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A-QbD-Optimized HPTLC Method for Quantitative Estimation and Impurity Profiling of Azilsartan Medoxomil in Bulk and Formulations

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Abstract

A novel, robust, and cost-effective high-performance thin layer chromatography (HPTLC) method has been developed and validated for the quantitative estimation and impurity profiling of Azilsartan Medoxomil Potassium (AZM), a widely used antihypertensive agent. Following an Analytical Quality by Design (A-QbD) strategy in conjunction with a Central Composite Design (CCD), the procedure was optimized in a methodical way to guarantee precision, reproducibility, and compliance with regulations. The chosen mobile phase—toluene, ethyl acetate, methanol, and formic acid (6:2:1.5:0.5, v/v/v/v)—resulted in crisp resolution and maximum peak response. The procedure was validated as per ICH Q2(R1) guidelines, showing high linearity ($R^2 = 0.9952$, 100–500 ng/spot), precision (RSD < 2%), and sensitivity (LOD: 28.75 ng/spot; LOQ: 87.15 ng/spot). Forced degradation studies also established the specificity and stability-indicating nature of the method. This confirmed HPTLC method provides a quick, consistent, and regulatory-acceptable option for regular quality control of AZM in bulk and pharmaceutical products. This leads to improved impurity identification and product quality in pharmaceutical analysis.

Keywords: High-Performance Thin Layer Chromatography (HPTLC), Central Composite Design (CCD), Impurity Profiling, Method Validation, Forced Degradation Studies, Pharmaceutical Quality Control, ICH Q2(R1) Guidelines

1. Introduction

With the advancement of technology and the merger of sophisticated computational instrumentation, the revolution in analytical chemistry has also introduced changes, particularly in qualitative and quantitative analysis [1]. Thin layer chromatography (TLC) has advanced immensely, from being a necessary tool for qualitative analysis to a more universal and advanced technique. HPTLC is an advanced form of TLC and is capable of yielding precise, sensitive, and efficient analysis [2]. HPTLC is distinctive in that it employs pre-coated plates, controlled solvent systems, and computerized detection to achieve proper separation and analysis of complex mixtures with minimal sample preparation. It is thus optimally suited for pharmaceutical use, environmental monitoring, or food safety applications [3].

HPTLC separation is developed on a uniform layer of adsorbent, like silica gel, to provide analysis of the chemical properties of compounds, including potency, impurity, and stability. These features play a vital role in drug development as they allow bioavailability, degradation products, and impurities to be analyzed [4]. Analytical method development for HPTLC is usually performed in a systematic process, especially if pharmacopeial methods are not readily available or are protected by patents [5]. An analytical quality by design (A-QbD) approach enables the optimization of the development of HPTLC methods toward critical method variables, thus supplying reliable conditions of high robustness within a broad range of analytical conditions [6].

A recent study by M. K. Munde *et al* (2023) utilized the A-QbD strategy to optimize and validate a method for simultaneous estimation of metformin hydrochloride and empagliflozin using HPTLC in bulk and tablet formulations. By using Silica gel 60 F254 plates with ethyl acetate: methanol: water in an optimized ratio as the mobile phase were used [7]. Important method parameters like mobile phase composition, saturation time, and detection wavelength were optimized with the help of Design of Experiments (DoE). The approach exhibited good linearity, accuracy, and ruggedness, and was properly used for routine quality control, demonstrating the effectiveness and reliability of A-QbD-guided HPTLC method development for multi-drug formulations [8].

The current research aims to develop and validate a robust A-QbD-driven HPTLC method for the quantitative estimation of AZM and its related impurities. Due to the low aqueous solubility and degradation susceptibility of AZM, a stability-indicating analytical technique is required to provide accurate quantitation as well as efficient quality control. Using the A-QbD strategy, this research seeks to optimize essential method parameters systematically and develop a robust, reproducible, and stability-indicating HPTLC method applicable for routine examination of AZM throughout the stages of pharmaceutical formulation development.

2. Materials and methods

2.1 Chemicals and Reagents

A gift sample of 99% AZM was acquired from Alembic Pharmaceutical Ltd. Hyderabad, Telangana. The solvents used for the HPTLC analysis were toluene, ethyl acetate, methanol, acetone, chloroform, ammonia, and formic acid, all of which were of HPTLC grade. All of the solvents were filtered using Millipore 0.45 µm filters. HPTLC analysis was performed using

precoated silica gel 60 F₂₅₄ aluminum-backed plates (Merck, Germany), which served as the stationary phase.

2.2 Instrumentation

The study used a Shimadzu UV1800 double-beam UV-visible spectrophotometer to perform UV-visible spectroscopy investigations to develop the AZM method. Throughout the studies, a 10 mm square cell cuvette made of silica quartz with a transmittance of $55.4 \pm 0.2\%$ was utilized. HPTLC analysis was conducted using a CAMAG HPTLC system, which included a Linomat V sample applicator and a CAMAG TLC Scanner III. Data acquisition and analysis were performed using WinCATS software (version 3.15). A Systronics pH meter (Ahmedabad, India) and a Citizen Cy220 analytical balance were among the other tools used in the investigation.

3 Methods

3.1 Chromatographic conditions

A pre-coated silica gel 60F254 plate (0.2 mm thickness) was employed for the HPTLC analysis. The plate was equilibrated with a mobile phase consisting of toluene, ethyl acetate, methanol, and formic acid in a ratio of 6:2:1.5:0.5 (v/v) [9]. For the best separation of AZM, the development was done according to the ascending technique in a twin trough glass chamber over a distance of 80 mm. The process was conducted at $26 \pm 5^\circ\text{C}$ temperature and $36 \pm 5\%$ relative humidity [10]. Under such conditions, the drug showed peak symmetry and maximum separation when the eluate was detected at 249 nm. For further improvement and enhancement of the HPTLC procedure for AZM, the central composite design (CCD) strategy was used, taking into account several parameters along the way [11].

3.2 Preparation of reference stock solution and analysis of AZM using HPTLC

For the preparation of the stock solution, 20 mg AZM was dissolved in 20 mL of methanol to get a concentration of 1000 $\mu\text{g/mL}$. The stock solution was diluted to prepare a sub-stock of concentration 100 $\mu\text{g/mL}$. A 1 mL of sub-stock solution was diluted to 10 mL with methanol to get the working solution of the concentration 10 $\mu\text{g/mL}$ [12].

The HPTLC analysis was carried out using a mobile phase consisting of toluene, ethyl acetate, methanol, and formic acid in a ratio of 6:2:1.5:0.5. The plates were developed using the ascending method up to a distance of 80 mm, under conditions of $26 \pm 5^\circ\text{C}$ temperature and 36

± 5% relative humidity, in a CAMAG twin trough glass chamber equipped with a stainless-steel top [13]. After development, the plates were dried with a dryer, and the chamber was allowed to saturate for 20 minutes before scanning. Derivatization and scanning were completed within 20 minutes. The CAMAG TLC Scanner III, controlled by WinCATS software, was used to scan the plates in all detected peak mode [14].

The optimal detection wavelength was determined by scanning a 10 µg/mL AZM solution within the 200–400 nm range, and the λ_{max} was found to be 249 nm, which was selected as the detection wavelength for the analysis.

3.3 Selection of and optimization of the mobile phase

The selection and optimization of the mobile phase are crucial for effective separation and quantification in HPTLC analysis. An A-QbD strategy was used to evaluate systematically different solvent systems for the best mobile phase composition that would effectively resolve AZM and impurities (Table 1).

Table 1: Optimization of mobile phase

Sr. No	Mobile Phase	Rf Value	Remark
1	Ethyl acetate: Acetone (7:3)	Not detected	No peak detected
2	Ethyl acetate: Acetone: Methanol (6:3:1)	Not detected	No Sharp peak
3	Chloroform: Methanol: Formic Acid (5:4:1)	Not detected	No peak detected
4	Ethyl acetate: N-Hexane (7:3)	0.81	No Sharp peak
5	Toluene: Ethyl Acetate: Ammonia (5:3:2)	0.76	Poor separation
6	Toluene: Ethyl Acetate: Glacial Acetic Acid (6:3:1)	Not detected	No peak detected
7	Toluene: Ethyl acetate: Methanol (6:2:2)	0.62	Tailing
8	Toluene: Ethyl acetate: Methanol: Formic Acid (6: 2: 1.5: 0.5)	0.62	Sharp peak detected

3.4 Analytical quality by design (A-QbD) approach for optimization of developed HPTLC method

Design expert version 13 was used to optimize the HPTLC technique of AZM using a CCD based on three factors and three levels using the A-QbD method [15]. The analytical quality target product profile (QTPP) was created to establish quality standards for achieving precision and repeatability in the analytical process, ensuring that it leads to high analyst compliance and strong analyst trust. Retention factor, peak area, peak resolution, and accuracy were the key factors taken into consideration while constructing QTPP for the HPTLC technique (Supplementary Table 1). By taking these factors into account, the developed method may achieve a satisfactory quality standard while maintaining robustness concerning the replicability of the analytical process [16].

QTPP is directly affected by method parameters, which have been recognised as CQA. To obtain the requisite QTPP response range, the optimized mobile phase composition was identified as the most important element that requires careful monitoring. Maintaining these established CQAs within their desired ranges could be beneficial in ensuring the method's stability and reliability [17].

The effect of critical process parameters (CPP) and CMA on CQAs was assessed as these factors directly affect CQAs and the method's overall effectiveness. To get reliable analytical results, these characteristics must be tracked or managed. The risk associated with each CMA's effect on CQAs was evaluated (Supplementary Table 2 & 3). Based on their potential to impact method reproducibility, attributes were categorised as high, medium, or low risk (Supplementary Table 4). The proposed HPTLC technique's reproducibility and regulatory compliance were made possible by sufficient control for CPP variability [18].

3.5 Method development as per the experimental design

A CCD incorporating three components at three levels was employed to optimize the proposed method (Table 2). The A-QbD approach was used to define both the outputs and the independent and dependent variables, selected based on existing research [19]. The independent variables involved the concentrations of toluene (mL) (X1), ethyl acetate (mL) (X2), and methanol (mL) (X3). These factors were optimized to influence the dependent variables: Retention Factor (R1) and Peak area (R2). The concentration range for toluene (X1) was set from 5 to 7 mL, ethyl acetate (X2) ranged from 1 to 3 mL, and methanol (X3) varied from 1 to 3 mL. The concentration of formic acid was maintained constant.

Response Surface Methodology (RSM) was utilized to optimize these parameters following several preliminary trials [20]. To provide accurate data, 17 experimental runs with different factor combinations were carried out (Table 3). The following model was used to generate the response's regression equation:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_1X_2 + b_5X_1X_3 + b_6X_2X_3 + b_7X_1^2 + b_8X_2^2 + b_9X_3^2$$

..... Equation 1

By utilizing the principles of CCD and A-QbD, this design approach ensures the robustness of the HPTLC method for AZM analysis. It allowed for the evaluation of the interaction and quadratic effects of the mobile phase composition on critical analytical parameters, such as peak area and retention factor [21].

Table 2: Independent and Dependent Variables

Type	Variable	Optimization levels used		
		Low (-1)	Medium (0)	High (+1)
Independent	X1: Toluene Concentration	5	6	7
	X2: Ethyl Acetate Concentration	1	2	3
	X3: Methanol Concentration	1	2	3
Dependent	R1: Retention Factor	Optimum		
	R2: Peak Area	Optimum		

Table 3: Composition for trial runs (R1-R17)

Run	Factors			Response	
	(X1) Toluene Concentration (mL)	(X2) Ethyl acetate Concentration (mL)	(X3) Methanol Concentration (mL)	(R1) Retention Factor	(R2) Peak area
R1	6	2	0.318207	0.62 ± 0.02	3118.23 ± 25
R2	7	3	1	0.65 ± 0.05	2885.64 ± 30
R3	6	0.318207	2	0.64 ± 0.01	2807.58 ± 27
R4	5	3	1	0.63 ± 0.07	2876.54 ± 32
R5	5	1	3	0.65 ± 0.04	3113.79 ± 29
R6	6	2	2	0.65 ± 0.09	4058.98 ± 30
R7	6	2	2	0.65 ± 0.01	4056.87 ± 34
R8	5	3	3	0.65 ± 0.02	3942.69 ± 21
R9	7	1	3	0.64 ± 0.1	3096.83 ± 27
R10	7	3	3	0.65 ± 0.08	3893.47 ± 22
R11	5	1	1	0.62 ± 0.07	3009.31 ± 20
R12	7	1	1	0.63 ± 0.05	3069.12 ± 29
R13	4.31821	2	2	0.66 ± 0.04	4050.08 ± 33
R14	7.68179	2	2	0.65 ± 0.05	3190.82 ± 31
R15	6	3.68179	2	0.66 ± 0.03	3351.92 ± 27
R16	6	2	3.68179	0.65 ± 0.09	4054.65 ± 21
R17	6		0.318207	0.62 ± 0.01	3118.23 ± 32

*All values expressed as mean, n=6

3.6 Evaluation of experimental results and selection of final method conditions

The robustness of the CCD technique conditions was rigorously evaluated. A comprehensive evaluation of parameters such as peak area and retention factor helped establish precise chromatographic conditions specifically optimized for AZM [22]. There are situations where deliberate, controlled modifications in method parameters do not compromise the quality, as the established conditions remain within permissible operational limits. This indicates a reduced likelihood of method failure during validation [23]. If the initial modeling attempts yield suboptimal results, further optimization of variables at different levels is required to achieve acceptable response ranges. subsequently, design expert software will be employed to determine the optimal chromatographic conditions, ensuring the method's accuracy and reliability [24].

Three 3D surface response plots (A-B, B-C, and A-C) were created for each response parameter (Rf value and peak area) to determine the optimal chromatographic conditions using three continuous factors (Factor A: Toluene concentration, Factor B: Ethyl acetate concentration, and Factor C: Methanol concentration). With the third component at the center, these plots made it possible to see how each pairwise combination of factors affected replies. In the end, the contour plots' overlapping design space was used to choose the region where each CQA met the previously established acceptance criteria. Using numerical desirability optimization, this overlap region was further optimized. Derringer's desirability function approach was used to optimize a multi-response. Every response was given a significant weight, and a composite desirability rating was created by fusing each desirability function. The ideal operating situation was the best point linked to the most important composite desire. First, a small ideal space was suggested by a desirability value of 1.0. Constraints were further loosened to increase the robustness region, which produced a new optimal desirability of 0.97 and achieved both method robustness and implementation simplicity.

3.7 Risk-Based Method Optimization and Lifecycle Management

Following ICH Q8 and Q9 guidelines, a risk-based approach grounded in A-QbD principles was implemented to develop a dependable and stable analytical method. It ensures the robustness and resilience of the technique throughout the product's lifecycle, maintaining its effectiveness under varying conditions, such as different laboratories, analysts, reagents, and instruments [25]. Once the method is completed, the analytical target profile (ATP) is employed to establish a comprehensive control strategy. This strategy sets predefined controls based on

an in-depth understanding of key parameters, method suitability, analytical techniques, and associated risks. This approach ensures that analytical results consistently meet ATP requirements through controls implemented at various stages, such as sample preparation, measurements, and replication, ensuring the accuracy and reliability of the analysis [26].

Continuous improvement strategies are also critical for the smooth functioning of the analytical stages. This would entail arranging maintenance for HPTLC computers and instruments, calibrating equipment related to the HPTLC instrument, updating software, and continuously checking factors like quality. The analytical method is kept operating at its best with these continuous improvements, ensuring consistency and dependability over time.

3.8 Analytical Method Validation

Method validation is a critical process that confirms an analytical technique is both reliable and suitable for its intended use. In this research, the HPTLC method for the determination of AZM was validated by the ICH Q2 (R1) guidelines. The validation covered key parameters including linearity, precision, accuracy, repeatability, limit of detection (LOD), and limit of quantification (LOQ) [27].

Linearity was assessed by performing linear regression analysis across a concentration range of 100–500 ng/spot, where the peak area was plotted against the AZM concentration. The mobile phase consisted of toluene: ethyl acetate: methanol: formic acid in the ratio 6:2:1.5:0.5 v/v. Five standard concentrations of 100, 200, 300, 400, and 500 ng/spot were used to prepare calibration curves.

Precision and accuracy were tested by repeatability and intermediate precision experiments. Repeatability was tested by analyzing three replicate runs in three different concentrations on a single day, and intermediate precision was conducted by analyzing the runs on three separate days. The concentrations chosen, which are low, medium, and high levels, were investigated at 100, 300, and 500 ng/spot levels, respectively. The values were tested using standard deviation (SD) and percentage relative standard deviation (%RSD) [28].

To confirm system suitability, analyses were performed at a concentration of 500 ng/spot in triplicate, with the retention factor (R_f) and peak area used to assess consistency. Robustness was tested by making slight adjustments to the mobile phase composition. The concentrations of 10, 30, and 50 ng/spot were evaluated for these tests, with the results being interpreted through %RSD and recovery data to assess the method's stability [29].

The LOD and LOQ were determined using the standard deviation and slope of the calibration curve, following established equations, and their validity was verified through appropriate statistical methods [30].

$$LOD = \sigma/S \times 3.3 \quad \text{Equation...2}$$

$$LOQ = \sigma/S \times 10 \quad \text{Equation...3}$$

3.9 Application of Developed Analytical Method in Marketed Formulation

The validated HPTLC method was employed to determine the quantitative amount of AZM in two commercially available tablet formulations, Zolahart-40 and Nexsart-40. Initially, twenty tablets were weighed with precision, ground into a fine powder, and an amount corresponding to 10 mg of AZM was accurately measured. This was dissolved in a suitable solvent, shaken thoroughly, and the volume adjusted to the required level. The prepared solution was subjected to sonication for 5 minutes and filtered through whatman filter paper. A portion of the filtrate was then diluted with the mobile phase to reach a concentration of 100 µg/ml. To achieve this concentration, 10 ml of the filtrate was utilized. Both standard and sample solutions were applied to the TLC plate using a 1 µl capillary, ensuring a spot concentration of 100 ng/spot.

3.10 Forced Degradation Studies

Forced degradation studies were conducted following the ICH guidelines Q1A(R2) for stability testing and Q1B for photostability to assess the specificity of the developed HPTLC method. AZM was utilized at a concentration of 10 µg/mL for all degradation tests [31].

To induce acid degradation, 2 mL of 0.1 N hydrochloric acid was mixed with 2 mL of the AZM working solution in a 10 mL volumetric flask, and the mixture was left to stand at ambient temperature for five days. The solution was then neutralized, and the volume adjusted to 10 mL [32].

For alkaline hydrolysis, a similar procedure was followed using 0.1 N sodium hydroxide, with the mixture maintained at room temperature for 2 hours before adjusting the volume [33].

Oxidative degradation was performed by combining 2 mL of AZM solution with 2 mL of 3% hydrogen peroxide, and the mixture was kept at room temperature for 2 hours before being diluted to 10 mL [34].

For thermal stress testing, 50 mg of AZM was placed in a petri dish and heated at 105°C for six hours; the resulting sample was appropriately diluted and analyzed [35]. Neutral

degradation was determined by exposing 50 mg of AZM to room temperature in a petri dish for five days, then it was diluted and tested [36].

Photostability experiments were carried out by exposing the AZM powder to sunlight for 8 hours, and samples were withdrawn at 0, 1, 3, and 6 hours. The stressed samples were then scanned by HPTLC to study the degradation pattern of the drug and peak purity [37].

3.11 System Suitability and Statistical Analysis

The system suitability was tested by analyzing six replicates of AZM using standard solutions. Retention factor and area under the curve (AUC) were the most important parameters that were determined in triplicate for every analysis. Results were expressed as mean $\pm$ SD. One-way ANOVA followed by Newman-Keuls post hoc was used to perform statistical analysis, and the results with p-values less than 0.05 were taken to be statistically significant [38].

4. Results and discussion

4.1 Preliminary method development

A sensitive and validated HPTLC method was established for the determination of AZM to ensure accurate stability testing and exact detection at trace levels. The procedure offers higher sensitivity than the usual UV spectrophotometry, enabling detection of AZM even at trace amounts. Solubility was tested using solvents like methanol, acetone, toluene, and other polar solvents. AZM exhibited acceptable solubility in methanol, and hence, the choice of the mobile phase to be used in chromatographic analysis was toluene, ethyl acetate, methanol, and formic acid. Different solvent combinations were tested as indicated in Table 1. At first, the retention factor (R_f) could not be detected, and the peaks were not well-defined. Optimal peak separation and sharpness were, however, obtained by using a ratio of mobile phase toluene: ethyl acetate: methanol: formic acid as (6:2:1.5:0.5) and had an R_f value of 0.62 (Table 4 & Figure 1). The chromatographic conditions were conducted at a temperature of $26 \pm 5^\circ\text{C}$ with $36 \pm 5\%$ relative humidity and at a detection wavelength of 249.0 nm.

Table No 4. Chromatographic Parameters for AZM Estimation Using HPLC

Sr. No.	Retention Time	Peak Area
1	0.620 ± 0.008	3090 ± 28.5
2	0.620 ± 0.012	3013 ± 42.1
3	0.630 ± 0.010	3076 ± 31.4
4	0.620 ± 0.009	3082 ± 37.2

5	0.650 ± 0.015	3024 ± 40.6
Mean	0.628	3057
Standard Deviation (SD)	0.013038	35.70714
Relative Standard Deviation(%RSD)	2.07596	1.168045

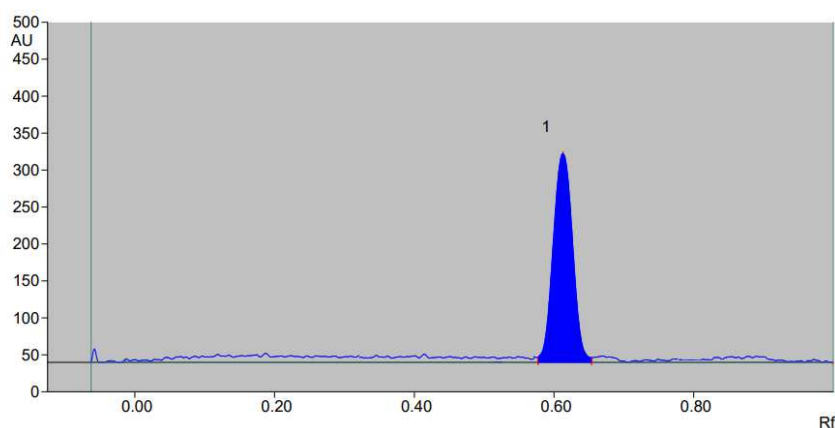


Figure 1 Chromatogram of AZM

4.2 Optimization of the developed HPTLC method for AZM

A CCD method was employed in this study. The selection of the response variables was cautious concerning the key factors influencing chromatographic performance [39]. Retention factor (Rf) and Peak Area were chosen as response variables due to their significance in analyte detection sensitivity and separation quality. The Rf value, indicative of analyte migration behavior, is critical to resolution and ensures distinct component identification, whereas the Peak Area is associated with analyte concentration, affecting the method's accuracy and quantitation. Both parameters are key to the establishment of an appropriate HPTLC method with enhanced reproducibility, sensitivity, and ruggedness [40].

For the optimization of the HPTLC process of AZM, a CCD was employed to evaluate the impact of three independent parameters, toluene concentrations (X1), ethyl acetate concentrations (X2), and methanol concentrations (X3), which is estimated at three levels (−1, 0, +1). Dependent parameters, retention factor (R1) and peak area (R2), were taken into consideration for investigation.

Experimental data were evaluated by Design expert® software to gain a suitable model. Among the various models attempted, the quadratic model had the best fit for R1 and R2. The relationships between the input variables and the responses were defined using multiple linear regression analysis (MLRA) [41]. The statistical significance of the model was assessed using analysis of variance (ANOVA) through design expert®, with a p-value below 0.05 indicating

statistical significance at a 95% confidence level. Most individual model terms were found to be significant ($P < 0.05$). Positive coefficients indicated a direct (synergistic) effect on the outcomes, while negative coefficients suggested an inverse (antagonistic) relationship (Table 5) [42]. In this model, the symbols A, B, and C represent the concentrations of toluene, ethyl acetate, and methanol, respectively.

Table 5: Response summary of ANOVA for data analysis of response variables using the quadratic mode.

Dependent Variable	Source	Sum of Squares	df	Mean Square	F-value	p-value	
Rf Value	Model	0.0023	9	0.0003	15.52	0.0017	significant
	A- Toluene Concentration	0.0001	1	0.0001	5.04	0.0659	
	B- ethyl acetate concentration	0.0004	1	0.0004	24.35	0.0026	
	C- Methanol concentration	0.0009	1	0.0009	54.79	0.0003	
		0.0001	6	0.0000			
	Lack of Fit	0.0000	4	7.790E-06	0.2337	0.8985	not significant
	Pure Error	0.0001	2	0.0000			
	Cor Total	0.0024	15				
Peak Area	Model	3.696E+06	9	4.107E+05	48687.43	< 0.0001	significant
	A- Toluene Concentration	36.23	1	36.23	4.30	0.0836	
	B- ethyl acetate concentration	3.582E+05	1	3.582E+05	42468.51	< 0.0001	
	C- Methanol concentration	1.054E+06	1	1.054E+06	1.250E+05	< 0.0001	
	Residual	50.61	6	8.43			
	Lack of Fit	7.35	4	1.84	0.0850	0.9789	not Significant
	Pure Error	43.26	2	21.63			
	Cor Total	3.696E+06	15				

4.2.1 Effect of Independent Factors on Rf

The HPLC method developed exhibited Rf values ranging from 0.62 (for R2) to 0.66 (for R11). Statistical analysis of the Rf values obtained from all 17 experimental runs was conducted using ANOVA, revealing significant differences among them ($p < 0.05$). The derived model expressed the Rf values through the following polynomial equation:

$$(\text{Rf Value}) = 0.6537 + 0.0002A + 0.0053B + 0.0080C + 0.0025AB - 0.005AC - 0.0025 BC - 0.0008A^2 - 0.0025B^2 - 0.0078C^2 \dots\dots\dots \text{Equation 4}$$

The above equation demonstrates that the independent variable has a substantial influence on the Rf, as shown in Equation 4. Here, A represents the concentration of Toluene, B the concentration of Ethyl Acetate, and C the concentration of Methanol. All values are in milliliters. The 3D surface plot visualizes the combined effect of Toluene and Ethyl Acetate on the Rf value, holding Methanol constant at 2 mL, while the equation itself reveals the broader trends, including the influence of Methanol as a variable.

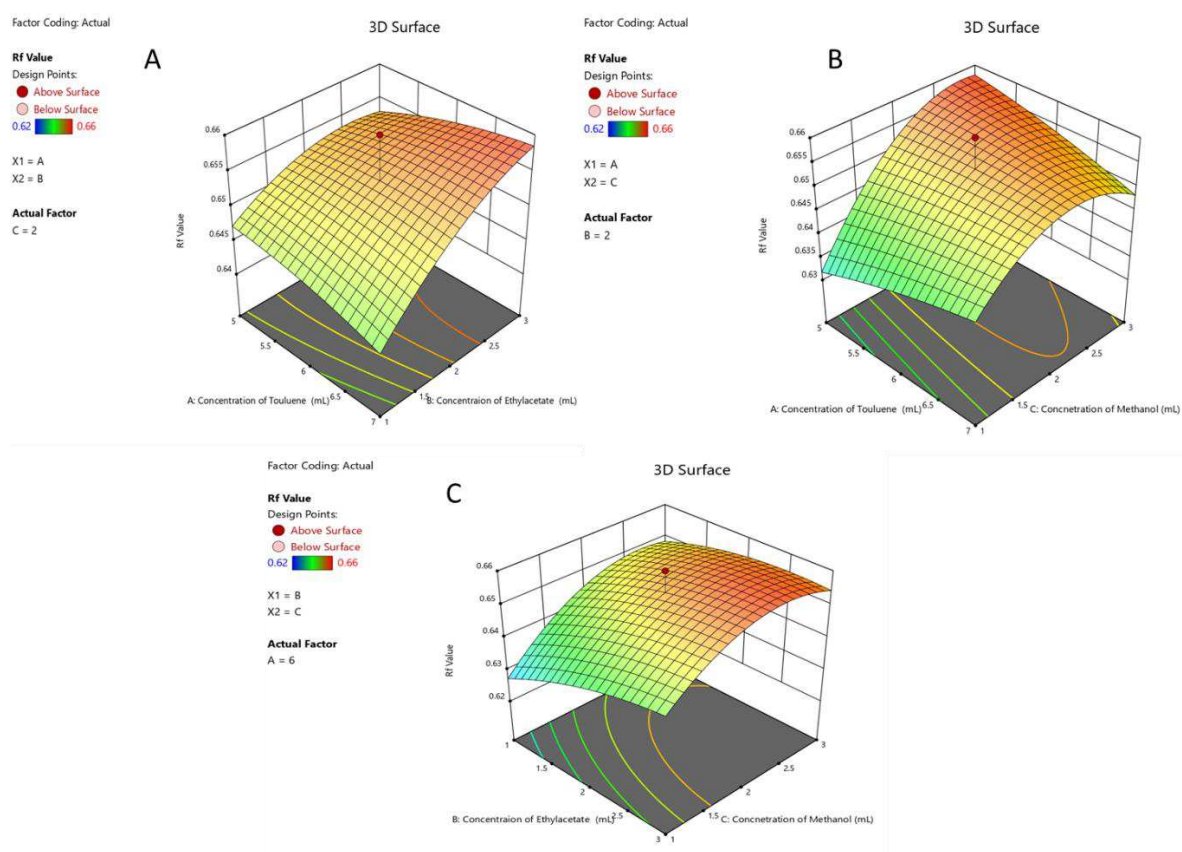


Figure 2. 3D Response surface plot for Rf Value

As the concentration of Toluene increases, the Rf value tends to decrease. This inverse relationship is subtle but evident, supported by the negative coefficient of the quadratic term $-0.0008A^2$ and the negative interaction with Methanol $-0.005AC$. This can be the attribute of the polarity of Toluene, which is a non-polar solvent.

When its proportion in the mobile phase increases, the overall polarity of the solvent system decreases. This reduction in polarity makes the mobile phase less effective at moving polar analytes, which then interact more strongly with the polar stationary phase (commonly silica), leading to reduced migration and thus a lower Rf value [43].

In contrast, the concentration of Ethyl Acetate exerts a mild positive influence on the Rf value, as indicated by the coefficient $+0.0053B$. Ethyl Acetate is moderately polar, and increasing its amount slightly enhances the mobile phase's ability to carry polar compounds, allowing for better migration. However, the presence of the quadratic term $-0.0025B^2$ suggests that this effect plateaus or even reverses slightly at higher concentrations, implying there is an optimal range beyond which additional Ethyl Acetate does not significantly increase Rf.

Methanol, a highly polar and protic solvent, exerts the most pronounced effect on the Rf value. Its linear coefficient $+0.0080C$ shows that, initially, increasing Methanol raises the Rf value, consistent with its role in enhancing the polarity of the mobile phase and weakening the analyte's interaction with the stationary phase. However, the significant negative quadratic term $-0.0078C^2$ reveals a non-linear, parabolic relationship: beyond a certain concentration, the effect of Methanol starts to reverse. This is likely due to excessive hydrogen bonding with either the analyte or the stationary phase, which can anchor the analyte more strongly and reduce its mobility.

The interaction terms in the equation further illustrate the complexity of the system. The negative AC ($-0.005AC$) and BC ($-0.0025BC$) terms suggest that the presence of Methanol modifies the influence of both Toluene and Ethyl Acetate, likely through changes in overall solvent polarity and competitive hydrogen bonding effects. These non-additive interactions mean that the effect of increasing one solvent component cannot be fully understood without considering the others.

In conclusion, the Rf value is not only dependent on individual solvent concentrations but also on their interactions. Toluene reduces Rf due to its non-polarity, Ethyl Acetate modestly increases Rf, and Methanol exhibits a dual-phase effect—initially increasing and then decreasing Rf beyond an optimal concentration. The combined behavior of these solvents defines the separation efficiency in TLC, underscoring the importance of carefully optimizing solvent ratios for desired chromatographic outcomes.

These interacting effects are better illustrated in the cube plot Figure 2(C), which clearly shows how the concentration of toluene, ethyl acetate, and methanol all interact to produce changes in the tailing factor. The cube plot visually represents the predicted Rf values across different combinations of Toluene (A), Ethyl Acetate (B), and Methanol (C) concentrations. It shows how variations in all three solvent components affect the Rf value in a three-dimensional interaction space. Notably, the central point (A = 6 mL, B = 2 mL, C = 2 mL) yields the highest

R_f(0.6557), suggesting an optimal solvent balance for compound migration. The determination coefficient (R²) for response Y1 was found to be 0.7341, indicating an acceptable correlation between the influencing variables. Three-dimensional response surface plots (Figure 2 A, B & C) and a cubic plot (Figure 3 A) visually represent how the concentrations of toluene, ethyl acetate, and methanol impact the tailing factor in the HPTLC analysis. An F-value of 6.01 suggests the model's significance, with only a 1.37% probability that such a value could result from random error. P-values below 0.0500 (<0.0001) confirm the significance of certain variables, specifically B, C, and C², while values above 0.1000 suggest non-significant factors.

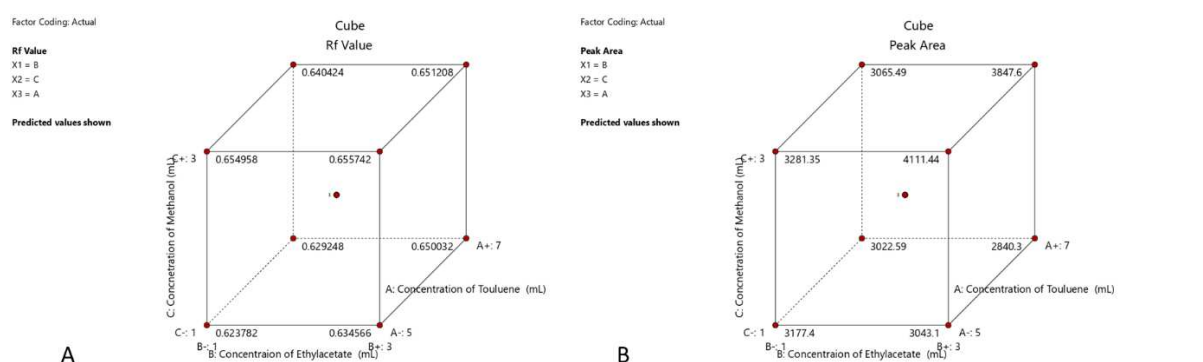


Figure 3. Cube plot for (A) R_f value and (B) Peak area

4.2.2 Effect of Independent Factors on Peak Area

The established HPTLC method demonstrated peak areas ranging from 2807.58 (Run 11) to 4056.87 (Run 1). Analysis of the tailing factor across all 17 experimental runs using ANOVA revealed statistically significant differences ($p < 0.05$). The resulting model expressed the tailing factor through the following polynomial equation:

$$(\text{Peak Area}) = 4064.49 - 104.66 A + 161.95 B + 277.81 C - 11.99 AB - 15.26 AC + 241.09 BC - 187.54 A^2 - 378.71 B^2 - 199.57 C^2 \dots\dots\dots \text{Equation 5}$$

The 3D surface plot (figure 4) illustrates the interactive influence of Toluene (A) and Ethyl acetate (B) concentrations on the Peak Area, while holding Methanol (C) constant at 2 mL. A clear curvature is observed, indicating a nonlinear relationship. The maximum peak area of 4056.87 is achieved at high levels of Ethyl acetate and moderate levels of Toluene. The polynomial regression model (Equation 5) accurately captures this response behavior, highlighting significant linear, interaction, and quadratic effects among the three solvents. The strong statistical significance ($p < 0.05$) confirms the reliability of the model in predicting peak area outcomes across various solvent combinations.

Toluene exhibits a predominantly negative influence on peak area, both in its linear ($-104.66A$) and quadratic ($-187.54A^2$) contributions. This implies that as Toluene concentration increases, the peak area tends to decrease significantly. Being non-polar, Toluene may reduce the solubility or elution efficiency of polar analytes within the mobile phase, leading to diminished detector response. At higher concentrations, this effect intensifies, likely due to stronger analyte retention on the polar stationary phase or a general suppression of chromatographic efficiency.

In contrast, ethyl acetate presents a more favourable yet non-linear effect. Its positive linear coefficient ($+161.95B$) suggests that increasing Ethyl Acetate improves peak area, likely through enhanced solubility and migration of moderately polar analytes. However, the substantial negative quadratic term ($-378.71B^2$) indicates that beyond an optimal concentration, further increases begin to reduce the peak area. This could stem from changes in the partitioning dynamics or increased baseline noise, which may reduce peak sharpness and area.

Methanol, the most polar and protic of the three solvents, shows the strongest positive linear influence on peak area ($+277.81C$), demonstrating its critical role in improving analyte response. Its capacity to form hydrogen bonds and to modify the polarity of the mobile phase helps to desorb analytes more efficiently from the stationary phase, leading to higher signal intensities. Nevertheless, its effect is also non-linear, as shown by the negative quadratic coefficient ($-199.57C^2$). This suggests that at high Methanol concentrations, peak area begins to decline, possibly due to peak broadening, interference from co-eluting species, or oversaturation effects at the detector.

The interaction terms in the model reveal further intricacies. Negative coefficients for AB (-11.99) and AC (-15.26) indicate that combining Toluene with either Ethyl Acetate or Methanol leads to a lower peak area than would be predicted by their individual effects alone. These antagonistic interactions could be attributed to disrupted solvent balance or altered retention mechanisms. Conversely, the strong positive interaction between Ethyl Acetate and Methanol ($BC = +241.09$) suggests a synergistic effect, where their combined use significantly boosts the peak area. This is most probably because of an ideal polarity profile that ensures good analyte solvation and elution with minimal retention or band broadening.

In general, the model effectively shows that peak area is not controlled by a straightforward additive function of solvent concentrations. Rather, it relies on a subtle balance among polarity, hydrogen bonding, and solute-stationary phase interactions [44]. Toluene tends to decrease

peak response, Ethyl Acetate does so within bounds, and Methanol greatly enhances it up to an optimal level. The dominant quadratic and interaction terms necessitate meticulous optimization of solvent proportions to achieve optimal chromatographic performance and analytical sensitivity. The results reaffirm the importance of optimizing mobile phase composition to the finest degree to achieve reproducible and high-quality chromatographic outcomes in intricate mixtures. The statistical reliability of the response variable Y2 was established through a high coefficient of determination ($R^2 = 0.8348$), indicating a strong correlation between the selected independent variables and the observed retention time. The model exhibited a significant F-value of 9.98, implying that there is only a 0.31% probability that the observed outcome resulted from random chance. Moreover, the model terms demonstrated statistical significance with P-values less than 0.0500. Among these, variables B, C, the interaction term BC, and the quadratic terms A^2 , B^2 , and C^2 were identified as the most influential factors affecting the retention time. These relationships are visually represented in the cube plot provided in Figure 3(B).

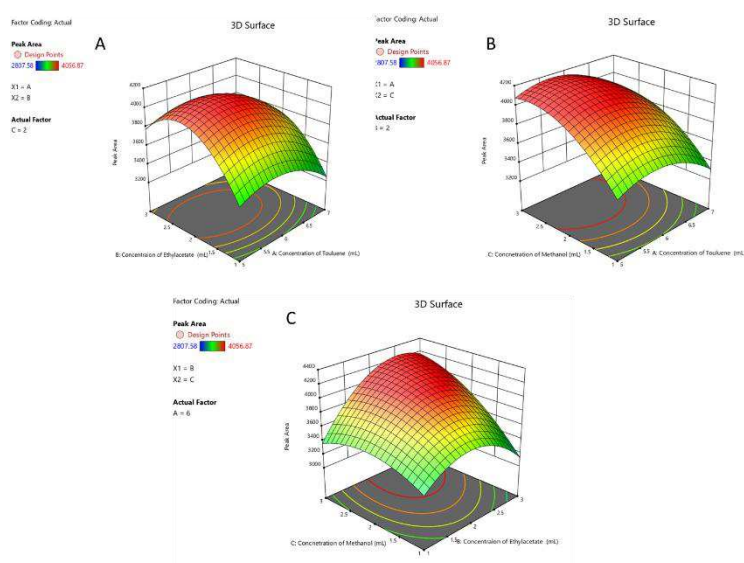


Figure 4. 3D Response surface plot for Peak Area

4.3 Validation of the Developed Response Surface Methodology (RSM) Model

Theoretical values of Y_1 (Rf value) and Y_2 (peak area) for the optimized HPTLC method were calculated by putting the chosen levels of independent variables A and B in the polynomial equations obtained through design expert® software [45]. For optimized chromatographic conditions, the calculated Rf value (Y_1) was 0.6573 ± 0.05 , which agreed with the corresponding experimentally measured value of 0.6521 ± 0.02 ($n = 6$). Similarly, the anticipated peak area (Y_2) was 4148.65 ± 25 , while the observed actual peak area was 4113 ± 32 ($n = 6$).

The similarity of the predicted and observed values shows the precision and credibility of the RSM model, thus validating its potential for method optimization and prediction in this analytical work [46].

4.4 Optimization and Selection of the Ideal HPTLC Method

The chromatographic method was optimized using the point prediction algorithm within design expert® software, applying a CCD comprising three factors at three levels and encompassing seventeen experimental runs. This design strategy was adopted to enhance both the precision and reproducibility of the analytical method [47]. Comprehensive analysis using Design Expert® identified Trial Run R9 as fulfilling all the critical parameters for robustness and reproducibility. The optimized mobile phase was composed of 6.57 mL toluene, 2.76 mL ethyl acetate, and 2.76 mL methanol. This method yielded an R_f value of 0.65 and a peak area of 4069.043, achieving a desirability score of 1.0. The outcomes of the numerical optimization for this method are detailed in Table 6.

Table 6: Numerical optimization solutions for HPTLC method

Sr. No.	Toluene Concentration (mL)	Ethyl Acetate Concentration (mL)	Methanol Concentration (mL)	R _f	Peak area	Observations for Desirability
1.	6.577	2.762	2.762	0.655	4069.043	1.000 Selected
2.	6.035	2.825	2.829	0.656	4193.600	1.000
3.	6.294	2.565	2.225	0.657	4068.017	1.000

4.5 Analytical Method Validation

The analytical technique developed was validated as per the ICH Q2(R1) guidelines, measuring parameters like linearity and range, precision, accuracy, ruggedness, robustness, LOD, and LOQ [48]. Linearity was checked by plotting a calibration curve showing the correlation between peak area and different concentrations of AZM spotted on the HPTLC plate [49]. A range of 100–500 ng/spot was used for this purpose. The calibration plot obtained had a regression equation of $y = 10.686x + 2117.4$, with a correlation coefficient (R^2) of 0.9952 (Figure 2), which reflects very good linearity. The increasing trend in peak response with higher AZM concentrations confirmed the method's suitability for quantitative analysis.

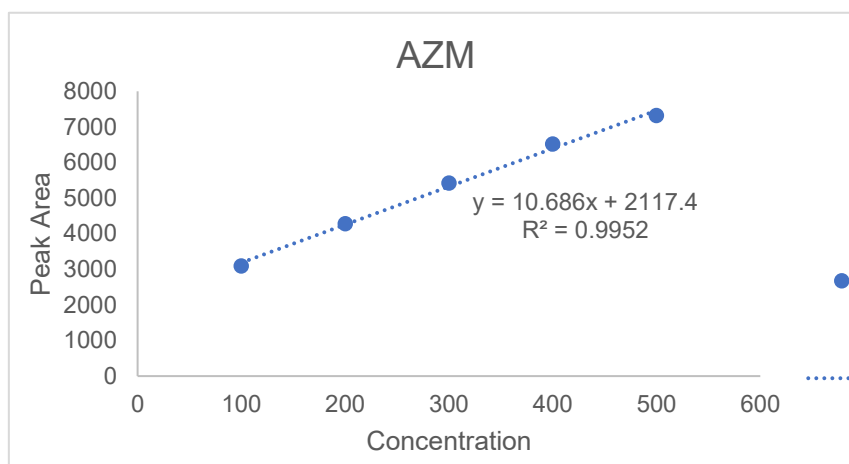


Figure 5: Calibration curve of Azilsartan Medoxmil

Linearity, as defined by the ICH Q2(R1) guideline, refers to an analytical method's capability to yield test results that are directly proportional to the concentration of the analyte within a specified range [50]. In the present study, the linearity of the method was evaluated by analyzing AZM across a concentration range of 100–500 ng per spot.

Table 7: Linearity performance parameter for AZM

Sr. No	Parameter	Results
1	Linear dynamic range (ng/mL)	100-500
2	Slope	10.686
3	Y-intercept	2117.4
4	Correlation coefficient	0.9952

The precision of the developed method was evaluated by analyzing AZM at three concentration levels—10 ng/spot (low), 30 ng/spot (medium), and 50 ng/spot (high)—using the mobile phase, as outlined in Table 8. Repeatability was assessed through same-day analysis, while intermediate precision was determined by performing the analysis over three consecutive days [51]. The %RSD values at all levels remained below 0.5%, confirming that the method provides highly precise and consistent results.

Table 8: Precision data of AZM

Sr No.	Drug Concentration (ng/mL)					
	100		300		500	
	Intraday	Interday	Intraday	Interday	Intraday	Interday
1	2987.43	3125.21	5429.66	5646.56	7332.16	7485.12
2	2991.42	3128.02	5432.14	5643.24	7329.59	7483.06
3	2989.21	3126.11	5434.38	5648.83	7326.29	7488.11
Statistics						
Mean	2989.35	3126.44	5432.06	5646.21	7329.34	7485.43

$\pm$ SD	1.9988	1.434	2.361	2.811	2.942	2.539
RSD %	0.0668659	0.045896	0.0434	0.04979	0.04014	0.0339

The limit of detection (LOD) and limit of quantification (LOQ) were calculated using the standard deviation of the response and the slope derived from the linear regression of the calibration curve [52]. With a sample size of $n = 6$, the LOD was determined to be 11.02 ng/spot, while the LOQ was found to be 33.40 ng/spot, indicating satisfactory precision and accuracy.

4.6 Application of the analytical method in pharmaceutical formulation

The optimized method was effectively utilized for quantifying AZM in tablet formulations. The assay outcomes revealed that the AZM content in Zolahart 40 was $98.59 \pm 0.4\%$ of the label claim, while Nexart 40 showed a content of $99.61 \pm 1.4\%$, confirming that both formulations meet the required quality standards.

4.7 Forced Degradation

The forced degradation studies were conducted following the ICH Q1A (R2) and ICH Q1B guidelines, specifically for photostability, to assess the selectivity of the developed assay method [53]. The degradation of AZM was analyzed under various stress conditions as outlined in Table 9. In the acidic hydrolysis test, AZM underwent a degradation of 21.27%, resulting in a single degradation product, impurity AZD1, and an assay value of 78.23% (Figure 6A). When subjected to alkaline hydrolysis, AZM exhibited a 19.62% degradation, leading to the formation of impurity AZD2 and an assay value of 80.21% (Figure 6B). Oxidative degradation caused a degradation of 24.79%, with impurity AZD3 formed, resulting in an assay value of 75.06% (Figure 6C). Under thermal stress, AZM produced two degradation products, impurities AZD4 and AZD5, with a total degradation of 27.63%, yielding an assay value of 72.13% (Figure 6D). In neutral degradation conditions, AZM experienced a degradation of 11.42%, producing impurity AZD6, and maintaining an assay value of 89.78% (Figure 6E). Lastly, photodegradation resulted in a 29.33% degradation, generating impurities AZD7 and AZD8, and an assay value of 69.29% (Figure 6F).

Table 9: Stress degradation study of AZM

Medium	Degradants%	AZM%
Acid Hydrolysis (0.1 N HCL)	21.27	78.23
Alkaline Hydrolysis (0.1N NaOH)	19.62	80.21
Oxidation (3% H2O2)	24.79	75.06
Thermal (3% H2O2)	27.63	72.13
Neutral Degradation	11.42	89.78
Photo Degradation	29.33	69.29

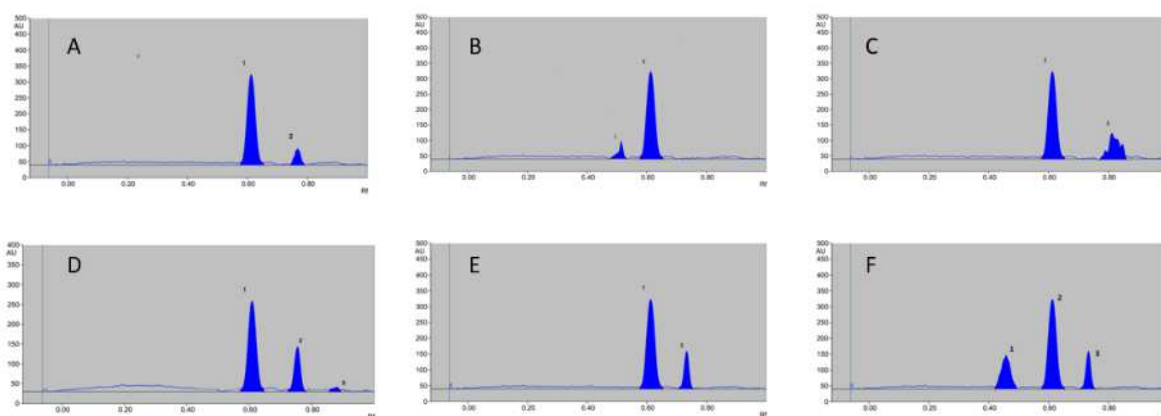


Figure 6: Chromatogram of AZM in (A) Acidic hydrolysis study (Impurity AZD1), (B) Alkaline hydrolysis study (Impurity AZD2), (C) Oxidative degradation (Impurity AZD3), (D) thermal degradation study (Impurity AZD4, AZD5), (E) Neutral degradation (Impurity AZD6), (F) Photodegradation (Impurity AZD7, ADZ8)

4.8 System Suitability Parameter

System suitability testing was conducted before sample analysis per the guidelines outlined in ICH Q2 (R1) and USP. Essential parameters, including the Rf value, area under the curve (AUC), and percent relative standard deviation (%RSD), were evaluated across six replicate injections [54]. The results of these tests complied with the established acceptance criteria, thereby confirming the chromatographic system's adequacy for the analysis (Table 10).

Table 10: System Suitability Parameter

Sr. No.	Concentration (ng/spot)	Rf Value	Area under curve
1	100	0.62	3090

2	200	0.62	3013
3	300	0.63	3076
4	400	0.62	3082
5	500	0.65	3024
Mean		0.628	3057
Standard Deviation		0.013038	35.70714
Relative Standard Deviation		2.07596	1.168045

5. Conclusion

The HPTLC method developed for Azilsartan Medoxomil Potassium, using the Analytical Quality by Design (AQbD) approach, was found to be accurate, precise, specific, and robust. The method efficiently identified and estimated the drug and impurities, meeting ICH validation parameters. Through the use of AQbD principles, the study effectively established a design space and optimized method variables, which ensured reproducibility and high performance under different analytical conditions. This method improves method reliability and allows regulatory acceptance. The established method is very applicable to regular quality control and impurity profiling of Azilsartan formulations for improved drug safety, efficacy, and pharmaceutical compliance.

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Ethics, Consent to Participate, and Consent to Publish declarations: not applicable.

Clinical Trial: not applicable.

Declaration Data Available: All data generated or analysed during this study are included within the manuscript and its supplementary information files.

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