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Research Article

ACHIEVING ANALYTICAL EXCELLENCE: QBD-DRIVEN DEVELOPMENT AND VALIDATION OF REVERSED PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY-BASED SIMULTANEOUS ESTIMATION METHOD FOR AZILSARTAN MEDOXOMIL AND CILNIDIPINE

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ABSTRACT

Objectives: This article aimed to develop a method for the simultaneous estimation of Azilsartan medoxomil (AZT) and cilnidipine (CIL) by incorporating a quality-by-design approach, which is used to develop the most accurate and precise analytical method in comparison with traditional method development.

Methods: At the initial phase, researchers conducted high-performance liquid chromatography (HPLC) trials as per the traditional method development protocol, and factors such as mobile phase, flow rate, and column temperature were taken into consideration for finalizing the most suitable trial. Quality-by-Design approach is then implemented for further study. As per the Box–Behnken design (BBD) system, the method was optimized. The most suitable optimized solution is used for validation.

Results: Based on traditional method development, methanol and 0.1% v/v ortho phosphoric acid selected as a mobile phase in an 82:18 ratio, the flow rate was selected as 1 mL/min, and column temperature was selected at 40°C. Based on these factors, 17 HPLC runs were performed as per BBD protocol the p-values for quadratic model for all factors was found <0.0500, which shows the quadratic model is best for proposed study by which method get optimized, and the validation was carried out on the most appropriate optimized solution. The R^2 for AZT and CIL was found to be 0.9998 and 0.9999, respectively. The method was linear for both the drugs, and accuracy was found to be 100.45% for AZT and 99.49% for CIL. Other validation parameters were also found within limits.

Conclusion: At the end of the study, it was found that the developed method was accurate, precise, linear, specific, and reproducible. Method is cost-effective and can be able to used for routine analysis in laboratories as well as in industries.

Keywords: Azilsartan, Cilnidipine, Quality by design, Box–Behnken design, Antihypertensive agents, High-performance liquid chromatography, Simultaneous estimation, Method development, Validation, Design Expert 13.

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INTRODUCTION

Pharmaceutical analysis plays a very prominent role in quality assurance as well as quality control of bulk drugs and pharmaceutical formulations [1]. As a consequence, analytical method development has become the basic activity of analysis. Analytical chemistry is separated into two predominant classes: a qualitative evaluation which is to say the identification with regard to the chemical additives exists in the sample, whereas a quantitative evaluation estimates the amount of positive detail or compound within the substance [2]. High-performance liquid chromatography (HPLC) is most accurate methods widely used for the qualitative and quantitative analysis of drug products. Analytical method development and validation play important roles in the drug discovery, drug development, and manufacturing of pharmaceuticals. It involves the detection of the purity and toxicity of a drug substance. A number of chromatographic parameters have been evaluated to optimize the methods in the analysis of method development in HPLC. An appropriate mobile phase, column, column temperature, wavelength, and pH. HPLC most widely applied analytical technique because of its highly selective and high reliability, especially in pharmaceutical formulations [3,4]. Regulatory authorities give utmost importance to analytical methods in manufacturing. Drug approval by regulatory authorities requires the applicant to prove control of the entire process of drug development by using validated analytical methods [5]. The main objective of the method validation process is to prove that an analytical method is acceptable for its intended purpose [6]. There are several internationally renowned organizations offering guidelines on method validation and related topics. Basic references are the Association of Official Analytical Chemists, the American Society for Testing and Material, the Codex Committee on Methods of Analysis and Sampling, the European Committee for Normalization, the Cooperation on International Traceability in Analytical Chemistry, the European Cooperation for Accreditation, the Food and Agricultural Organization, the United States Food and Drug Administration, the International Conference on Harmonization, the International Laboratory Accreditation Cooperation, The World Health Organization, the International Organization for Standardization, the International Union of Pure and Applied Chemistry, the United States Pharmacopeia, the analytical chemistry group EURACHEM, etc. [7]. As per guidelines, practical approaches for validation are selectivity, specificity, limit of detection, limit of quantitation, linearity, range accuracy, precision, recovery solution stability, ruggedness, and robustness of liquid chromatographic methods to support the routine, in-process, and stability analysis [8]. The validated method was successfully applied to the commercially available pharmaceutical dosage form, yielding very good and reproducible results [9].

The pharmaceutical industry is rapidly adopting the quality by design (QbD) principles for the fabrication of safe, effective, and quality products; however, we are still on a journey, and the process of gathering all experience and metrics required for connecting and demonstrating QbD benefits to all stakeholders is still in progress [10]. QbD is a systematic approach to development that begins with predefined objectives and emphasizes product and process

understanding and process control, based on sound science and quality risk management [11]. Benefits of Analytical QbD:

- Increased understanding and control
- Beyond the traditional ICH procedure of method validation
- Flexibility in the analysis of API, impurities in dosage forms, and stability of samples
- Reduction in variability in analytical attributes for improving the method's robustness
- To keep the values of analytical attributes within the pharmacopeial monographs,
- Avoid Out of Specification limits
- To develop the most preferable method with the help of QbD [12].

For implementing QbD approach, the Box-Behnken Design (BBD) is used. The BBD is a widely used response surface methodology design that is particularly useful for establishing cause-and-effect relationships between factors and responses in experiments [13].

According to the previous studies, we got bioanalytical method development, reversed phase HPLC method development and their validation, stability indicating method development, etc., but nobody went for a statistical approach such as QbD due to that we selected method development by QbD for Azilsartan Medoxomil (AZT) and Cilnidipine (CIL) [14] for QbD approach. An initial risk assessment will be carried out to identify the various attributes [15].

Cardiovascular disease accounts for more expenditures on health care than any other diagnostic category, and hypertension is the most prevalent cardiovascular condition [16]. AZT (5-methyl -2-oxo-1,3-dioxol-4-yl)-2-ethoxy-3- [[4-[2-(5-oxo-4H-1,2,4-oxodiazol-3-yl] phenyl] methyl] benzimidazole4-carboxylate and CIL 3-0-(2- methoxyethyl) 5-0[(E)-3-phenylprop-2-enyl]-2-enyl] 2,6-dimethyl-4-(3-nitrophenyl)-1,4-dihydropyridine -3,5-dicarboxylate falls under a category of anti-hypertensive drugs called ARB blocker and calcium channel blocker, respectively [17]. Figs. 1 and 2 describe the chemical structure of AZT and CIL, respectively.

METHODS

Instrument

Details about the HPLC used for this experimental work are given in Table 1.

Materials used

AZT and CIL APIs were made available as a Gift sample from Hetero Labs Ltd, Baddi, Himachal Pradesh, and Lupin Ltd, Chhatrapati Sambhajinagar, respectively. Tablet formulation of the same combination was available as a brand name of Myotan CN, Mfg-Synokem Pharmaceuticals Ltd, and marketed by J.B. Chemicals Pvt. Ltd, which is purchased from the local market.The list of chemicals is given in Table 2.

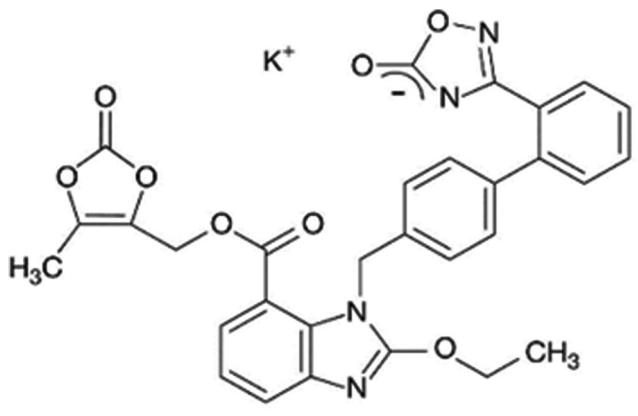

Fig. 1: Chemical structure of azilsartan medoxomil [18]

Preparation of stock solution

AZT 20 mg and CIL 5 mg were diluted up to 10 mL of methanol in separate volumetric flasks, by which 2000 µg/mL and 500 µg/mL solutions for AZT and CIL, respectively. Take above-prepared, 1 mL AZT solution, dilute it up to 20 mL in a separate volumetric flask, by which a 100 µg/mL AZT stock solution is formed, and 1 mL of above-prepared CIL solution is diluted up to 20 mL in another volumetric flask, by which a 25 µg/mL stock solution of CIL is formed. Same dilutions were carried out for tablet formulation with the consideration of equivalent weight.

Plan of method development

Schematic representation of the QbD-based method development plan is given in Fig. 3.

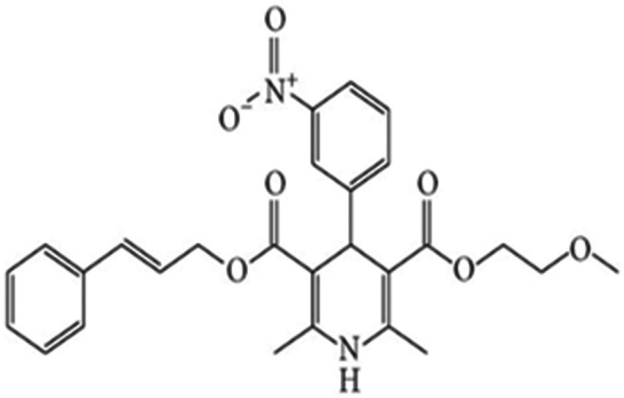

Fig. 2: Chemical structure of cilnidipine [19,20]

Table 1: Details about HPLC instrument

Parts of instruments	Model	Information
System	UHPLC	Ultra-high-performance liquid chromatograph
Model no.	LC 20 AD	---------
Company	-----	Shimadzu Japan
Pump	LC-20 AD	Quaternary Gradient
Column	Make: G.L Sciences Model: Inertsil ODS-3V	C-18, 250×4.6 mm, 5 µ
Detector	SPD-20 A	Dual Wavelength ultraviolet-visible
Column Oven	CT0-10 AS VP	Max. Temp. 80°c
Autosampler	SIL-20AC HT	0.1–100 µL (20 µL injection volume is used in this study)
Software	Version DB 6.110	Lab Solution

HPLC: High-performance liquid chromatography,
UHPLC: Ultra-high-performance liquid chromatography

Table 2: List of chemicals and reagents

S. No.	Chemical	Grade	Manufacturer
1	Methanol	HPLC	Rankem Labs
2	Acetonitrile	HPLC	Rankem Labs
3	Water	HPLC	Molychem
4	Ammonium dihydrogen ortho-phosphate	AR	Ozone Int. India
5	Potassium dihydrogen ortho-phosphate	AR	Ozone Int. India
6	Triethyl Amine (pH Adjustment)	AR	Ozone Int. India
7	Acetic Acid	AR	Ozone Int. India
8	Ortho Phosphoric Acid (pH Adjustment)	AR	Ozone Int. India

HPLC: High-performance liquid chromatography, AR: Analytical reagent

RESULTS AND DISCUSSION

Implementation of traditional method development approach

At the initial stage, the appropriate method was finalised with the help of a traditional method development approach, the critical method parameters (CMPs) such as mobile phase composition, flow rate, temperature, and pH are discussed in Table 3. In this whole study, 20 µL injection volume is used. The isosbestic point for AZT and CIL was found out by ultraviolet-visible spectroscopy and the point is 248 nm which is used in whole study and is given in Fig. 4. An isosbestic point is observed in overlaid spectra when a chromophoric precursor is converted to a product with a different spectrum, so that it is often assumed that an isosbestic point occurs only when the precursor is quantitatively converted to a single product [21].

On the basis of above 14 trials, the most suitable trial was trial number 09 and trial number 14. Trial number 14 is superior than trial number 9. The MeOH and 0.1% ortho phosphoric acid (OPA) v/v is used as a mobile phase, 1 mL/min is flow rate and 40°C is the temperature for this trial. pH of all trials is maintained/adjusted by OPA or by triethyl amine buffer solution. Trial number 14 is shown in Fig. 5, this trial is used for further QbD approach, which is mainly implemented by Design of Experiment (DoE), and the software used for this is Design Expert-13. The system suitability parameters for trial number 14 are given in Table 4. For further study, three factors are selected from the most appropriate trial that is concentration of mobile phase, temperature, and flow rate. Finalized parameters are given in Table 5.

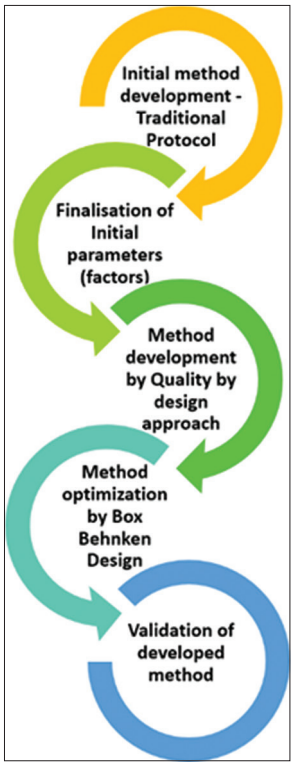

Fig. 3: Plan of quality by design-based method development

Implementation of QbD approach for optimization of method

DoE (BBD) is implemented for optimizing the final method, for which that finalized parameters in the traditional method were used. Based on 3 factors, BBD given 17 runs protocol with 5 center points. All 17 Runs are evaluated based on 6 responses, i.e., retention time, area, symmetry, number of theoretical plates, resolution, and selectivity. Results of all 17 runs are given in Table 6.

ANOVA for Quadratic Model suggests that all F-values shown by the model is significant for all Responses. The *P*-values are < 0.0500 confirms the Quadratic Model is Significant for the study.

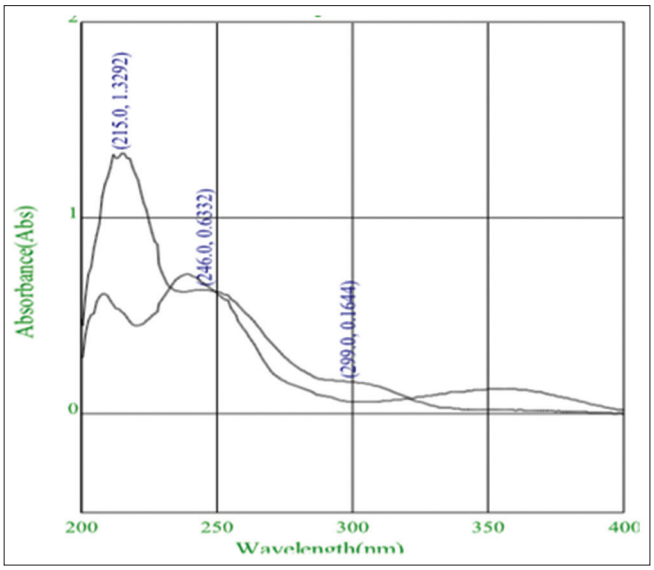

Fig. 4: Isosbestic point wavelength is 248 nm for the estimation of Azilsartan Medoxomil and Cilnidipine

Table 3: Initial trials for finalizing critical method parameters/factors for quality by design

Trials no (column)	Mobile phase	Ratio	Flow rate	Temp	pH	Inference
1 (C8)	MeOH: OPA	82:18	0.7	25°C	3	Tailing, separation was not good, excessive peaks
2 (C8)	MeOH: OPA	75:25	0.7	25°C	3	Same as trial 1
3 (C18)	MeOH: OPA	80:20	0.7	25°C	3	Peak resolution was not Proportionate
4 (C18)	MeOH: OPA	80:20	0.7	25°C	3	Repeated once again, but the same results
5 (C18)	MeOH: OPA	80:20	0.9	25°C	3	Peak resolution was not Proportionate
6 (C18)	MeOH: OPA	85:15	0.9	25°C	3	Peak resolution, intensity improved by increasing MeOH Conc.
7 (C18)	MeOH: OPA	90:10	0.9	25°C	3	Excessive band arrived in spectra
8 (C18)	MeOH: OPA	75:25	1	25°C	3	Prolong retention, tailing, excessive bands
9 (C18)	MeOH: OPA	82:18	1	25°C	3	Resolution, RT, and symmetry were good
10 (C18)	ACN: OPA	82:18	1	25°C	3	Excessive bands, tailing
11 (C18)	ACN: KH2PO4 Buffer	82:18	1	25°C	3	Same as the previous one excessive bands, tailing
12 (C18)	ACN: Acetic acid	82:18	1	25°C	3	Same as the previous one
13 (C18)	MeOH: OPA	82:18	1	25°C	5	pH change is not suitable
14 (C18)	MeOH: OPA	82:18	1	40°C	3	By increasing the temperature RT and resolution get improved

OPA: Ortho phosphoric acid, n=14, data in above table contains total 14 experimental runs

The F-Values for Lack of Fit study shown Not Significant and Non Significant Lack of Fit is necessary for the study now it shows that the Quadratic model fits for further study.

The summery of ANOVA for Quadratic Model is given in Table 7.

The Predicted R^2 of all responses is in reasonable agreement with the Adjusted R^2 of respective responses, i.e., the difference is <0.2. Adequate precision measures the signal-to-noise ratio. A ratio >4 is desirable. The study shows that all ratios of all responses indicate an adequate signal-to-noise ratio, which suggests that this quadratic model is suitable for further study. Details about the study of Fit Statistics for the Quadratic Model are given in Table 8.

On the basis of the ANOVA study and fit statistics study, it was concluded that the Quadratic model is suitable for finding out the optimization parameters for the proposed method development and validation.

All responses of BBD are theoretically in considerable limits, but for finding out the most suitable solution, this study focuses on retention time, selectivity, and resolution.

Retention time, selectivity, and resolution of all trials are taken into consideration for finalizing the most suitable solution while other responses are in a considerable range. The minimum values of 2.483, 5.16, and 1.27 are set for retention time, resolution, and selectivity, respectively, for finding out most suitable solution which having the least retention time, least value with better resolution, and least value with precise selectivity.

On the basis of the given input, Design Expert 13 suggested the most suitable solution, which shows specified criteria in above paragraph. Figs. 6 and 7 supports the most suitable solution giver by BBD.

The optimized parameters by BBD are given in Table 9, which is based on 17 runs of BBD. The values given by BBD (i.e., Design Expert 13) are not practically achievable that's why, for practical purposes, values are rounded off.

In comparison with traditional method development values and Box Behnken optimized values the results of retention time, resolution, and selectivity get improved, this suggests that the Box Behnken optimized parameters are advantageous over conventionally developed method. QbD-based method gets finalized and the validation protocol is implemented for further study.

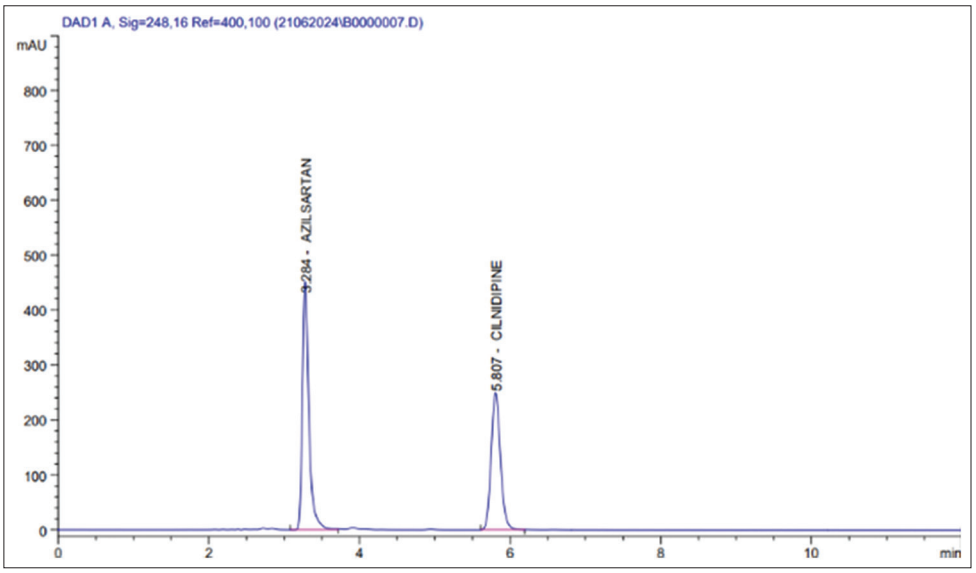

Fig. 5: Trial number 14

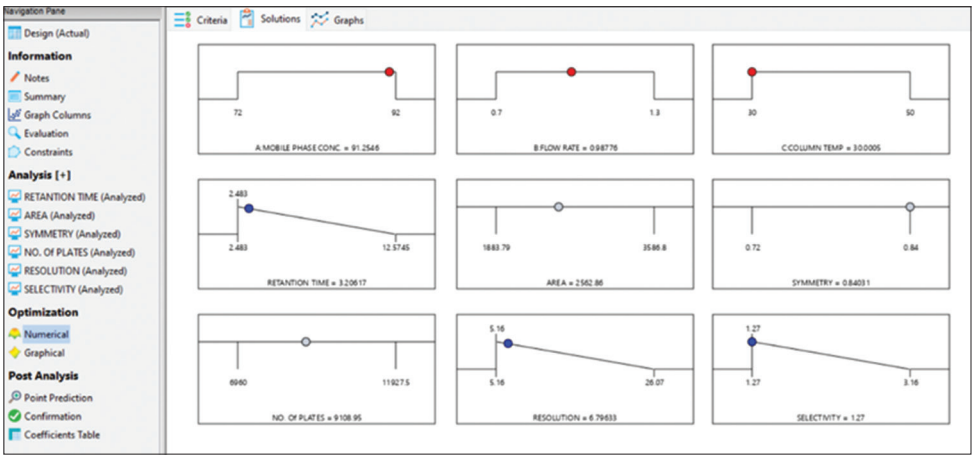

Fig. 6: Most appropriate solution by Box Behnken design

Table 4: System suitability parameters for trial number 14

Parameter	Results for Azilsartan Medoxomil	Results for Cilnidipine
Retention time (min)	3.284	5.807
Area (mAU*s)	2724.97705	2151.32080
Height (mAU)	424.75809	246.36357
Symmetry	0.77	0.88
Width (min)	0.0883	0.1300
Plates	7657	11054
Resolution	-	13.58
Selectivity	-	1.77

Table 5: Finalized parameters (factors) for design of experiment (Box Behnken design)

S. No.	Important parameters	Values
1	MeOH: OPA (0.1%) v/v	82:18
2	Flow rate	1 ml/min
3	Temperature	40°C

OPA: Ortho phosphoric acid

Method validation

Accuracy

Accuracy is the true value or closeness to the true value. This value is found out at 80%, 100% and 120%. Details about accuracy values are given in Table 10 for AZT and Table 11 for CIL.

Range linearity

The linearity study was based on the concentration versus absorbance area. Different concentrations were used for this study, i.e., 20, 40, 60, 80, 100, and 5, 10, 15, 20, 25 for AZT and CIL, respectively. Figs. 8 and 9 represent the range linearity for AZT and CIL, respectively.

All details about validation parameters, i.e., intraday and interday precision, LOD, LOQ. Percentage recovery, repeatability, linearity, regression equation, correlation coefficient, and slope are given in Table 12.

The analytical method was developed using a QbD framework to guarantee efficiency, reliability, and robustness. At the beginning of

Table 6: Results of Box Benken design

Run	Factor 1	Factor 2	Factor 3	Response 1	Response 2	Response 3	Response 4	Response 5	Response 6
	Mobile phase (%)	Flow rate (mL/min)	Column temp (°C)	Retention time (min)	Area (mAU)	Symmetry	No. of plates	Resolution	Selectivity
1	72	1	30	9.5535	2474.13	0.77	9767.5	25.86	3.02
2	82	1	40	4.6335	2471.61	0.82	9285.5	13.86	1.79
3	82	1	40	4.623	2466.41	0.81	9238	14.81	1.89
4	92	1	50	3.2065	2512.78	0.82	9659.5	5.71	1.28
5	82	0.7	30	6.577	3519.43	0.81	11829	15.56	1.79
6	82	1	40	5.828	2665.57	0.84	9229	13.76	1.79
7	82	1	40	4.621	2465.91	0.815	9093	13.71	1.79
8	72	1.3	40	7.309	1883.79	0.78	7518.5	24.11	3.02
9	82	0.7	50	6.605	3523.44	0.805	11927.5	15.64	1.79
10	72	0.7	40	12.5745	3483.39	0.72	11925	24.73	3.16
11	82	1	40	4.6195	2474.53	0.82	9385	13.86	1.79
12	72	1	50	9.418	2448.17	0.77	9332	26.07	3
13	92	1.3	40	2.483	1941.61	0.8	6960	5.16	1.28
14	82	1.3	50	3.584	1910.18	0.83	7255	12.33	1.8
15	92	1	30	3.2115	2533.6	0.84	9010	5.85	1.28
16	82	1.3	30	3.5825	1903.74	0.84	7229.5	12.3	1.8
17	92	0.7	40	4.55	3586.8	0.81	11836	6.55	1.27

n=17, above data are given based on 17 experimental runs

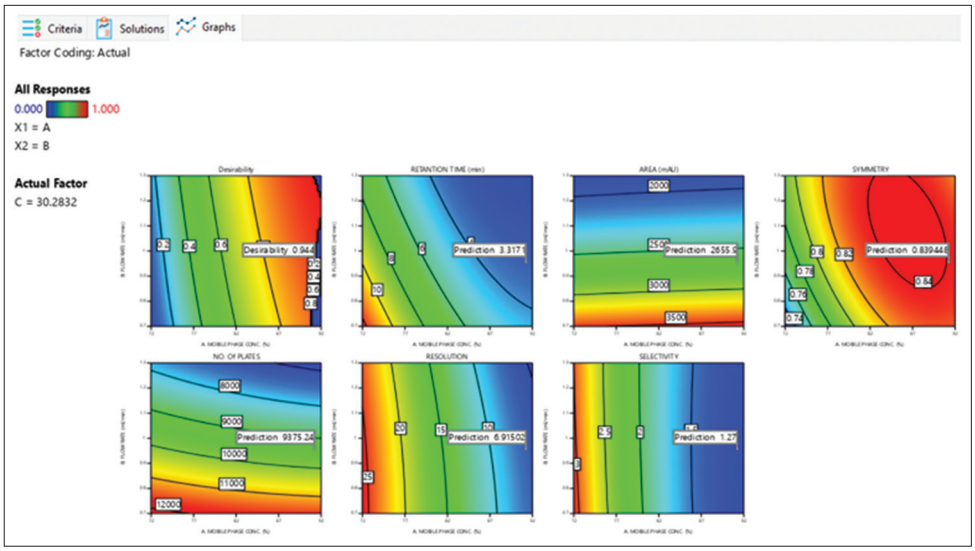

Fig 7: Contour plot for most suitable solution by Box Behnken design

Table 7: Summary of analysis of variance study for quadratic model

Response number	Response	Source	Summary of analysis of variance for quadratic model					
			Sum of Squares	df	Mean square	F-value	p-value	Remark
1	Retention time	Model	116.41	9	12.93	65.10	<0.0001	Significant
		Lack of Fit	0.2314	3	0.0771	0.2662	0.8472	Not significant
2	Area	Model	5.451	9	6.057	135.09	<0.0001	Significant
		Lack of Fit	613.20	3	204.40	0.0266	0.9933	Not significant
3	Symmetry	Model	0.0149	9	0.0017	21.54	0.0003	Significant
		Lack of Fit	0.0000	3	6.250	0.0481	0.9841	Not significant
4	No. of plates	Model	4.385	9	4.872	661.14	<0.0001	Significant
		Lack of Fit	6939.56	3	2313.19	0.2073	0.8866	Not significant
5	Resolution	Model	771.09	9	85.68	126.30	<0.0001	Significant
		Lack of Fit	3.91	3	1.30	6.23	0.0547	Not significant
6	Selectivity	Model	6.84	9	0.7605	367.76	<0.0001	Significant
		Lack of Fit	0.0065	3	0.0022	1.08	0.4528	Not significant

n=17, above data are given on the basis of 17 experimental runs

Table 8: Study of fit statistics for model

Parameters for fit statistics	Responses					
	Retention time	Area	Symmetry	No. of plates	Resolution	Selectivity
Standard deviation	0.4457	66.96	0.0088	85.84	0.8236	0.0455
Mean	5.70	2603.83	0.8059	9440.00	14.70	1.97
C.V. %	7.81	2.57	1.09	0.9094	5.60	2.30
R^2	0.9882	0.9943	0.9652	0.9988	0.9939	0.9979
Adjusted R^2	0.9730	0.9869	0.9204	0.9973	0.9860	0.9952
Predicted R^2	0.9532	0.9894	0.9280	0.9959	0.9176	0.9831
Adeq. Precision	28.3386	32.9038	18.1132	75.2079	34.0669	53.4382

n=17, above data are given based on 17 experimental runs

Table 9: Optimized parameters by Box Behnken design

S. No.	Important parameters	Traditional method values	Values by design expert 13 software	Final values for experimentation
1	MeOH: OPA (0.1%)	82:18	91.2546: 8.7454	91.20: 8.80
2	Flow rate	1 mL/min	0.98776 mL/min	0.98 mL/min
3	Temperature	40°C	30.0005°C	30°C

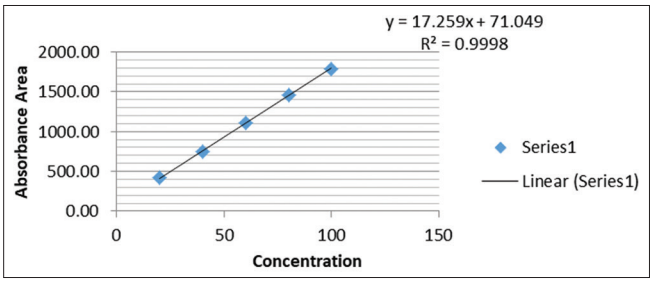

Fig. 8: Linearity details about Azilsartan Medoxomil

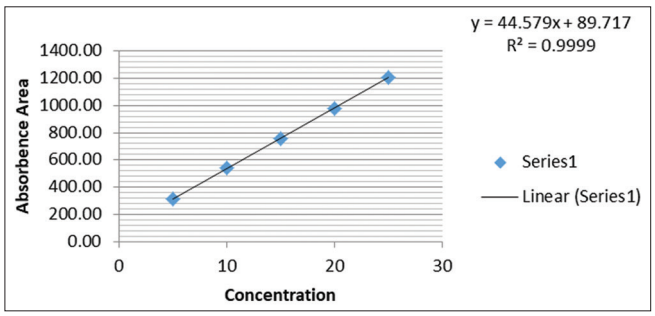

Fig. 9: Linearity details about Cilnidipine

the study, the isosbestic point was identified at 248 nm, which was consistently employed for detection purposes throughout the entire work.

Based on literature reports, preliminary trials were performed to evaluate different solvent systems. Out of these, a mixture of methanol and 0.1% v/v OPA was found to be the most appropriate, as confirmed through 14 exploratory experiments. These trial-and-error investigations also highlighted three CMPs that significantly affected performance: mobile phase composition, flow rate, and column temperature.

For systematic optimization and control of these CMPs, the QbD strategy was applied using Design Expert® version 13 software. A BBD was chosen to structure the DoEs, which included 17 experimental runs. The Quadratic model was followed by all the responses. This statistical design allowed a detailed assessment of factor interactions and their combined influence on the method. Consequently, the optimized conditions were defined as: mobile phase composition of methanol: 0.1% OPA (91.20: 8.80, v/v), flow rate of 0.98 mL/min, and column temperature of 30°C.

With these optimized parameters, the developed method exhibited consistent and reliable performance. A full validation study was then carried out, covering accuracy, precision, linearity, robustness, and system suitability which are given in Table 12. All evaluated criteria met the acceptance requirements, with values showing close agreement to theoretical expectations, thereby confirming the reliability and validity of the method.

Table 10: Details about accuracy for Azilsartan Medoxomil

S. No	% Accuracy	Amount added µg/mL	Amount recovered µg/mL	% recovery	Mean	SD	% RSD
1	80	32	32.18	100.57	100.43	0.20	0.20
2		32	32.09	100.29			
3	100	40	40.44	101.12	100.86	0.37	0.36
4		40	40.24	100.60			
5	120	48	47.97	99.94	100.08	0.20	0.20
6		48	48.10	100.23			

n=6, total 6 runs have been carried out for accuracy study

Table 11: Details about accuracy for Cilnidipine

S. No.	% Accuracy	Amount added µg/mL	Amount recovered µg/mL	% recovery	Mean	SD	% RSD
1	80	8	7.95	99.37	99.71	0.47	0.47
2		8	8.00	100.00			
3	100	10	9.94	99.42	99.53	0.16	0.16
4		10	9.96	99.64			
5	120	12	11.93	99.45	99.24	0.30	0.30
6		12	11.88	99.03			

n=6, total 6 runs have been carried out for accuracy study

Table 12: Different validation parameters with results

S. No.	Parameters	Azilsartan Medoxomil	Cilnidipine
1	Linearity Range	20–100 µg/mL	5–25 µg/mL
2	Regression Equation	y=17.259x+71.049	y=44.579x+89.717
3	Correlation Coefficient (R2)	0.9998	0.9999
4	Slope	17.259	44.579
5	Specificity	Specific	Specific
6	Accuracy (assay)	100.45	99.49
	Recovery		
7	80%	100.43	99.71
	100%	100.86	99.53
	120%	100.08	99.24
8	Precision (Intraday % RSD)		
	Low	0.23	0.19
	Middle	0.12	0.15
	High	0.03	0.02
	Precision (Interday % RSD)		
9	Low	0.15	0.3
	Middle	0.58	0.29
	High	0.03	0.15
10	Repeatability	0.3	0.05
11	LOD	0.35 µg/mL	0.06 µg/mL
12	LOQ	1.08 µg/mL	0.19 µg/mL

CONCLUSION

In the present work, a QbD-driven strategy was employed for the development of a robust analytical method for the simultaneous estimation of AZT and CIL. The method was systematically optimized through risk assessment and DoEs, followed by validation in accordance with ICH guidelines. All validation parameters were found to be within the acceptable limits, confirming the reliability and reproducibility of the method. The QbD-based approach thus ensured a scientifically sound, well-optimized, and regulatory-compliant method, making it highly suitable for routine application in quality control laboratories and pharmaceutical industries.

AUTHORS CONTRIBUTIONS

All authors have contributed equally.

CONFLICTS OF INTEREST

The authors declare that they have no conflict of interest for this research work.

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