The Bioscan
AN INTERNATIONAL QUARTERLY JOURNAL OF LIFE SCIENCES
21(2): S.I (2), 727-744, 2026
www.thebioscan.com

ANALYTICAL QUALITY BY DESIGN (AQbD)-BASED RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF AZILSARTAN

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DOI: https://doi.org/10.63001/tbs.2026.v21.i02.S.I(2).pp727-744

KEYWORDS
Azilsartan, RP-HPLC, AQbD, Central Composite Design, Method Validation, ICH Q2(R2) Guidelines

Received on:
14-04-2026
Accepted on:
03-05-2026
Published on:
12-05-2026

Abstract

A good and effective consistent analytical method of quantitative determination of azilsartan in bulk and tablet dose forms was developed by the application of Analytical Quality by Design (AQbD) principles. A Central Composite Design (CCD) has been applied to optimize the method and to determine the impact of the critical method parameters on the chromatographic performance. Separations were done in Agilent Zorbax C18 column (250 mm × 4.6 mm, 5 µm) using a mobile phase of phosphate buffer and methanol in the proportions of 50:50 (v/v) at a flow rate of 1.0 mL/min. The wavelength of detection was 256 nm, and the retention time of azilsartan was about 2.9 minutes. The constructed method was confirmed as per the ICH Q2(R2) guidelines as it exhibits excellent linearity (r2 = 0.9999), accuracy, precision, specificity and robustness. The percentage relative standard deviation (percentage RSD) was less than 2.0 percent indicating that it is highly precise and recovery studies yielded an average recovery of 100.36 percent. The limit of detection (LOD) and the limit of quantitation (LOQ) were calculated to be 3.26 µg/mL and 9.86 µg/mL, respectively, calculated per ICH Q2(R2) using standard deviation and slope method (sigma/S). The proposed approach is easy, quick, and cost-effective, which is why it can be applied to routine studies of azilsartan in pharmaceutical preparations. The use of AQbD offered a better understanding of the method and its robustness that could be successfully implemented in the area of quality control.

1. INTRODUCTION

Cardiovascular diseases, stroke, heart attack and kidney failure are major causes of death in the world with hypertension being one of the major risk factors in their development [1]. Reduction of blood pressure is imperative in thwarting complications and lessening mortality. The angiotensin II receptor blockers (ARBs) are widely recognised as effective and safe antihypertensive agents [2]. Azilsartan,

another high-efficiency ARB, is a selective antagonist of the angiotensin II type 1 (AT 1) receptor, which causes an opening of blood vessels, a decrease in the production of aldosterone, and ultimately, a decrease in blood pressure [3]. Azilsartan has also received a lot of attention as a treatment option of hypertension in light of its desirable pharmacokinetic profile and enhanced binding affinity on the AT1 receptor [4].

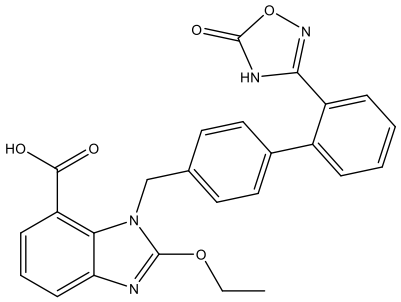

Figure 1: Chemical structure of Azilsartan

Sound and high quality of analytical methods is very critical in quality assurance and control of pharmaceutical products [5]. Reverse-phase high-performance liquid chromatography (RP-HPLC) is a widely used technique in the quantitative analysis of drugs in raw materials and formulations, due to its sensitivity, accuracy and precision [6]. Analytical Quality by Design (AQbD) is a systematic approach to the development of an analytical method, which focuses on the robustness of a method, risk management, and understanding the process [7], [8].

AQbD is a risk-based, science-based approach to help identify and optimise critical method parameters (CMPs) and their effects on critical quality attributes (CQAs) [9]. Central Composite Design (CCD) is a popular experimental design method that is used to optimize a method by exploring the effects of multiple variables with a minimum number of experimental trials [10]. CCD can be used to create predictive models and can also aid in identifying optimal chromatographic conditions with few experiments [11].

Our research group has successfully used the AQbD methodology in the development and validation of RP-HPLC methods of various pharmaceutical compounds. Baste et al. designed and validated an AQbD-based RP-HPLC-method to determine the antidiabetic agent pioglitazone using the CCD optimization with a phosphate bufferacetonitrile mobile phase system, which yielded excellent validation parameters (r 2 = 1.0000, recovery = 99.84%, LOD = 0.24 ug/mL) [12]. Likewise, the same AQbD/CCD methodology has been used to febuxostat, a

xanthine oxidase inhibitor used to treat gout, and has shown the power and adaptability of this technique to different classes of drugs (r 2 = 0.9997, recovery = 100.17% LOD = 0.12 ug/mL) [13].

Although there are a number of methods that can be used to quantify azilsartan, many of them do not consider the use of AQbD, which is essential in the development of methods and acceptance by regulatory authorities [14], [15]. The present study is therefore aimed at developing and validating a simple, efficient and specific RP-HPLC method of the estimation of azilsartan using AQbD principles. The method is optimized based on CCD and tested according to International Council for Harmonisation (ICH) Q2(R2) guidelines in order to prove its applicability in routine quality control [16].

2. MATERIALS AND METHODS

2.1. Chemicals and Reagents

The source of azilsartan reference standard was Arni Analytical Laboratory, Nashik, India. The Methanol and potassium dihydrogen orthophosphate of HPLC grade were sourced commercially. The quality of all reagents that were used in the study was of analytical standard and ultrapure water was used throughout the experiments.

2.2. Instrumentation

The chromatographic analysis was done using a Thermo Ultimate 3000 HPLC system and Chromeleon 7.3 software. The separation was achieved on an Agilent Zorbax C18 column (250 mm × 4.6 mm, 5 µm particle size). The same software was used for data acquisition and processing.

2.3. Chromatographic Conditions

The mobile stage was a mixture of phosphate buffer and methanol at a 50:50 (v/v) ratio, but filtered using a 0.45 µm membrane filter and degassed prior to use. The flow rate was kept at 1.0 mL/min and it was detected at a wavelength of 256 nm. The volume of injection was adjusted to 20 µL and analysis performed under isocratic conditions at ambient temperature.

2.4. Preparation of Buffer Solution

A 0.01 M buffer solution containing potassium dihydrogen orthophosphate was prepared by dissolving the correct quantity of the salt in distilled water and filtering with a 0.45 µm membrane filter.

2.5. Preparation of Standard Solution

Weigh 40 mg of azilsartan and put it into a 50 mL volumetric flask. Put in 30 mL of methanol and sonicate at 5 minutes to fully dissolve. Add methanol to make the volume up to 50 mL. Further dilute 5 mL of this solution to 100 mL using methanol to obtain the working standard solution.

2.6. Preparation of Sample Solution

Weigh accurately 10 tablets and determine the average weight. Powder the tablets into fine powder. Measure the weight of an equivalent quantity of powder of the weight of an average tablet and add to a 100 mL volumetric flask. To extract the drug, add 60 mL of methanol and sonicate the mixture (10 minutes). Make up the volume to 100 mL with methanol. Pass solution through Whatman filter paper No. 41. To make the sample solution, dilute 5 mL of the filtrate to 50 mL with methanol.

2.7. Experimental Design (Central Composite Design)

A Central Composite Design (CCD) was utilized to determine the effects of important parameters of the method. As the independent variable, the percentage of methanol in the mobile phase and flow rate were selected, and the retention time of azilsartan was chosen as the response variable.

The CCD model consisted of 13 experimental runs, including factorial points, axial points and center points. Design-Expert 11 Software (Stat-Ease Inc., Minneapolis, USA) was used to perform experimental design and statistical analysis. The design enabled construction of a quadratic regression model to examine the effects of interaction between variables and to determine optimum chromatographic conditions.

2.8. Optimization of Chromatographic Conditions

Preliminary tests were carried out to see what the right composition of the mobile phase was. The combination of phosphate buffer and methanol gave a satisfactory peak shape and resolution. The optimum

chromatographic conditions were established with the help of CCD analysis in order to achieve the minimum retention time with acceptable peak symmetry and resolution.

Table 1: Central composite design showing 13 randomized trial runs

Run	% Methanol	Flow rate (mL/min)	Rt azilsartan (min)
1	50	1.0	2.924
2	60	1.2	1.954
3	50	1.0	2.941
4	40	0.8	3.639
5	35	1.0	4.791
6	50	1.0	2.951
7	50	1.0	2.888
8	50	0.7	3.431
9	50	1.0	2.954
10	40	1.2	2.888
11	50	1.2	2.246
12	64	1.0	1.954
13	60	0.8	2.884

Table 2: Experimental factors and levels used in central composite design

Name	Low (-1)	Medium (0)	High (+1)
Independent factor			
A: Methanol %	40	50	60
C: Flow rate (mL/min)	0.8	1.0	1.2
Dependent factor			
R1	Retention time of Azilsartan		

Table 3: Experimental results and optimized conditions

Parameters	Description
Program	Isocratic
Mobile phase	Phosphate buffer:Methanol (50:50 v/v)
Wavelength	256 nm
Injection volume	20 µL
Flow rate	1 mL/min

2.9. Method Validation

The developed method was validated in accordance with the International Conference on Harmonisation (ICH Q2(R2)) guidelines [16]. The validation parameters evaluated were specificity, linearity, accuracy, precision, limit of detection (LOD), limit of quantitation (LOQ), and robustness.

2.10. Specificity

Specificity was evaluated by comparing chromatograms of blank, standard, and sample solutions to ensure that there was no interference at the retention time of azilsartan.

2.11. Linearity

Linearity was assessed within the concentration range of 80 to 120% by plotting peak area versus concentration. A calibration curve was constructed, and the correlation coefficient (r^2) was calculated.

2.12. Precision

Precision was evaluated in terms of system precision and intermediate precision. The results were expressed as percentage relative standard deviation (%RSD).

2.13. Accuracy

Recovery studies at three levels (80%, 100%, and 120%) were conducted to determine accuracy, and percentage recovery was calculated.

2.14. Limit of Detection (LOD) and Limit of Quantitation (LOQ)

LOD and LOQ were calculated using the standard deviation of the response and the slope of the calibration curve according to

ICH Q2(R2) guidelines using the σ/S method.

2.15. Robustness

Robustness was evaluated by making small deliberate variations in chromatographic conditions such as flow rate and mobile phase composition, and the impact on method performance was assessed.

3. RESULTS AND DISCUSSION

3.1. Optimization of Chromatographic Conditions

To evaluate the effect of key method parameters on the retention time of azilsartan, a Central Composite Design (CCD) was employed to optimize the chromatographic conditions by analyzing the effects of critical method parameters, including the percentage of methanol in the mobile phase and flow rate. Initial experiments demonstrated that a blend of phosphate buffer and methanol provided superior peak symmetry and resolution compared to other solvent system.

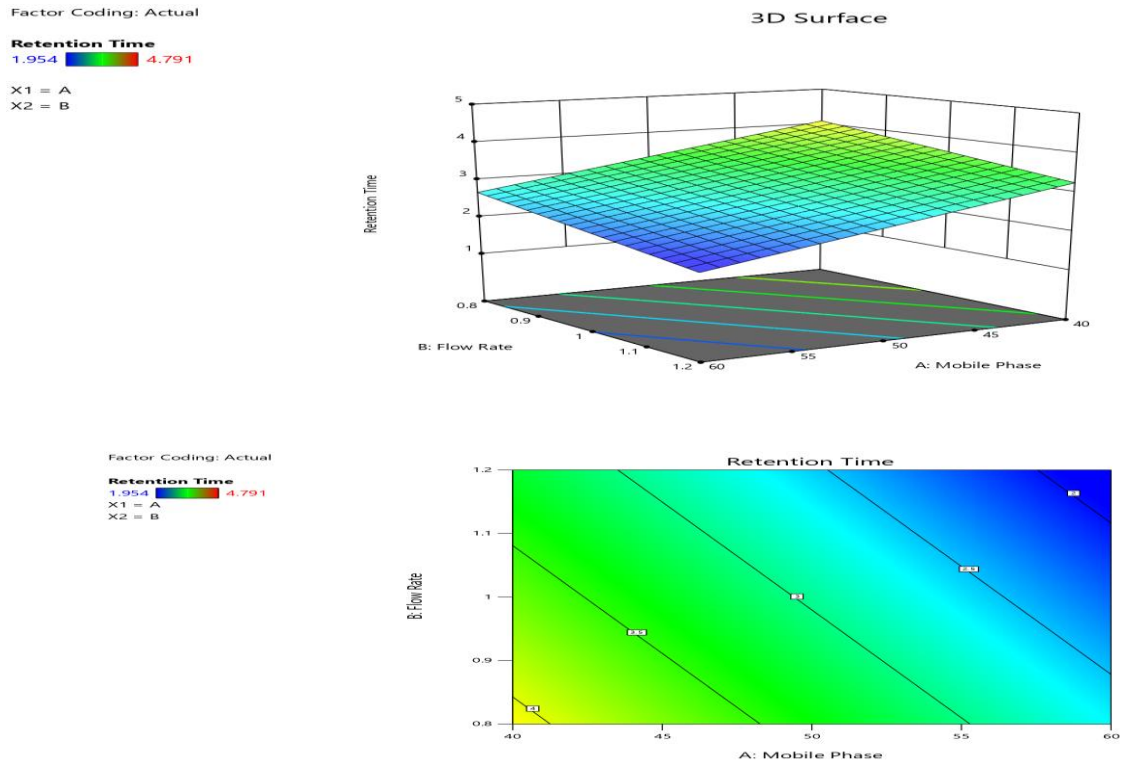

Figure 2: 2D and 3D plots showing the interaction effect of critical factors on retention time of azilsartan

The CCD model generated 13 experimental runs, and the results obtained were utilized to create a quadratic regression model. The optimized chromatographic conditions consisted of a mobile phase of phosphate buffer and methanol (50:50 v/v) at a flow rate of 1.0 mL/min, which produced a sharp and symmetrical peak at a retention time of approximately 2.9 minutes, indicating excellent separation of azilsartan with minimal analysis time.

3.2. Effect of Method Parameters on Retention Time

Response surface methodology was used to systematically determine the effect of independent variables on retention time. As observed, increasing the percentage of methanol in the mobile phase resulted in decreased retention time due to increased elution strength. Similarly, retention time decreased with an increase in flow rate, as the analyte traveled faster through the column.

The interaction between these variables was visualized using 2D contour plots and 3D response surface plots. These graphical representations revealed that retention time was significantly and negatively affected by both methanol concentration and flow rate. The CCD model enabled the determination of a design space within which optimal chromatographic performance could be achieved.

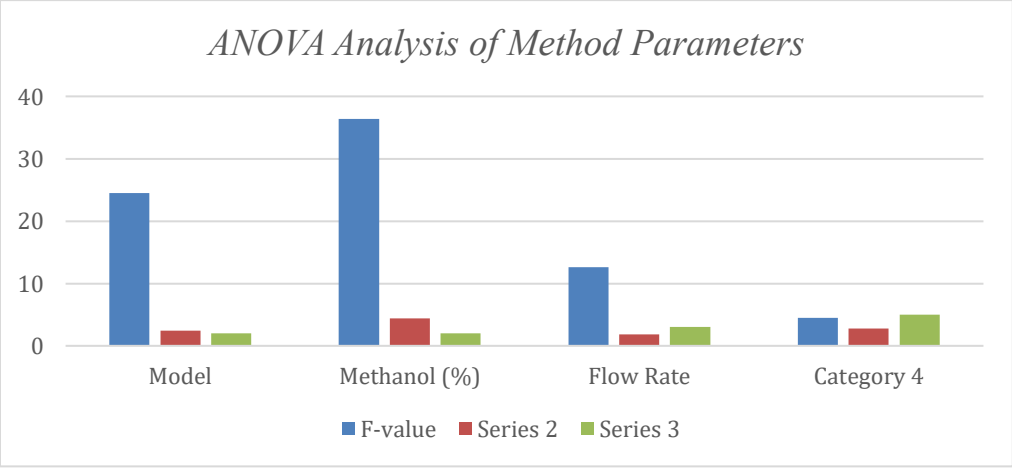

Figure 3: ANOVA analysis

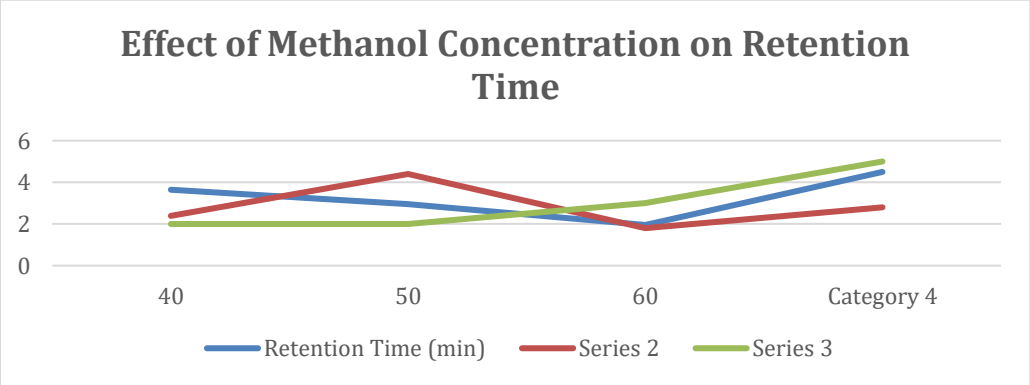

Figure 4: Effect of methanol concentration on retention time

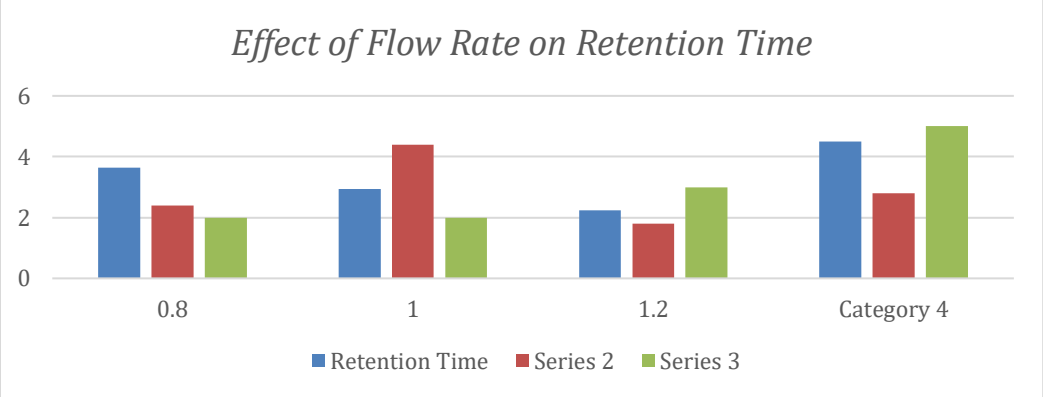

Figure 5: Effect of flow rate on retention time

3.3. Statistical Analysis (ANOVA)

Analysis of variance (ANOVA) was performed to evaluate the statistical significance of the model. The model F-value indicated that the developed model was statistically significant, with a very low probability of error. The p-values of the model terms were determined to be less than 0.05, demonstrating that the selected independent variables had a significant effect on the response.

Table 4: Statistical parameters obtained from ANOVA

Source	Sum of Squares	df	Mean Square	F-value	p-value	Significance
Model	5.47	2	2.74	24.52	0.0001	significant
A-Mobile Phase	4.06	1	4.06	36.41	0.0001	-
B-Flow Rate	1.41	1	1.41	12.62	0.0052	-
Residual	1.12	10	0.1116	-	-	-
Lack of Fit	1.11	6	0.1855	253.61	< 0.0001	significant
Pure Error	0.0029	4	0.0007	-	-	-
Cor Total	6.59	12	-	-	-	-

The Model F-value of 24.52 indicates that the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise. P-values less than 0.0500 indicate that model terms are significant.

The regression model showed a high level of agreement between the predicted and observed values, demonstrating that the model is adequate. The lack-of-fit test was also evaluated, and the findings indicated that the model was appropriate to explain the relationship between the variables and response. These results demonstrate the relevance of the AQbD approach in method optimization.

3.4. Response Surface and Optimization Analysis

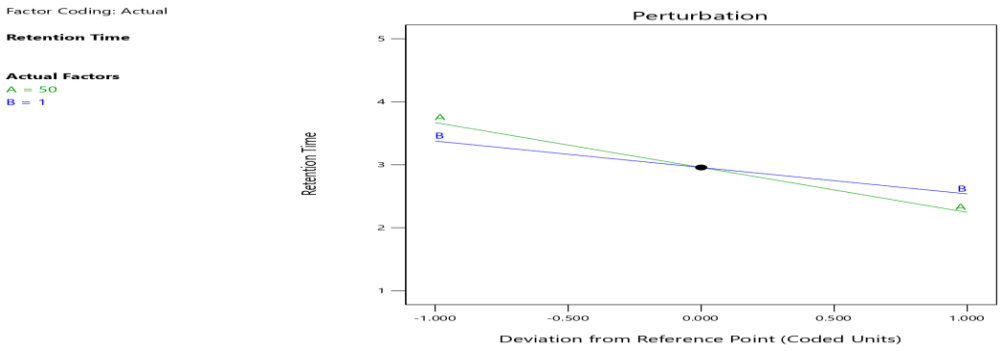

Figure 6: Perturbation plot showing effect of factors on retention time

The perturbation plot demonstrated the comparative contribution of each factor to retention time, with both methanol concentration and flow rate being important parameters. As shown in Figure 6, methanol percentage and flow rate had the most significant negative effect on retention time of azilsartan, with retention time decreasing as both flow rate and mobile phase methanol concentration increased.

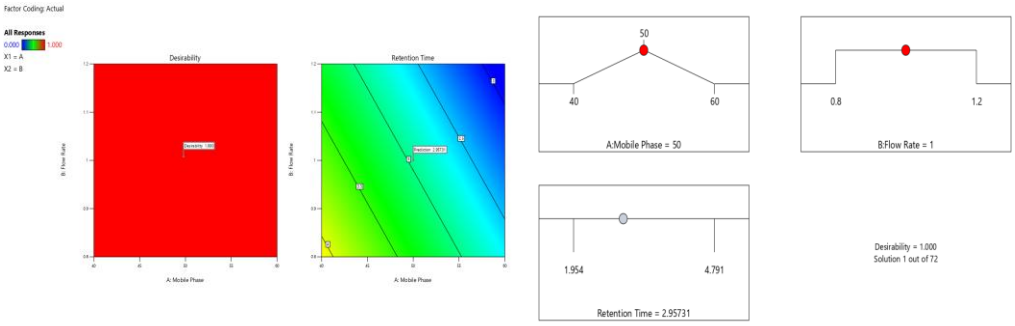

Figure 7: Graphical representation of constraints accepted for determination of global desirability and obtained optimal condition

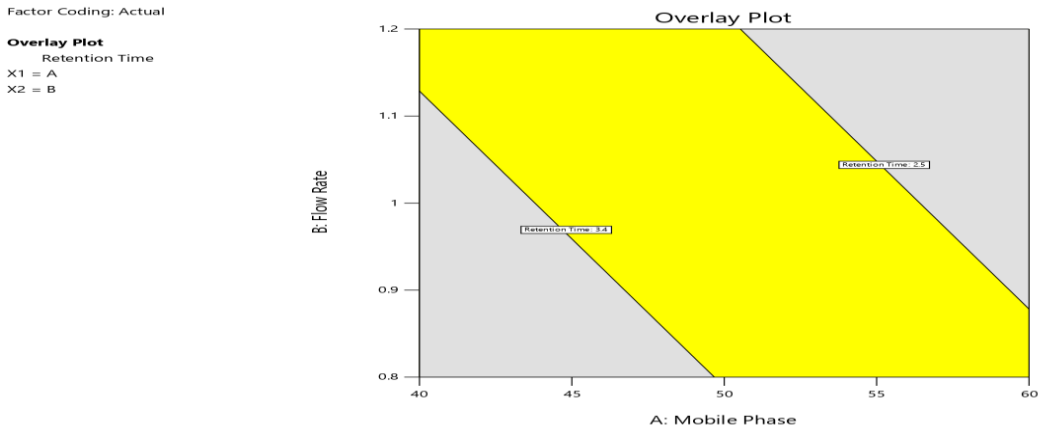

Figure 8: Overlay plot showing the sweet spot where the desired response is met

The overlay plot was used to identify the design space (sweet spot) in which all method criteria were met simultaneously. The optimization procedure ensured that the selected chromatographic conditions offered a balance of minimum retention time, acceptable peak symmetry, and reproducibility. The application of CCD greatly minimized the number of

experimental trials needed while providing a high degree of method understanding.

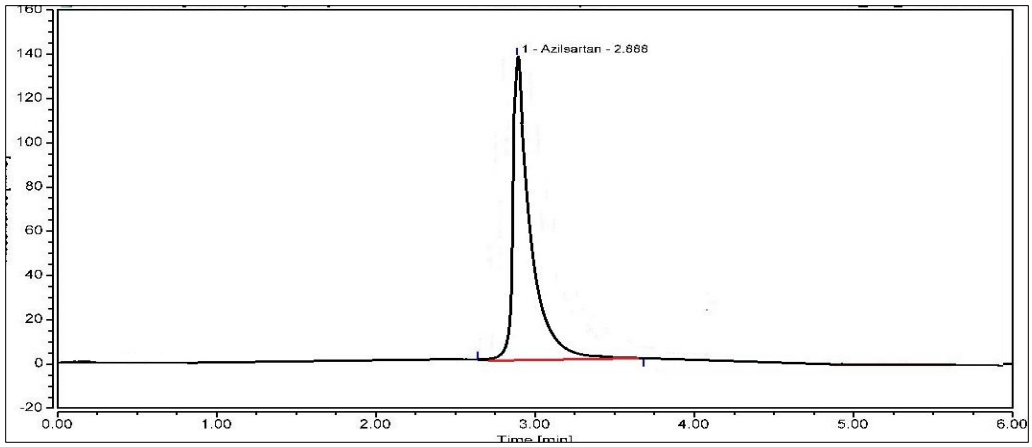

Figure 9: Optimized chromatogram of azilsartan

3.5. Validation of Developed Method

3.5.1. Specificity

The method was demonstrated to be specific as indicated by the comparison of chromatograms of blank, standard, and sample solutions. No interfering peaks were observed at the retention time of azilsartan, demonstrating that the method is specific and can be used to analyze the sample in the presence of excipients.

Table 5: Specificity results for azilsartan

Sr. No.	Azilsartan	Retention Time (min)
1	Blank	0.000
2	Standard Solution	2.925
3	Sample Solution	2.924

3.5.2. Linearity

The method was found to be highly linear within the concentration range of 80–120%. The calibration curve exhibited excellent linear correlation between peak area and concentration with

a correlation coefficient (r^2) of 0.9999. This confirms that the method is reliable for quantitative analysis.

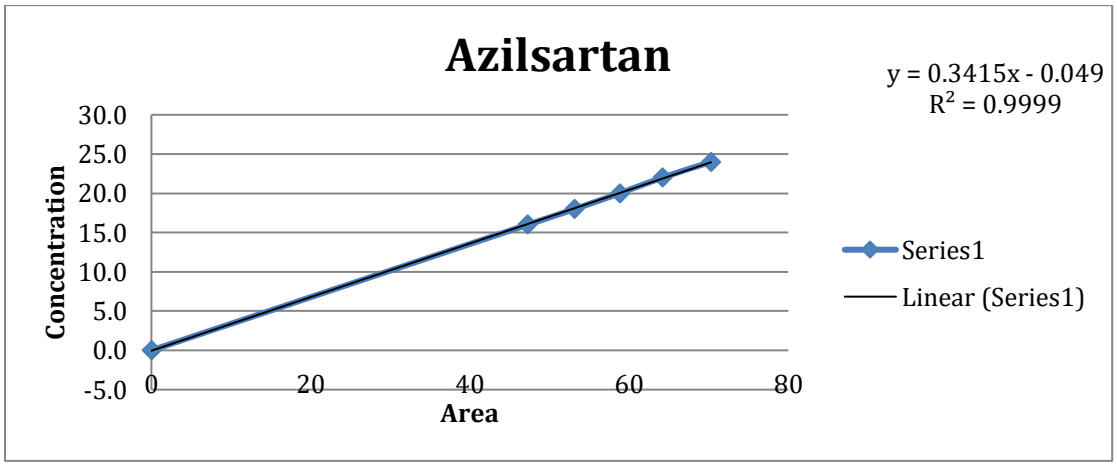

Figure 10: Calibration curve of azilsartan

Table 6: Linearity results for azilsartan

Sr. No.	Concentration (ppm)	Concentration of Solution	Diluted to (mL)	Area
1	16	80	50	47.267
2	18	90	50	53.157
3	20	100	50	58.827
4	22	110	50	64.183
5	24	120	50	70.291

3.5.3. Precision

The precision of the method was assessed through system precision and intermediate precision. The percentage relative standard deviation (%RSD) values were found to be less than 2.0%, indicating excellent reproducibility and consistency of the method under varying conditions.

3.5.4. Accuracy

Recovery studies were conducted at three levels (80%, 100%, and 120%) to determine the accuracy of the method. The percentage recovery of azilsartan was within acceptable ranges (approximately 99–101%), indicating the validity of the method.

Table 7: Accuracy results for azilsartan

Sr. No.	Level	mg of drug spiked	Area	mg of drug Recovered	% Recovery
1	80%	0.032000	36.692	0.031916	99.74
2	100%	0.040000	46.148	0.040141	100.35
3	120%	0.048000	55.724	0.048471	100.98
Average					**100.36**
Std. dev					**0.62**
%RSD					**0.62**

3.5.5. Solution Stability

The %RSD was found to be less than 2%, indicating that sample solutions containing azilsartan 40 mg are stable for up to 24 hours at room temperature.

Table 8: Solution stability results for azilsartan

Sr. No.	Hours	Area	% Assay	% Assay Difference
1	Initial	52.000	100.28	——
2	8 Hour	52.290	99.69	0.59
3	24 Hour	52.160	99.57	0.71

3.5.6. Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The LOD and LOQ values were calculated according to ICH Q2(R2) guidelines using the standard deviation of the response and the slope of the calibration curve (σ/S method). The LOD was determined to be 3.26 µg/mL and the LOQ was 9.86 µg/mL. These values indicate that the method is sufficiently sensitive and can detect and quantify low levels of azilsartan with high accuracy. While these values are higher than those reported for pioglitazone (LOD = 0.24 µg/mL, LOQ = 0.74 µg/mL) [12] and febuxostat (LOD = 0.12 µg/mL, LOQ = 0.35 µg/mL) [13], they are acceptable for the intended application in pharmaceutical quality control of azilsartan formulations.

3.5.7. Robustness

The robustness of the method was evaluated by introducing small deliberate changes in chromatographic conditions, including flow rate and mobile phase composition. The results indicated that there were no significant differences in retention time or peak area, confirming the robustness of the method.

Table 9: Validation results of azilsartan

Parameters	Azilsartan
Specificity	No interference observed in Standard & Sample due to Blank
Linearity	
Range (µg/mL)	80–120%
Y-Intercept	-0.0490
Slope	0.3415
Correlation coefficient	0.9999
Accuracy	
% recovery	100.36
Precision	
System Precision	RSD - 0.41%
Method Precision	% Assay - 100.31
Intermediate precision	% Assay – 99.65
LOD (µg/mL)	3.26 (calculated per ICH Q2(R2) using σ/S method)
LOQ (µg/mL)	9.86 (calculated per ICH Q2(R2) using σ/S method)
Stability of solution	RSD < 2%

3.6. Overall Method Performance

The developed RP-HPLC method demonstrated excellent performance with respect to sensitivity, precision, accuracy, and robustness. The application of AQbD

principles led to a comprehensive understanding of method variables and their interactions, enabling the creation of a highly reliable analytical method suitable for routine quality control. This systematic approach, consistent with the methodologies successfully applied by our research group for pioglitazone [12] and febuxostat [13], demonstrates the versatility and effectiveness of AQbD-based method development across diverse pharmaceutical compounds with different physicochemical properties and therapeutic applications.

4. CONCLUSION

A powerful and robust RP-HPLC method was successfully developed and validated to estimate azilsartan in bulk and tablet dosage forms by adopting an Analytical Quality by Design (AQbD) approach. The use of Central Composite Design (CCD) made it possible to optimize critically important method parameters systematically, and this has resulted in increased knowledge and control of the method. The optimized chromatographic conditions offered a fast analysis with a retention time of about 2.9 minutes and a good peak symmetry and resolution.

The method also has excellent performance in terms of linearity (r 2 = 0.9999), precision (percentage of RSD = 2.0%), accuracy (percentage of recovery = 100.36%), specificity and robustness and thus meets all the requirements of ICH Q2(R2) guidelines. The LOD (3.26 µg/mL) and LOQ (9.86 µg/mL), calculated per ICH Q2(R2) using the σ/S method are acceptable in pharmaceutical quality control applications. The presence of AQbD principles made sure that a design space was identified, which added to the enhanced reliability and repeatability of the method.

The resulting procedure is easy, inexpensive and can be applied in the process of routine quality control examination of azilsartan in pharmaceutical preparations. This work, along with the previous successful applications of the AQbD/CCD methodology in the development of the two high-quality analytical performance applications: of pioglitazone [12] and febuxostat [13], demonstrates the advantages of the AQbD-based approach to developing methods in achieving high-quality analytical performance and justifies its use in regulatory and industrial applications. The fact that the methodology

has been successful with a variety of different classes of drugs is a testament to its strength and the fact that it can be used to analyze pharmaceuticals.

5. DECLARATIONS

Acknowledgement

The authors wish to express their gratitude to the Dean and faculty of the School of Pharmaceutical Sciences, Sandip University, Nashik, for providing the facilities and support to conduct this study.

Conflict of Interest

None

Funding

None

Ethics Statement

None

Informed Consent

None

Data Availability

Data are available upon reasonable request from the corresponding author.

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