

AQbD-based stability indicating development and validation of azilsartan medoxomil and cilnidipine by UPLC method

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ABSTRACT

This work describes a chromatographic technique for quantifying azilsartan medoxomil (AZL) and cilnidipine (CIL) in bulk and pharmaceutical formulations using quality by design (QbD). The analytical targeting profile distribution and critical analytical attributes (CAA) are incorporated with analytical QbD. Risk evaluation studies and factor screening research facilitate the identification of critical method parameters (CMPs). The application of 2^2 full factorial designs was used to optimize the process. Selected CMPs, such as plate number of peak 1 (R1), resolution (R2), and tailing factor of peak 2 (R3) were evaluated. Utilizing statistical data and response surface plots, the individual and interaction effects of CMP on CAA were evaluated. The significance ($p < 0.05$) of the procedure parameters was shown by analysis of variance. Mobile phase-Acetonitrile and 1% triethylamine buffer (50:50 v/v), pH (2.5) adjusted with 0.1% ortho-phosphoric acid, and Waters X-Bridge C18 column, (50 × 4.6 mm, 2.5 μm), the flow rate is 0.5 ml/minute with photodiode array detector at 273 nm. According to ICH requirements, method validation and subsequent stress degradation experiments were carried out. All variables are within their bounds. The suggested method is effectively illustrated by using a QbD to perform extremely sensitive, stable, and suited for regular analysis and clinical applications.

INTRODUCTION

Azilsartan medoxomil (AZL) is a new angiotensin-II receptor blocker (ARB) suggested to treat high blood pressure. AZL is a prodrug that is broken down into azilsartan (Drug bank). It is recognized chemically as 2-ethoxy-3-[[4-[2-(5-oxo-4H-1,2,4-oxadiazol-3-yl) phenyl] phenyl]methyl] carboxy-4-benzimidazole acid (Pub chem) (Fig. 1A). AZL has been suggested to be more effective compared to other ARBs due to its greater reduction in blood pressure (WHO, 2021).

Cilnidipine (CIL) is a dihydropyridine calcium channel blocker used to treat high blood pressure that works on both N and L-type calcium channels (Rathod *et al.*, 2018). Its official name

in chemical terms is 1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridine carboxylic acid. 3-phenylpropenyl esters of 2E and 2M. Figure 1B illustrates the structure of CIL (Pub chem). It works by inhibiting long-acting Ca^{+2} channels, which prevent calcium ions from entering tiny blood capillaries. Inhibition of the cascade that causes vasoconstriction ultimately results in vasodilation when the Ca^{+2} entrance is blocked. It lowers blood pressure by lowering peripheral resistance (Drug bank).

Pharmaceutical quality by design (QbD) is a scientific approach to drug development that promotes comprehension and control of products and processes based on quality science and quality risk management and begins with predetermined goals (Juran, 1992). In the pharmaceutical QbD technique of analytical method development, the applicant identifies characteristics that are crucial to quality from a design point of view and develops them into critical quality attributes (CQAs) of the analytical method and establishes the relationship between the variables and the CQAs (U. S. Food and Drug Administration, 2009; Yu *et al.*, 2014).

Only a few approaches are described in the literature for the estimation of AZL and CIL (Andhalea and Nikalje, 2022;

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Acid degradation

Add 0.8 ml of sample stock solution into a 10 ml VF, add 1 ml of 1 N HCl heat for 30 minutes at 60°C, then cool and add 1 ml of 1 N NaOH to neutralize the solution and dilute to volume with diluent and mix. This solution is injected every 6 hours up to 24 hours.

Alkali degradation

Add 0.8 ml of sample stock solution into a 10 ml VF, add 1 ml of 1 N NaOH heat for 30 minutes at 60°C, then cool and add 1 ml of 1 N HCl to neutralize the solution and diluted to volume with diluent and mix. This solution is injected every 6 hours up to 24 hours.

Peroxide degradation

Add 0.8 ml of sample stock solution into a 10 ml VF, add 1 ml of 30% hydrogen peroxide solution, and heat the solution at 60°C for 30 minutes. After that, cool and dilute to volume with diluent and mix.

Reduction degradation

Add 0.8 ml of sample stock solution into a 10 ml VF, add 1 ml of 30% sodium bi-sulfate solution, and heat the solution at 60°C for 30 minutes. After that, cool and dilute to volume with diluent and mixed. This solution is injected every 6 hours up to 24 hours.

Hydrolysis degradation

Add 0.8 ml of sample stock solution into a 50 ml VF, add 3 ml of HPLC water, and heat on water bath at 60°C for 30 minutes. After that, dilute to volume with diluent and mix.

This solution is injected every 6 hours up to 24 hours.

Thermal degradation (105°C/72 hours)

Expose 100 mg of the sample to a hot air oven at 105°C for 72 hours. After that, take 19 mg of this sample and transfer it into a 10 ml VF and add 7 ml of diluent sonicate to dissolve and make up.

Further, add 0.8 ml of the above solution into 10 ml VF and dilute the volume with diluent. This solution is injected every 6 hours up to 24 hours.

Photolytic degradation

Expose 100 mg of sample was to a photostability chamber to expose samples of 1.2 million l × hour and 200 W hour/m² light. After that, take 19 mg of this sample and transfer it into a 10 ml VF and add 7 ml of diluent sonicate to dissolve and make up. Further, add 0.8 ml of the above solution into 10 ml VF and dilute the volume with diluent. This solution is injected every 6 hours up to 24 hours.

RESULTS AND DISCUSSION

Construction techniques by CCD

To determine the impact of different chromatographic conditions on the R1, R2, and R3, three main critical analytical attributes (CAAs) were created. There are a variety of 13 tests performed in the design. The investigation is carried out to support the design of statistical information utilizing the response surface methodology (RSM) with a systematic study of crucial components by evaluating their significant influence on acquiring critical method parameters (Desai and Nikalje, 2021; Jena *et al.*, 2021). Table 1 provides a summary of the design specifications for all identified CAAs and the corresponding replies. The independent variables, namely the mobile phase composition and rate of flow, are also described in depth in Table 2, together with the design factors and the results that were recorded.

Parameters for optimal chromatography

Column: Waters X-Bridge C18, 50 × 4.6 mm, 2.5 μm.

Detector: PDA at 273 nm

Injection volume: 10 μl

Flow rate: 0.5 ml/minute

Run time: 5 minutes

Mobile Phase: 1% TEA buffer at pH 2.5 with OPA and acetonitrile (50:50).

Retention Time: 1.354 and 2.443 minutes respectively (Fig. 2).

Utilizing RSM to improve the chromatographic procedure

The two most important variables for optimization were decided to be the organic phase's composition (A) and the mobile phase's pH (B). Thirteen different experimental tests were all sent through the Design Expert (Tables 1 and 2). Every experiment was conducted at random to reduce the impact of uncontrolled variables that can bring bias into the response (Andhalea and Nikalje, 2022). When comparing the various models, the design expert software chose the quadratic model since it had the largest least squares regression coefficients for each of the three responses (R1, R2, and R3). When the model was put through a lack of fit test, the results revealed a non-significant lack of fit value, which corresponds to a larger *p*-value than the model's *F*-value. The normal residual plot further demonstrated that there were no detectable outliers in the data and that all data were concentrated along the model fit line (Fig. 3A–C). ANOVA, which was used to validate the model, demonstrated its significance and further showed that it was valid. Following is the quadratic equation for all model responses, R1, R2, and R3:

$$R1 = 552.02157 - 3,927.40242 - 2.42228 + 1,404.30333$$

$$R2 = 0.292197 + 2.93964 - 0.015974 - 0.386869$$

$$R3 = -0.017841 + 0.420607 - 0.004500 + 0.000261 - 0.035500$$

Table 1. Experimental design matrix for factors and their obtained responses by 2² CCD.

Factor	Name	Type	Minimum	Maximum	Coded Low	Coded High	Mean	Std. dev.
A	Mobile Phase	Numeric	35.86	64.14	-1	+1	50	8.16
					40	60		
B	pH	Numeric	1.79	3.21	-1	+1	2.50	0.4082
					2	3		

Table 2. Experimental runs of selecting (2²) factors by CCD.

Std.	Run	Factor 1 A: mobile phase	Factor 2 B: pH	Response 1 PC of peak 1	Response 2 Resolution	Response 3 Tailing of peak 2
7	1	50.0	1.79	11,889	6.07	1.02
11	2	50.0	2.50	10,113	6.38	1.03
8	3	50.0	3.21	9,773	6.37	1.05
3	4	40.0	3.00	9,161	6.05	1.11
6	5	64.1	2.50	10,811	6.16	1.06
2	6	60.0	2.00	12,218	6.16	1.04
9	7	50.0	2.50	10,114	6.39	1.02
4	8	60.0	3.00	9,868	6.21	1.01
5	9	35.9	2.50	8,477	5.72	1.15
12	10	50.0	2.50	10,175	6.36	1.04
10	11	50.0	2.50	10,112	6.37	1.05
13	12	50.0	2.50	10,118	6.43	1.08
1	13	40.0	2.00	9,686	5.68	1.05

Results from an ANOVA for responses R1, R2, and R3 showed that the models were significant because their respective model *F*-values of 3,199.15, 110.70, and 7.80 were more than zero. The variables A and B are both significant in every case, according to the *p*-values (*p* < 0.05) for the model terms (Table 3). According to Table 4, the anticipated *R*-squares for all solutions R1 (0.9983), R2 (0.9331), and R3 (0.5595) are reasonable by the adjusted *R*-squared values of 0.9993, 0.9786, and 0.7391, respectively, which are all lower than 0.2 in each case. Accurate measurements of the signal-to-noise ratio were made. A sufficient signal (ratio >4.0) is indicated by a ratio of 202.4996, 28.2172, and 9.1991. The design space can be explored using these models. The quantitative analysis of AZL and CIL was unaffected by changes in the experimental settings, which demonstrated the analytical method's robustness. The statistical results for PC peak 1, resolution, and peak 2's tailing also supported this conclusion. All results fell within the acceptable level, and the experimental results of the proposed method are comparable to the suggested replies (NMT 2.0%). The optimized method is according to the design of the experiment shown in Table 5. These conditions are used for further validation studies.

Method validation

The chromatographic separation was evaluated for linearity, range, accuracy, precision, robustness, specificity, and system appropriateness by (Q2 R1) ICH criteria (ICH, 2005).

Suitability of the system

For the system appropriateness analysis, it was examined how theoretical plates, peak areas, and tailing factors would behave. Results from the six replicates of injections of AZL and CIL at 40 and 10 g/ml were displayed in Table 6. All of the outcomes fall within the acceptable range.

Linearity and range

The six different serial concentrations (10, 20, 30, 40, 50, and 60 µg/ml) were prepared for the standard calibration curve using acetonitrile. With each concentration, three replicate injections were used to test the linearity. The regression formula

was $Y = 2,425,977.50x + 20,890.89$ and $Y = 4,543,356.29x + 9,887.82$ respectively and the regression coefficient $R^2 = 0.99986$ and $R^2 = 0.99936$ (Fig. 5).

Precision

Repeatability was studied (system precision, method precision, and intermediate precision) within six replicate sets. The %RSD is within the limit. Results are shown in Table 7.

Accuracy

By incorporating a known quantity of the drug into the tablet formulation at three different concentrations—50%, 100%, and 150% with each concentration, three replicate injections were used to conduct the recovery study. At each level, the average percent recovery and percent RSD values were discovered to be within acceptable limits, proving the method's accuracy. Results are shown in Table 8.

Robustness

It measures how well it can withstand slight changes and shows that it was stable in routine use. For anticipated changes in technique, parameters like the organic phase ratio (45–55), pH (2.4–2.6), and flow rate (0.8–1.2 ml/minute) are listed in Table 9. This demonstrated that the analyzed independent variables did not influence the outcomes.

Limit of detection (LOD) and limit of quantification (LOQ)

The LOD and LOQ for AZL and CIL were determined using the slope technique and were observed to be 1.2 and 4 µg/ml and 0.3 and 1 µg/ml correspondingly.

Specificity

The specificity of the method was assessed to find out any interference in the chromatographic separation of the AZL and CIL with a blank sample and placebo. There is no interference was observed. It is shown in Figure 2.

Table 10 shows the summary of all validated parameters. The approach has been validated successfully under optimum conditions, and the validation parameters are also within acceptable bounds.

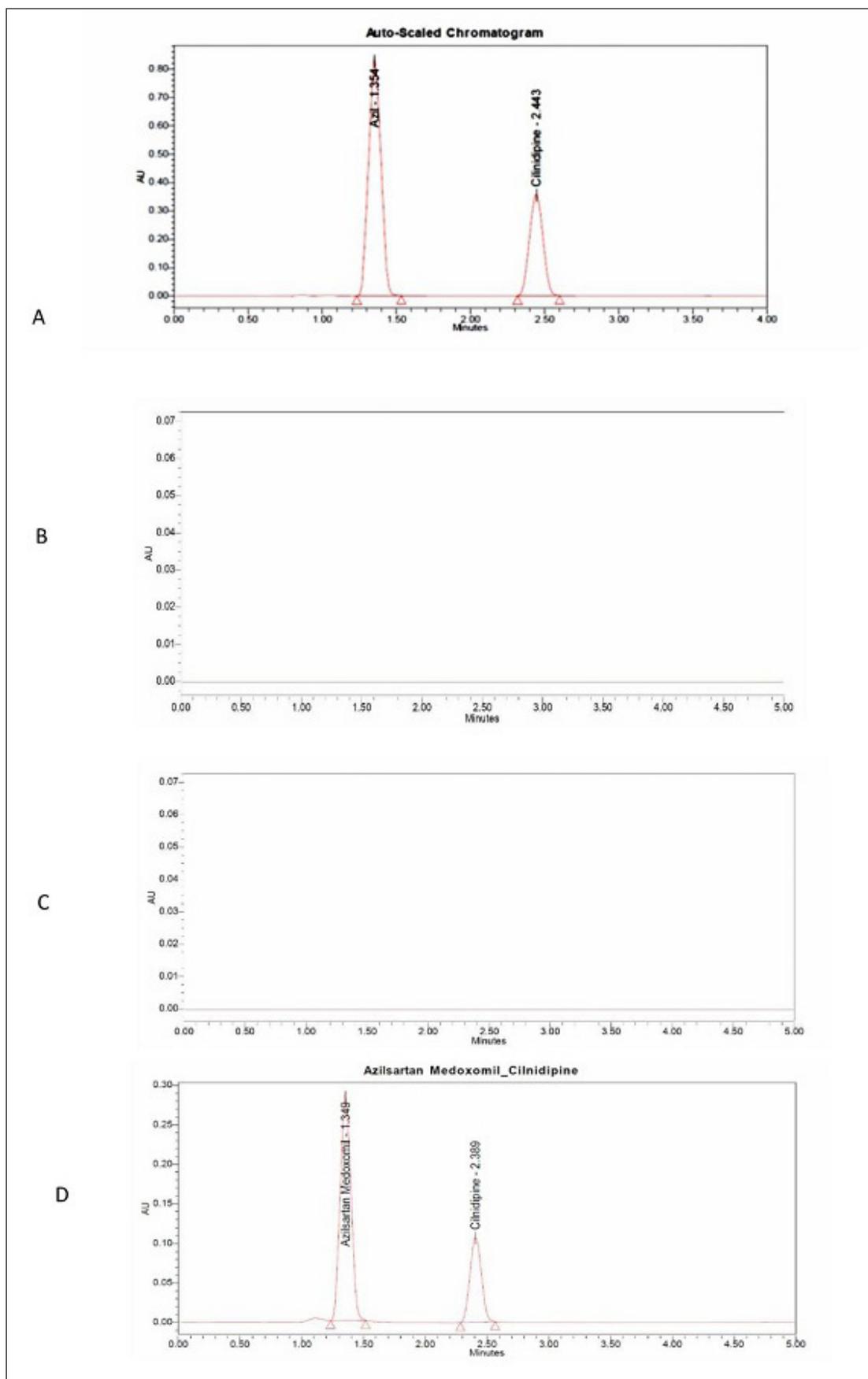


Figure 2. (A) Standard chromatogram of AZL and CIL, (B) blank chromatogram, (C) placebo, and (D) sample chromatogram.

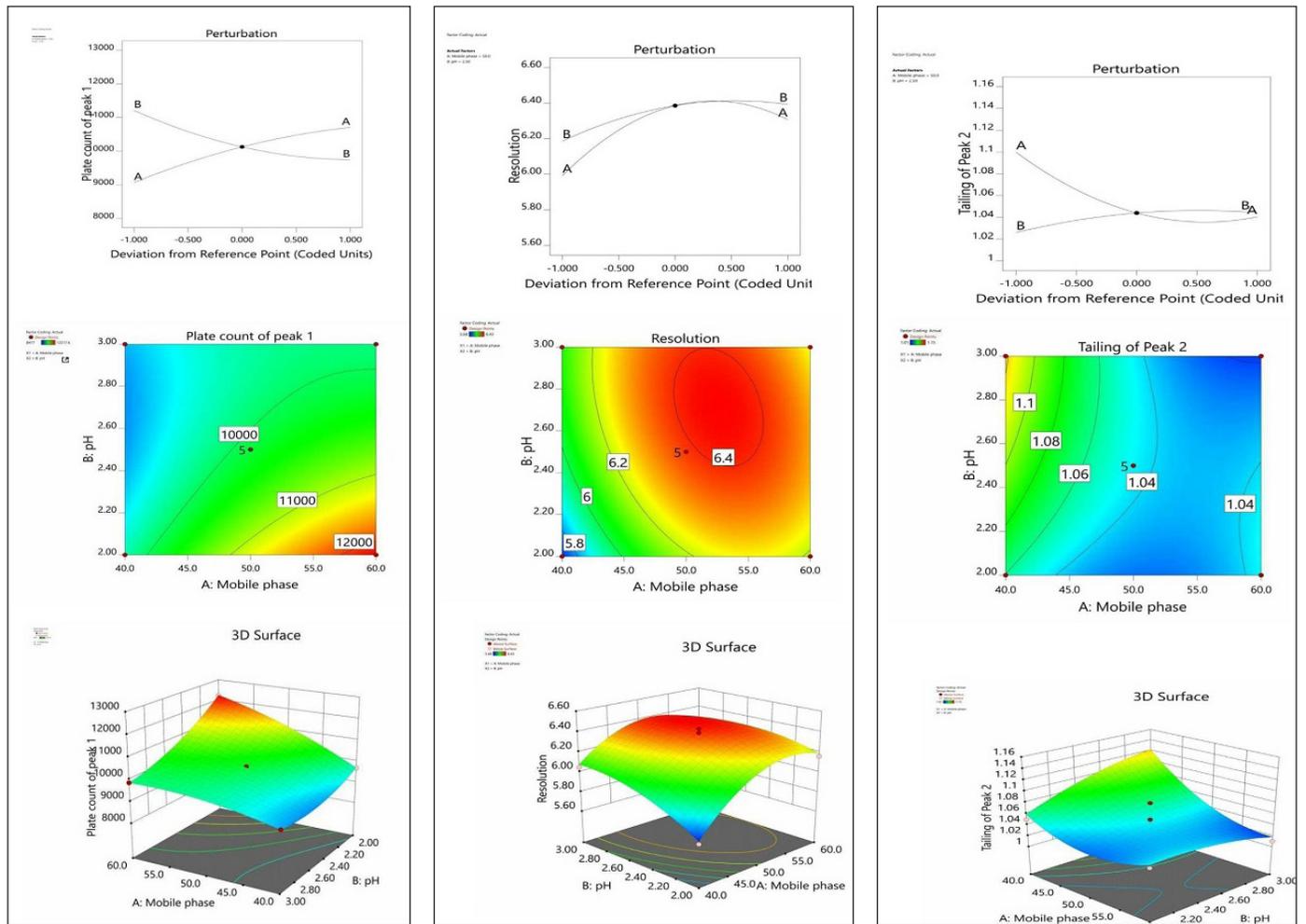


Figure 3. A) Perturbation, counter plot, and 3D response surfaces effect on R1. (B) Perturbation, counter plot, and 3D response surfaces effect on R2. (D) Perturbation, counter plot, and 3D response surfaces effect on R3.

Table 3. ANOVA results for PC of peak 1 (R1).

Source	Sum of squares	df	Mean square	F-value	p-value	
The plate number of peak 1						
Model	1.193E + 07	5	2.385E + 06	3,199.15	<0.0001	Significant
A-Mobile phase	5.346E + 06	1	5.346E + 06	7,170.86	<0.0001	
B-pH	4.303E + 06	1	4.303E + 06	5,771.39	<0.0001	
Lack of fit	2,245.71	3	748.57	1.01	0.4765	Not significant
Resolution						
Model	0.0155	5	0.0031	7.80	0.0088	Significant
A-Mobile phase	0.0070	1	0.0070	17.66	0.0040	
B-pH	0.0007	1	0.0007	1.65	0.2404	
Lack of fit	0.0063	3	0.0021	2.88	0.1666	Not significant
Tailing factor of peak 2						
Model	0.0155	5	0.0031	7.80	0.0088	Significant
A-Mobile phase	0.0070	1	0.0070	17.66	0.0040	
B-pH	0.0007	1	0.0007	1.65	0.2404	
Lack of fit	0.0007	3	0.0002	0.4208	0.7485	Not significant

Table 4. Summary statistics for responses R1, R2, and R3.

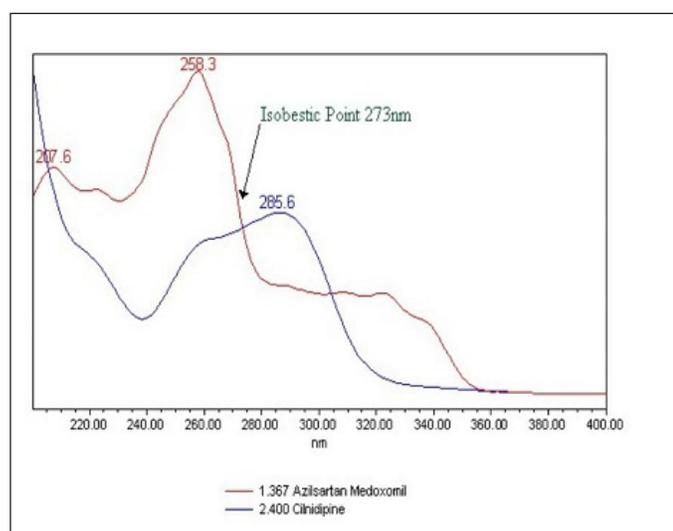
Response	R1 (PC of peak1)	R2 (Resolution)	R3 (Tailing factor)
Std. dev.	27.30	0.0363	0.0200
Mean	10,193.38	6.18	1.05
CV%	0.2679	0.5874	1.89
R^2	0.9996	0.9875	0.8478
Adjusted R^2	0.9993	0.9786	0.7391
Predicted R^2	0.9983	0.9331	0.5595
Adequate precision	202.4996	28.2172	9.1991

Table 5. The optimized method is according to the design of the experiment.

Mobile phase	pH	PC of peak 1	Resolution	Tailing of peak 2	Desirability
50.000	2.500	10,126.400	6.386	1.044	1.000

Table 6. System suitability.

AZL		CIL	
System suitability Parameters	Proposed method	System suitability Parameters	Proposed method
Retention time (Rt)	1.354	Retention time (Rt)	2.443
Theoretical plate (N)	10,113.5	Theoretical plate (N)	3,133.93
Tailing factor (T)	1.04	Tailing factor (T)	1.03

**Figure 4.** UV spectra of AZL (40 µg/ml) and CIL (10 µg/ml).

AZL and CIL tablet assay

AZL % assay was determined to be 99.9% and CIL % assay to be 99.5% when taken as tablets. It states that there was no interference and a high percentage of recovery in the excipients of the formulation during the drug's retention time, demonstrating the technique's suitability for the detection of AZL and CIL in the tablet dosage form.

Forced degradation

Degradation studies revealed that AZL and CIL degradative peaks were not observed except for peroxide

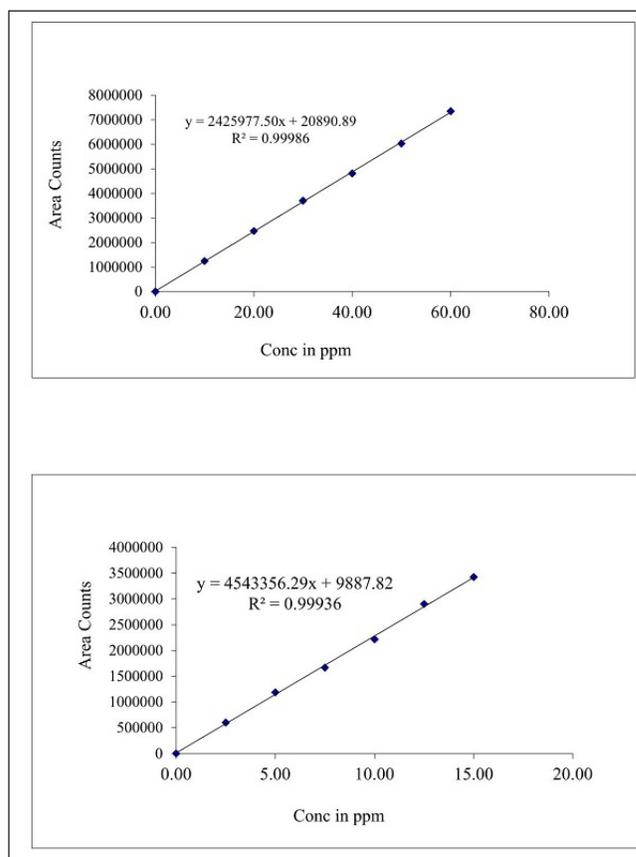
**Figure 5.** Linearity of AZL and CIL.

Table 7. Result of system precision method precision, intermediate precision, and ruggedness.

Sample MP/IP	AZL		CIL	
	% Release MP	% Release IP	% Release MP	% Release IP
1	100.8	100.3	99.1	99.9
2	100.3	99.6	100.4	99.0
3	99.7	100.9	100.1	98.3
4	100.4	101.1	101.7	99.3
5	99.7	100.7	100.1	98.5
6	99.3	100.5	98.4	99.7
Mean ± SD	100 ± 0.557	100.5 ± 0.531	100 ± 1.134	99.1 ± 0.64
%RSD	0.56	0.53	1.13	0.65
Overall mean ± SD	100.25 ± 0.544		99.55 ± 0.887	
Overall RSD (%)	0.54		0.89	

Table 8. Result of accuracy (recovery).

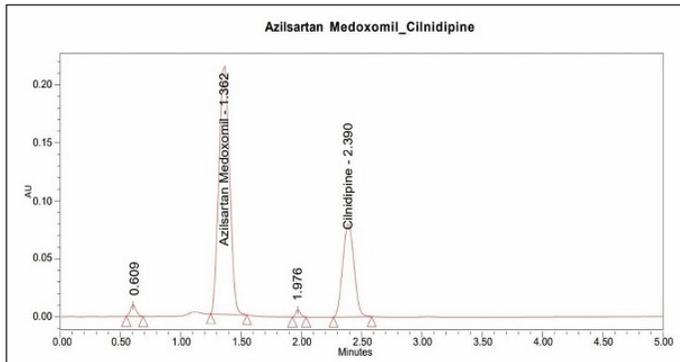
Recovery sample name	% Recovery AZL				% Recovery CIL			
	AZL amount added (mg)	AZL amount recovered (mg)	% Recovery	Mean	CIL amount added (mg)	CIL amount recovered (mg)	% Recovery	Mean
50% -1	2.50	2.51	100.4		0.60	0.60	100.0	
50% -2	2.50	2.49	99.6	100.4	0.60	0.61	101.7	100.5
50% -3	2.50	2.53	101.2		0.60	0.59	100.0	
100% -1	5.00	5.04	100.8		1.20	1.21	100.8	
100% -2	5.00	5.02	100.4	100.33	1.20	1.18	98.3	99.7
100% -3	5.00	4.99	99.8		1.20	1.20	100.0	
150% -1	7.50	7.43	99.1		1.80	1.77	98.3	
150% -2	7.50	7.42	98.9	98.9	1.80	1.78	98.9	98.8
150% -3	7.50	7.40	98.7		1.80	1.79	99.4	
		Mean ± SD	99.9 ± 0.839			Mean ± SD	99.5 ± 0.569	
		%RSD	0.84			%RSD	0.57	

Table 9. Result of robustness of AZL and CIL.

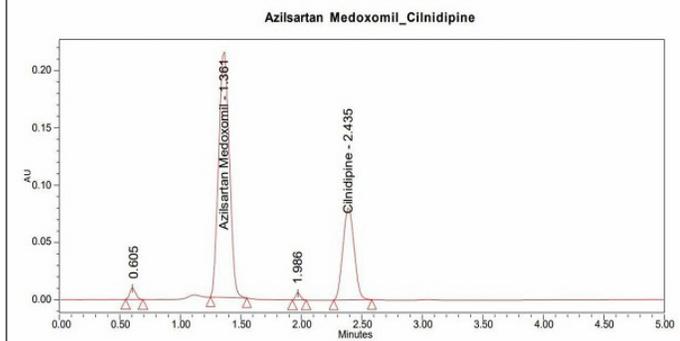
Parameter	Modification	AZL			CIL		
		RT ^a	%RSD of RT	% Assay ^a	RT ^a	%RSD of RT	% Assay ^a
Flow-rate	0.45 ml/minute	1.252	0.4	100.3	2.244	0.75	100.7
	0.55 ml/minute	1.414	0.3	100.7	2.575	0.66	101
pH of the mobile phase	2.25	1.327	0.25	101.9	2.349	0.1	100.5
	2.75	1.381	0.06	101.7	2.493	0.52	100.8
Ratio of the organic phase	45 v/v	1.454	0.25	100.1	3.834	0.1	100.5
	55 v/v	1.395	0.55	100.6	2.025	0.1	98.7

^a Mean of three determinations.**Table 10.** Summary of validation.

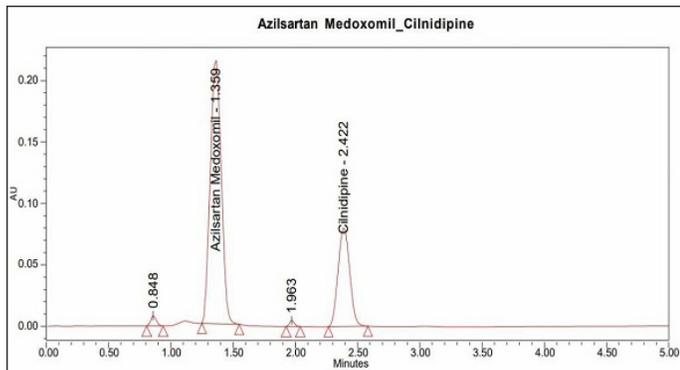
Summary of optimized UPLC	AZL	CIL
Parameter	Results	Results
Regression coefficient	0.99986	0.99936
Range	10–60 µg/ml	2.5–15 µg/ml
System precision	0.26	0.81
Method precision	0.54	0.89
Robustness	Robust	Robust
Specificity	Specific	Specific
Accuracy (recovery)	99.9%	99.5%



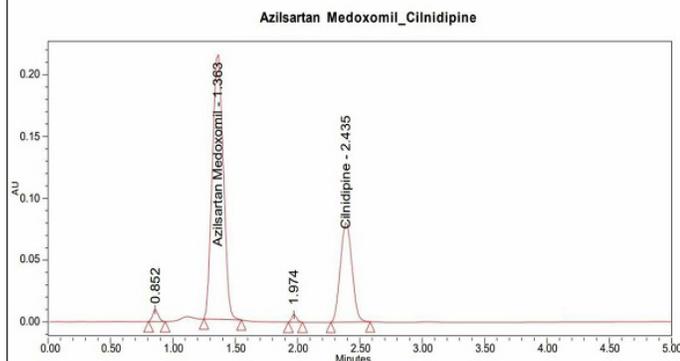
6A- Acid degradation at 18 hours



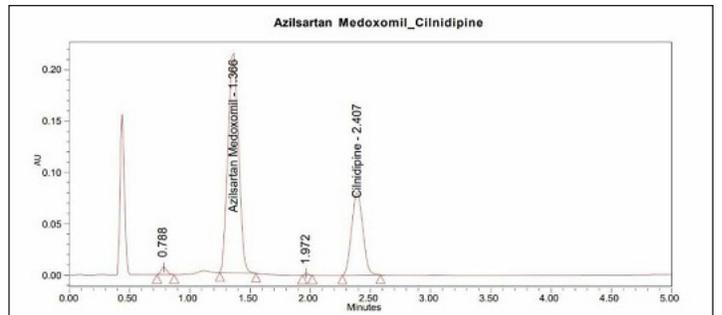
6 B- Acid degradation at 24 hours



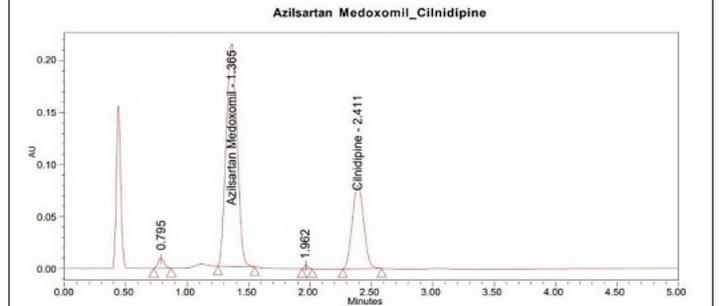
6 C- Alkali degradation at 18 hours



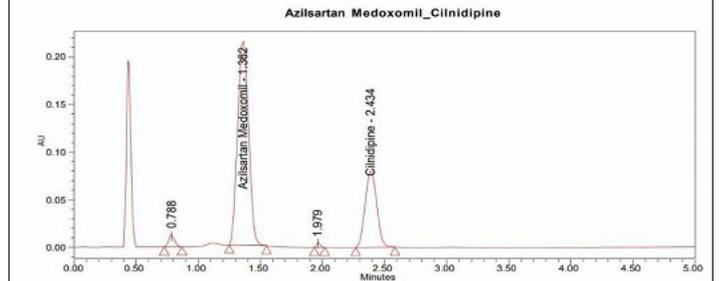
6 D- Alkali degradation at 24 hours



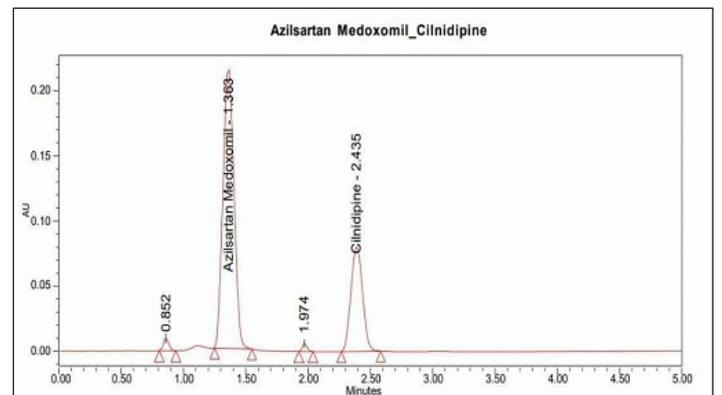
6 E- Peroxide degradation at 12 hours



6 F- Peroxide degradation at 18 hours



6 G- Peroxide degradation at 24 hours



6 H- Hydrolysis degradation at 24 hours

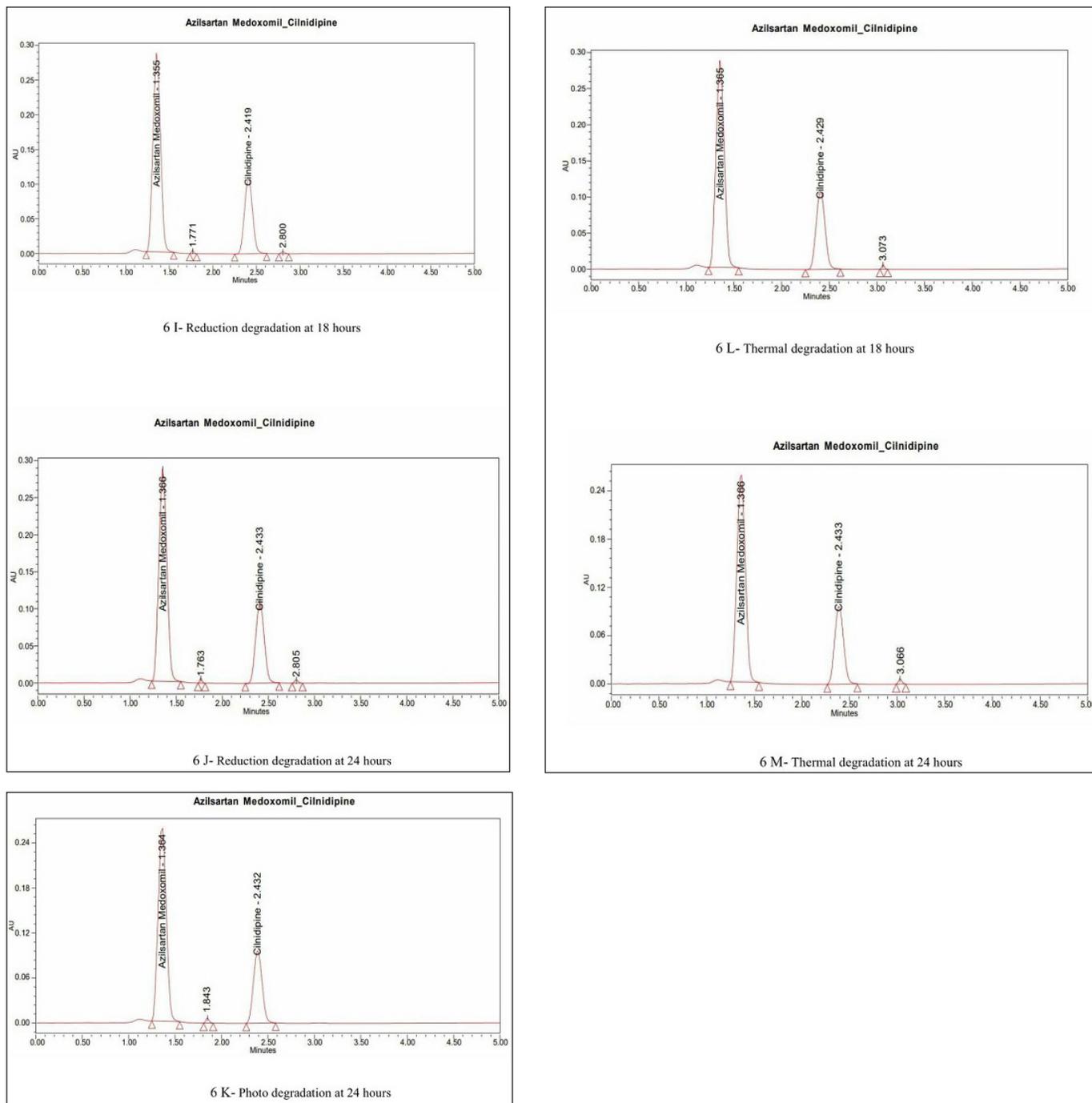


Figure 6. (A) Acid degradation at 18 hours. (B) Acid degradation at 24 hours. (C) Alkali degradation at 18 hours. (D) Alkali degradation at 24 hours. (E) Peroxide degradation at 12 hours. (F) Peroxide degradation at 18 hours. (G) Peroxide degradation at 24 hours. (H) Hydrolysis degradation at 24 hours. (I) Reduction degradation at 18 hours. (J) Reduction degradation at 24 hours. (K) Photo degradation at 24 hours. (L) Thermal degradation at 18 hours. (M) Thermal degradation at 24 hours.

degradation (up to 12 hours), after 18 hours different percentages of degradative peaks were observed under specified conditions (Fig. 6A–M and Table 11). Among all the stress studies, major degradation occurred in peroxide (25.8% and 25.2% for AZL and CIL respectively at 24 hours), and minimum in hydrolysis degradation (3.1% and 5.2% for AZL and CIL respectively at 24 hours). AZL and CIL were exposed to degradation conditions

degradation peaks were observed, Hence the formulation does not expose to critical conditions.

A QbD-based UPLC approach for AZL and CIL has not yet been developed. Some HPLC and UV spectrometric techniques have been published. However, there are other methods for estimating AZL and CIL utilizing different chromatography methods, such as HPTLC with QbD (Prajapati *et al.*, 2022), HPLC (Andhalea and Nikalje, 2022; Solanki *et al.*, 2022), and UV

Table 11. Forced degradation.

Sample exposure condition	Total no. of products with Rt	AZL		CIL	
		Degradation %	Recovery %	Degradation %	Recovery %
Acidic, 1N + Heat					
18 hours	2 (0.609, 1.976)	19.5	80.4	15.2	84.7
24 hours	2 (0.605, 1.986)	24.5	75.4	23	76.9
Alkali					
18 hours	2 (0.848, 1.963)	18.7	81.2	17.8	82.1
24 hours	2 (0.852, 1.974)	23.5	76.4	21.9	78
Peroxide					
12 hours	2 (0.788, 1.912)	16.8	83.1	15.2	84.7
18 hours	2 (0.795, 1.964)	21.7	78.2	21.4	78.5
24 hours	2 (0.788, 1.979)	25.8	74.1	25.2	74.7
Hydrolysis					
24 hours	2 (0.852, 1.974)	3.1	96.8	5.2	94.7
Reduction					
18 hours	2 (1.771, 2.800)	9.4	90.5	11.5	88.4
24 hours	2 (1.783, 2.805)	14.6	85.3	14.4	85.5
Photolytic					
24 hours	1 (1.843)	11.1	88.8	5.4	94.5
Thermal					
18 hours	1 (3.073)	5.3	94.6	12.2	87.7
24 hours	1 (3.066)	5.9	94	16.3	83.6

Spectroscopic methods (Jani and Patel, 2018a, 2018b), the present chemometrics-assisted chromatographic approach ensures reliable, accurate, and effective methodology while saving time and reagents.

CONCLUSION

For the assessment of AZL and CIL in bulk and pharmaceutical oral pharmaceutical formulations, a simple, precise, accurate, specific, robust, and stable UPLC technique was used by the DoE approach employing a CCD. The method of solvent used in this technique was cost-effective. Values of % RSD were within 2% and the accuracy of the procedure was confirmed by the 99.9% and 99.5% recovery. The outcomes for the UPLC technique were expressed as good. The UPLC technique is more sensitive, and precise than the techniques using spectroscopy. This approach is suitable for routine analysis of AZL with CIL in pharmaceutical dosage forms and bulk drugs.

AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal editors (ICMJE) requirements/guidelines.

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CONFLICTS OF INTEREST

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ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

DATA AVAILABILITY

All data generated and analyzed are included in this research article.

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