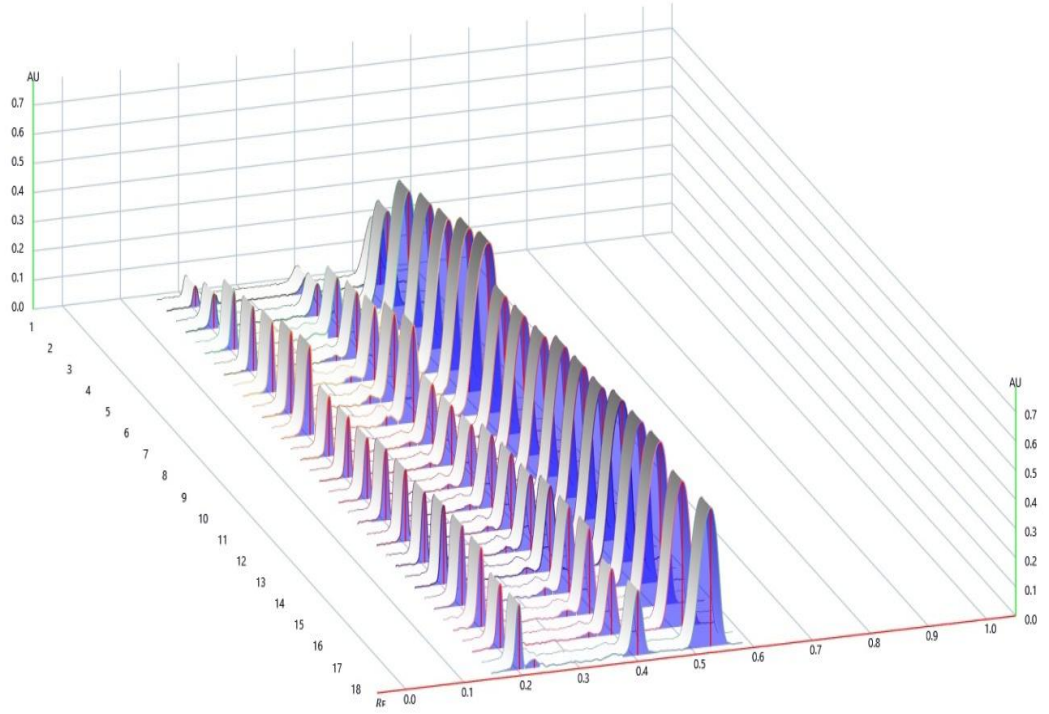

Figure 4. 3D Overlay Accuracy and Assay spectra of AZM, FLZ and OZ

Table 1. System Suitability Test Parameters

	Proposed method		
	AZM	**FLZ**	**OZ**
RF	0.202	0.382	0.522
Peak purity	0.9996	0.9998	0.9995

Table 2. Statistical Data of AZM, FLZ and OZ by HPTLC method

Parameters	**AZM**	**FLZ**	**OZ**
Linearity range	1000-5000	500-5000	100 - 500
Slope	4.506	9.7948	4.8778
Intercept	2189.4	3078.4	340.33
S.D of slope	0.065866	0.303173	0.088307
S.D of intercept	186.0797	207.4936	111.1364
LOD (µg/band)	0.20	0.0524	0.826
LOQ (µg/band)	0.146	0.150	0.168

Precision

Precision of the method was determined by injecting the standard and sample solutions of azithromycin, fluconazole and ornidazole separately for six times and measured % RSD with the help of peak area for all six injections. System precision was established by injecting six replicate injections of standard solutions into the chromatographic system by maintaining the optimized conditions. Method precision was established by injecting six replicate injections of sample solution into the chromatographic system by maintaining the optimized conditions.

The precision of the method was determined according to the ICH document by evaluating repeatability (intra-day) and intermediate precision (inter-day) at three levels covering low, medium, and higher concentrations. It was expressed as the relative standard deviation (RSD

%) of a series of measurements). The result obtained shows a RSD indicating good intra-day precision. Inter-series variability was calculated from assays on 3 independent series using the analysis of variance (ANOVA).

Intermediate precision (Inter-day and Intra-day precision)

The intra and inter-day precision was calculated by assay of the sample solution ones the same day and on different days at different time intervals respectively (n=3). Results obtained are given in table 9, 10 and 11

Table 3. Precision study of AZM

S. No.	**Intra day**			**Inter day**		
	1 hr.	**2 hr.**	**3 hr.**	**1 day**	**2 day**	**3 day**

Replica 1	6123.3	12453.5	17367.6	6123.3	12453.5	17367.6
Replica 2	6150.4	12156.4	17015.6	6288.6	12477.8	17555.5
Replica 3	6173.1	12164.3	17055.8	6267.5	12622.7	17478.4
Mean	6148.9333	12258.07	17146.33	6226.467	12518	17467.17
S.D	24.932375	169.2963	192.6739	89.96568	91.48328	94.45233
% R.S.D	0.4054748	1.381101	1.123703	1.444891	0.730814	0.540742

Table 4. Precision study of FLZ

S. No.	**Intra day**			**Inter day**		
	1 hr.	**2 hr.**	**3 hr.**	**1 day**	**2 day**	**3 day**
Replica 1	7552.5	14070.3	19223.6	7832.1	14325.8	19376.4
Replica 2	7532.7	14278.4	19344.6	7756.6	14233.7	19256.7
Replica 3	7549.5	14266.4	19322.7	7877.4	14256.8	19297.4
Mean	7544.9	14205.03	19296.97	7822.033	14272.1	19310.17
S.D	10.671457	116.8367	64.47405	61.02592	47.91837	60.86266
% R.S.D	0.1414393	0.822502	0.334115	0.78018	0.335749	0.315185

Table 5. Precision study of OZ

S. No.	**Intra day**			**Inter day**		
	1 hr.	**2 hr.**	**3 hr.**	**1 day**	**2 day**	**3 day**
Replica 1	2709.1	5483.3	7698.4	2725.6	5419.7	7612.9
Replica 2	2765.5	5419.1	7675.4	2787.4	5486.5	7698.5
Replica 3	2798.6	5438.5	7616.8	2786.8	5476.1	7691.4
Mean	2757.7333	5446.967	7663.533	2766.6	5460.767	7667.6
S.D	45.252661	32.92679	42.07438	35.50831	35.94292	47.50442
% R.S.D	1.6409368	0.604498	0.549021	1.283464	0.658203	0.619547

Table 6. Precision study of AZM

S. No.	Intra day			Inter day		
	1 hr.	2 hr.	3 hr.	1 day	2 day	3 day
Replica 1	6123.3	12453.5	17367.6	6123.3	12453.5	17367.6
Replica 2	6150.4	12156.4	17015.6	6288.6	12477.8	17555.5
Replica 3	6173.1	12164.3	17055.8	6267.5	12622.7	17478.4
Mean	6148.9333	12258.07	17146.33	6226.467	12518	17467.17
S.D	24.932375	169.2963	192.6739	89.96568	91.48328	94.45233
% R.S.D	0.4054748	1.381101	1.123703	1.444891	0.730814	0.540742

Table 7. Precision study of FLZ

S. No.	Intra day			Inter day		
	1 hr.	2 hr.	3 hr.	1 day	2 day	3 day
Replica 1	7552.5	14070.3	19223.6	7832.1	14325.8	19376.4
Replica 2	7532.7	14278.4	19344.6	7756.6	14233.7	19256.7
Replica 3	7549.5	14266.4	19322.7	7877.4	14256.8	19297.4
Mean	7544.9	14205.03	19296.97	7822.033	14272.1	19310.17
S.D	10.671457	116.8367	64.47405	61.02592	47.91837	60.86266
% R.S.D	0.1414393	0.822502	0.334115	0.78018	0.335749	0.315185

Table 8. Precision study of OZ

S. No.	Intra day			Inter day		
	1 hr.	2 hr.	3 hr.	1 day	2 day	3 day
Replica 1	2709.1	5483.3	7698.4	2725.6	5419.7	7612.9
Replica 2	2765.5	5419.1	7675.4	2787.4	5486.5	7698.5
Replica 3	2798.6	5438.5	7616.8	2786.8	5476.1	7691.4
Mean	2757.7333	5446.967	7663.533	2766.6	5460.767	7667.6

S.D	45.252661	32.92679	42.07438	35.50831	35.94292	47.50442
% R.S.D	1.6409368	0.604498	0.549021	1.283464	0.658203	0.619547

4. CONCLUSION

A simple, quick, and cost-effective way for the pharmaceutical business to simultaneously analyze multicomponent formulations without needing to separate or extract the analyte from the excipients or from itself is required. All of this taken into consideration, a novel, simple, rapid, cost-effective, precise, and selective high-performance thin-layer chromatography (HPTLC) method has been developed in the present study for the simultaneous quantitative determination of Paracetamol, Hydrochlorothiazide, and Enalapril maleate in bulk and tablet dosage forms

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