RESEARCH PAPER

Development and Validation of an RP-HPLC Method for Simultaneous Estimation of Azelnidipine and Chlorthalidone in Bulk and Fixed Dose Combination Using Analytical Quality by Design with Greenness Assessment

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ABSTRACT

The present study focuses on the development and validation of a robust, reliable, and eco-friendly RP-HPLC method for the simultaneous estimation of Azelnidipine (AZE) and Chlorthalidone (CTL) in bulk and fixed-dose combination formulations using an Analytical Quality by Design (AQbD) approach. The Analytical Target Profile (ATP) was defined to ensure the desired method performance, followed by systematic risk assessment using Ishikawa diagram and Failure Mode and Effects Analysis (FMEA) to identify critical method parameters (CMPs) and critical method attributes (CMAs). A Box–Behnken Design (BBD) was employed for optimization of chromatographic conditions. The optimized method utilized a Cosmosil C18 column with a mobile phase consisting of methanol:water (60:40 % v/v) at pH 3.0, a flow rate of 1.0 mL/min, and detection at 205 nm. The retention times were found to be 5.684 min for CTL and 7.379 min for AZE with satisfactory resolution and system suitability parameters. The Method Operable Design Region (MODR) was successfully established, ensuring robustness and regulatory flexibility. The developed method was validated according to ICH Q2(R2) guidelines and demonstrated excellent linearity (R^2 > 0.999), accuracy (99–102%), and precision (%RSD < 2%). The limits of detection and quantitation indicated high sensitivity of the method. The assay results for the marketed formulation were within acceptable limits, confirming the applicability of the method for routine quality control. Greenness evaluation using the AGREE tool yielded a score of 0.66, while the Analytical Eco-Scale score was 78, indicating excellent green analytical performance. The method minimizes environmental impact while maintaining high analytical efficiency. In conclusion, the proposed AQbD-based RP-HPLC method is simple, accurate, precise, robust, and environmentally sustainable. It offers regulatory flexibility, ease of method transfer, and suitability for routine analysis of AZE and CTL in pharmaceutical formulations.

Keywords: Azelnidipine, Chlorthalidone, RP-HPLC, Analytical Quality by Design, Box–Behnken Design, Method Validation, Green Analytical Chemistry, AGREE, Eco-Scale, MODR

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INTRODUCTION:

Hypertension is one of the most prevalent cardiovascular disorders worldwide and remains a major risk factor for stroke, myocardial infarction, and cardiovascular mortality despite advancements in pharmacotherapy [1]. Combination therapy using antihypertensive agents with complementary mechanisms of action has been widely recommended to achieve better blood pressure control and improved patient compliance [1]. Fixed-dose combinations (FDCs) of calcium channel blockers and diuretics have demonstrated superior therapeutic efficacy compared to monotherapy due to their synergistic pharmacological effects [1].

Azelnidipine (AZE) is a long-acting dihydropyridine calcium channel blocker that exerts antihypertensive activity by inhibiting calcium influx through L-type calcium channels, resulting in vasodilation and reduced peripheral resistance [2]. Chlorthalidone (CTL), a thiazide-like diuretic, reduces blood pressure by promoting sodium and water excretion, thereby decreasing plasma volume

and vascular resistance [3]. The combination of AZE and CTL has been approved for clinical use and has shown significant benefits in reducing cardiovascular risk factors and improving therapeutic outcomes in hypertensive patients [4,1].

Accurate and reliable analytical methods are essential for the quality control and routine analysis of pharmaceutical formulations, particularly for fixed-dose combinations [5]. Various analytical techniques have been reported for the estimation of AZE and CTL individually or in combination with other drugs, including UV spectrophotometry, spectrofluorimetry, HPLC, HPTLC, and LC-MS methods [6–10]. However, only a limited number of methods are available for the simultaneous estimation of AZE and CTL in combined dosage forms, and most of these methods are developed using conventional trial-and-error approaches [6,9,11]. Such traditional methods often lack robustness, flexibility, and comprehensive understanding of method variables, which may affect reproducibility and regulatory compliance [5].

In recent years, the concept of Analytical Quality by Design (AQbD) has emerged as a systematic and science-based approach for analytical method development [5]. AQbD emphasizes predefined objectives, method understanding, and risk management to ensure consistent analytical performance throughout the method lifecycle [5]. The Analytical Target Profile (ATP) forms the foundation of AQbD, defining the intended purpose of the analytical method along with critical quality attributes and acceptance criteria [12]. Risk assessment tools such as Failure Mode and Effects Analysis (FMEA) are employed to identify and control critical method parameters (CMPs) that may influence method performance [13].

Design of Experiments (DoE), particularly Box–Behnken Design (BBD), is widely used in AQbD to optimize chromatographic conditions and evaluate the interaction between variables [5]. This multivariate approach enables efficient method optimization with minimal experimental runs while ensuring robustness and reproducibility [5]. Furthermore, the establishment of a Method Operable Design Region (MODR) provides a multidimensional space within which method parameters can vary without affecting analytical performance, thereby offering regulatory flexibility and ease of method transfer [5,14].

Reverse Phase High-Performance Liquid Chromatography (RP-HPLC) is one of the most widely employed analytical techniques in pharmaceutical analysis due to its high sensitivity, selectivity, and reproducibility [15]. It is particularly suitable for the simultaneous estimation of multiple components in complex matrices such as fixed-dose combinations [15]. However, conventional RP-HPLC methods often overlook environmental concerns associated with solvent consumption, energy usage, and waste generation.

Green Analytical Chemistry (GAC) has gained increasing attention in recent years, aiming to minimize the environmental impact of analytical procedures while maintaining analytical performance [16]. The integration of green principles into analytical method development promotes the use of safer solvents, reduced energy consumption, and minimal waste generation [16]. Various tools have been developed to evaluate the greenness of analytical methods, including the Analytical GREEnness (AGREE) metric and Analytical Eco-Scale Assessment [17,18]. The AGREE tool evaluates analytical procedures based on twelve green chemistry principles and provides a comprehensive greenness score [17]. Similarly, the Eco-Scale approach assesses the environmental impact of analytical methods by assigning penalty points based on reagent hazards, energy consumption, and waste generation [18].

Regulatory authorities such as the International Council for Harmonisation (ICH) and the United States Pharmacopeia (USP) strongly encourage the adoption of AQbD principles and lifecycle approaches in analytical method development [5,14,19,20,21]. Validation of analytical methods as per ICH Q2(R2) guidelines ensures that the developed method meets predefined criteria for accuracy, precision, specificity, linearity, robustness, and sensitivity [19]. Incorporation of AQbD along with green analytical chemistry principles not only enhances method performance but also ensures sustainability and regulatory compliance.

Despite the availability of several analytical methods, there is a lack of AQbD-based RP-HPLC methods for the simultaneous estimation of AZE and CTL that also consider environmental sustainability [4]. Therefore, the present study aims to develop and validate a robust, reliable, and eco-friendly RP-HPLC method for simultaneous estimation of Azelnidipine and Chlorthalidone in bulk and fixed-dose combination using an Analytical Quality by Design approach. Additionally, the greenness of the developed method is evaluated using AGREE and Analytical Eco-Scale tools to ensure compliance with green analytical chemistry principles [17,18].

MATERIALS AND METHODS

Azelnidipine and Chlorthalidone were provided by reputed pharmaceutical company in Nashik, Maharashtra, INDIA. HPLC grade methanol and water were used; analytical reagent (AR) grade O-Phosphoric Acid was used. HPLC 3000 Series binary gradient system, Analytical Technologies Ltd. was used and data were processed by

HPLC workstation software. Statistical software used was design expert (version 13) for design optimization purposes.

METHODOLOGY

Pre-development Studies

Initially, the target analytes were characterized by evaluation of organoleptic properties, Fourier Transform Infrared (FTIR) spectroscopy, and melting point determination to confirm identity and purity of the drugs [22,23]. The solubility studies were performed using the shake flask method, which is a standard and widely accepted technique for determining equilibrium solubility of pharmaceutical compounds [24,25].

Analytical Target Profile (ATP)

The Analytical Target Profile (ATP) was established for the simultaneous estimation of Azelnidipine (AZE) and Chlorthalidone (CTL), defining the intended purpose, analytes, analytical technique, and method performance characteristics along with acceptance criteria [26,27]. ATP serves as the foundation of Analytical Quality by Design (AQbD) and ensures that the method consistently meets predefined objectives [26].

Chromatographic Conditions

Based on solubility data and analytical requirements, preliminary chromatographic conditions were optimized by selecting suitable detection wavelength, mobile phase composition, and stationary phase [28]. The detection wavelength of 205 nm was selected based on UV spectral analysis of both analytes [29]. Methanol and water were selected as mobile phase components considering solubility and chromatographic behavior of analytes [28]. Acidic pH conditions were preferred due to improved peak shape and ionization characteristics of the drugs [30]. A Cosmosil C18 column (250 mm × 4.6 mm, 5 µm) was used due to its high efficiency and suitability for reverse-phase separations [28].

Preparation of Standard and Sample Solutions

Standard and sample stock solutions were prepared as per standard analytical procedures for quantitative analysis of pharmaceutical dosage forms [31]. Filtration through a 0.45 µm membrane filter was performed to remove particulate matter and ensure system compatibility [31].

Risk Assessment and Identification of CMAs and CMPs

Risk assessment was performed according to quality risk management principles to identify critical factors affecting analytical method performance [32]. Ishikawa (fishbone) diagram was used to systematically identify potential variables influencing method development [33]. Failure Mode and Effects Analysis (FMEA) was applied to evaluate risks and assign Risk Priority Number (RPN) based on severity, occurrence, and detectability [32,34]. The Critical Method Attributes (CMAs) and Critical Method Parameters (CMPs) were identified based on RPN scoring and risk matrix evaluation [26].

Control Strategy

A control strategy was developed based on risk assessment and prior knowledge to ensure consistent analytical performance throughout the method lifecycle [26,35]. This strategy ensures robustness and regulatory compliance of the analytical method [35].

Box–Behnken Design (BBD)

Box–Behnken Design, a response surface methodology, was employed for optimization of chromatographic conditions using three levels of CMPs [36]. The design required 17 experimental runs to evaluate interaction effects and optimize responses [36]. Statistical analysis using ANOVA was performed to determine significance and adequacy of the model [36].

Optimization and Method Operable Design Region (MODR)

Optimization was performed using numerical and graphical approaches to achieve maximum desirability [36]. The Method Operable Design Region (MODR) was established to define a multidimensional space where method performance remains unaffected [26,35]. MODR provides regulatory flexibility and ensures robustness during method transfer [35].

System Suitability Parameters

System suitability tests including peak area, resolution, theoretical plates, and tailing factor were evaluated to ensure proper functioning of the chromatographic system [31,37]. Acceptance criteria were defined based on analytical performance requirements and regulatory expectations [37].

Analytical Method Validation (ICH Q2(R2))

Linearity

Linearity was evaluated by preparing calibration curves over a specified concentration range and performing regression analysis to establish correlation between concentration and response [38].

Specificity

Specificity was assessed by comparing chromatograms of blank, standard, and sample solutions to ensure absence of interference at retention times of analytes [38].

Accuracy

Accuracy was determined using the standard addition method at multiple levels, and percentage recovery was calculated to evaluate trueness of the method [38].

Precision

Precision was evaluated in terms of repeatability and intermediate precision, expressed as percentage relative standard deviation (%RSD) [38].

Limit of Detection (LOD) and Limit of Quantitation

(LOQ)

LOD and LOQ were calculated using standard deviation of response and slope of calibration curve as per validation guidelines [38].

Robustness

Robustness was studied by deliberate variation in method parameters such as wavelength and pH to evaluate method reliability under small changes [38].

Assay

Assay of pharmaceutical dosage form was performed using the validated analytical method and calculated as percentage of label claim [31].

Greenness Assessment

AGREE Tool

The Analytical Greenness (AGREE) tool evaluates analytical methods based on 12 principles of green analytical chemistry and provides a comprehensive greenness score [39]. A score above 0.60 indicates acceptable green analytical performance [39].

Analytical Eco-Scale Assessment

Analytical Eco-Scale evaluates greenness by assigning penalty points for reagent hazards, energy consumption, and waste generation [40]. The total score is calculated by subtracting penalty points from 100, where a higher score indicates a greener method [40].

Classification of greenness:

- ≥75: Excellent green analysis
- ≥50: Acceptable green analysis
- <50: Inadequate green analysis [40]

RESULTS AND DISCUSSION:

Pre-Development Studies:

The predevelopment studies results are mentioned in Table 1.

Table 1 Pre-development studies of Azelnidipine and Chlorthalidone

Pre development Studies	Azelnidipine	Chlorthalidone
Organoleptic Properties	Pale yellow coloured solid powder ; odourless	White colored solid crystalline powder; Odourles
Melting Point	123-125ºC	238-242ºC
FTIR Analysis	Based on the FTIR analysis the drugs were identified to be Azelnidipine and Chlorthalidone.	
Solubility analysis	Sparingly soluble in methanol, Slightly soluble in ethanol, freely soluble in DMSO and Acetonitrile, and very slightly soluble in the water	Soluble in methanl, DMSO,sparinglysoluble in ethanol and acetonitrile, and very slightly soluble in water.

Analytical Target Profile:

The ATP was prepared for the analytical method development. The ATP is shown in the Table 2.

Table 2 Analytical Target Profile (ATP) for simultnaeouse stimation of azelnidipine and chlorhtalidone in bulk and dosage form.

Parameters	Details and Acceptance Limits
Objective	Simultaneous Estimation of Azelnidipine and Chlothalidone
Selection of Target Analyte	Azelnidipine and Chlorthalidone
Selection of Analytical Method	RP-HPLC

Accuracy	%RSD <2%
Precision	%RSD <2%
Resolution	>2
Number of Theoretical Plates	>2000
Peak Area	%RSD <2%
Tailing Factor	<2

Preliminary Method Development:

From preliminary studies, it was found that Methanol:Water (60:40%v/v) composition, pH 3 of mobile phase and flow rate of 1ml/min with 30°C column oven temperature at 205 nm gives results with good separation and system suitability parameters.

Risk Assessment:

The potential risk factors in the method development were identified by ishikawa fishbone cause and effect diagram shown in Figure 1.

The risk analysis was carried out using FMEA was carried out using the failure modes and their RPN scoring as shown in Figure 2. The Risk Prioritization number given to each failure mode between 1-10 based on their effect on the method performance characteristics. 10 being the highest value of severity, occurrence, probability of detection while 1 was the lowest value. The factors with RPN more than 300 were considered as the high risk. While the factors with RPN 150-300 were considered as medium risk. Risk matrix was used to do the risk evaluation shown in Figure 3.

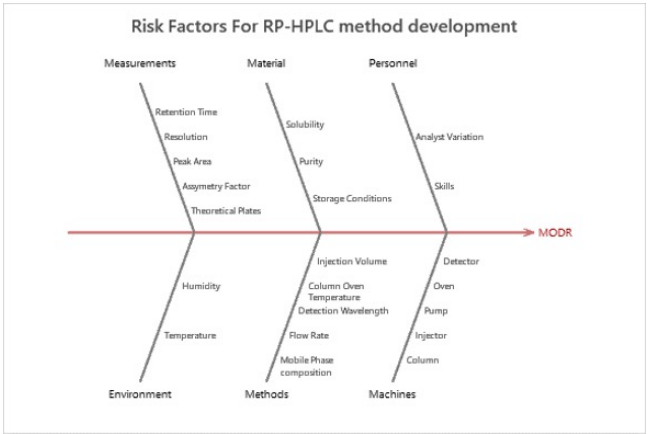

Figure 1 Risk Identification using Ishikawa fishbone diagram to identify the potential risk factors which affect the analytical method performance and MODR

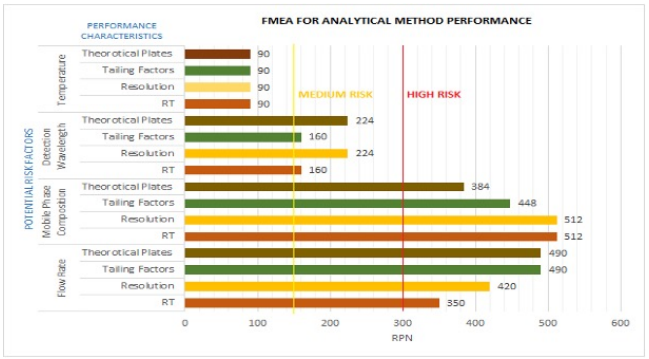

Figure 2 Risk analysis using FMEA based on RPN. Where, RPN >300 = High risk factors, RPN (150-300) = Medium risk factors, RPN (<150)=Low risk factors

RISK MATRIX BASED ON RPN

AMPCs / POTENTIAL RISK FACTORS	RT	Resolution	Theoretical Plates	Tailing Factor
Flow Rate	High	High	High	High
Mobile Phase Composition	High	High	High	High
Detection Wavelength	Medium	Medium	Medium	Medium
Temperature of column	Low	Low	Low	Low

Figure 3 Risk Evaluation using Risk Matrix based on Risk Ranking and Filtering using RPN.

Based on the whole risk assessment process, three Control Method Parameters were identified i.e. Factor A: % Composition of Methanol in Mobile Phase (%v/v); B: flow rate (ml/min) C: Detection wavelength (nm). The critical method attributes (CMAs) were selected i.e. Retention Time (R1, R2), Resolution (R3), Number of Theoretical Plates (R4, R5), Tailing Factor (R6,R7).

Control Strategy:

Mobile phase composition, flow rate, detection wavelength were found to be control method parameters. So in order to reduce risk appropriate control strategies were used to maintain the method performance throughout the method development procedure. The control strategy involved maintaining the flow rate, accurate preparation of the mobile phase, confirming the detection wavelength.

Box Behenken Design

The factors and their ranges levels were introduced to design expert software, to generate a randomized Box Behenken Design for optimization. The factors and their levels are shown in Table 3.

Table 3 The input factors (CMPs) and their levels for box-behenken design; where (-1): minimum level , (+1): maximum level

Factor	Unit	Level (-1)	Level(+1)
Composition	%	60	80
FlowRate	ml/min	0.8	1
Wavelength	Nm	203	207

Table 4 Box-behenken design optimization layout

	Factor1	Factor 2	Factor 3	R1	R2	R3	R4	R5	R6	R7
Run	A:Composition	B:Flowrate	C:Wavelength	RT CHL	RT AZL	Resolution	TP CHL	TP AZL	AF CHL	AF AZL
	%	ml/min	nm	min	min	Units	Units	Units	Units	Units
1	70	0.9	205	4.683	5.344	1.54	4053	4438	1.3	1.31
2	60	0.9	203	6.118	7.914	3.41	7439	7401	1.29	1.3
3	70	0.9	205	4.683	5.344	1.54	4053	4438	1.3	1.31
4	70	1	203	4.238	4.84	1.45	3787	4156	1.31	1.32
5	70	1	207	4.653	5.37	1.56	3897	4014	1.28	1.29
6	80	0.9	203	3.958	4.178	0.67	3202	2026	1.32	1.27
7	80	0.8	205	4.432	4.678	0.67	3247	2048	1.29	1.25
8	70	0.9	205	4.683	5.344	1.54	4053	4438	1.3	1.31
9	70	0.9	205	4.683	5.344	1.54	4053	4438	1.3	1.31
10	70	0.9	205	4.683	5.344	1.54	4053	4438	1.3	1.31
11	60	0.9	207	6.518	8.451	3.43	7440	7881	1.29	1.3
12	60	1	205	5.684	7.379	3.59	7931	8109	1.28	1.29
13	80	1	205	3.595	3.798	0.68	3243	2052	1.39	1.28
14	70	0.8	207	5.666	6.471	1.61	3218	3657	1.29	1.32
15	80	0.9	207	3.95	4.173	0.54	1243	1159	1.26	1.29
16	70	0.8	203	5.355	6.101	1.49	2036	2259	1.3	1.34
17	60	0.8	205	6.826	8.829	3.47	7537	7536	1.28	1.31

Based on input factors, box behenken design matrix was generated which is shown in Table 4 along with obtained responses. The suggested chromatographic experimental runs were performed and obtained responses were introduced into the design. The analysis of each response were performed. The quadratic model was suggested for analysis of the all responses. The statistical parameters for all factors were obtained from ANOVA shown in Table 5, which confirmed that model for all parameters is significant as the P value was <0.05.

Table 5 Statistical Validation of model using ANOVA; Where, RT: Retention Time, TP: number of theoretical plates, AF: Assymetry factor, C.V.: Coefficient of Variation

ANOVA Parameters	RT CTD	RT AZL	Resolution	TP CTD	TP AZL	AF CTD	AF AZL
R-Square	0.998213	0.999258	0.998767	0.966614	0.990588	0.662338	0.83437
Adjusted R-square	0.995914	0.998304	0.997183	0.92369	0.978486	0.45974	0.621417
F-value	434.3622	1047.671	630.27	22.51907	81.85609	3.269231	3.918092
P-value	8.95×10^{-9}	4.1×10^{-10}	2.44×10^{-9}	0.000231	2.94×10^{-6}	0.04781	0.042726
C.V. %	1.186586276	1.067453	3.097971	12.51726	7.355273	1.535319	0.996162

Multivariate analysis and Identification of Optimum method Condition:

The influence of Control Method Parameters (CMPs) on retention time, resolution, theoretical plates, and asymmetry factor for peaks of both drugs was analyzed through the 3D surface plots as shown in Figure 4, Figure 5, Figure 6, Figure 7 respectively. This analysis led to improved method understanding and better understanding of the relation between input (CMPs) and output factors (CMAs).

The 3D surface plots show optimum retention times for both drugs in case of lower to middle methanol percentage values, high flow rate and the detection wavelength was not found to be having significant effect on the retention time (Figure 4). The resolution between two peaks was higher at low % Methanol, lower flow rate and the detection wavelength was not found to be having significant effect on the resolution (Figure 5). Theoretical Plates for both drugs were found high at low methanol percent, high flow rate, and middle values of detection wavelength of the set values (Figure 6). The asymmetry factor was found to be low, for chlorthalidone, at lower % methanol and higher flow rate. While for azelnidipine , it was found to be low at, higher % methanol and lower flow rate. The detection wavelength was not found to be having significant effect on the asymmetry factor. (Figure 7)

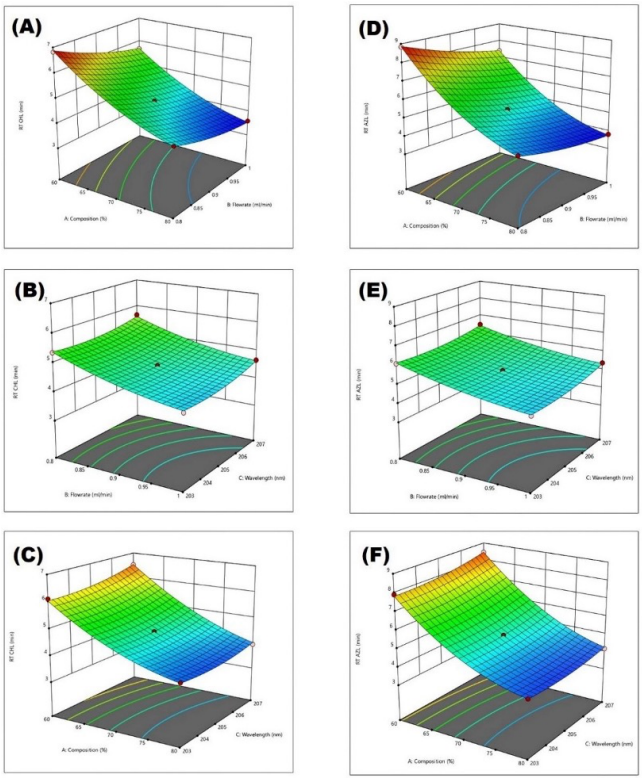

Figure 4 3D response surface plots for effect of various Control Method Parameters (CMPs) on the Retention Time (RT) of the Azelnidipine and Chlorthalidone. Where, Factor A: Mobile Phase Composition Methanol (%) Vs Factor B: Flow Rate (ml/min) Factor C: Detection Wavelength (nm). Z axis: Retention Time for CTD (4A, 4B, 4C), Retention Time for AZE (4D, 4E, 4F); XY axis: Figure 4 A) AB 4 B) BC 4 C) AC for Chlorthalidone, Figure 4 D) AB 4 E) BC 4 F) AC for Azelnidipine.

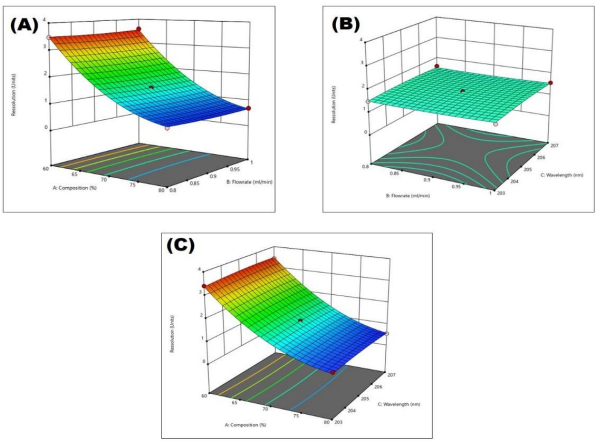

Figure 5 3D response surface plots for effect of various Control Method Parameters (CMPs) on the Resolution between two peaks. Where, Factor A: Mobile Phase Composition Methanol (%) Vs Factor B: Flow Rate (ml/min) Factor C: Detection Wavelength Where. Z axis: Resolution between two peaks XY axis: 7A) AB, 7B) BC, 7C) AC.

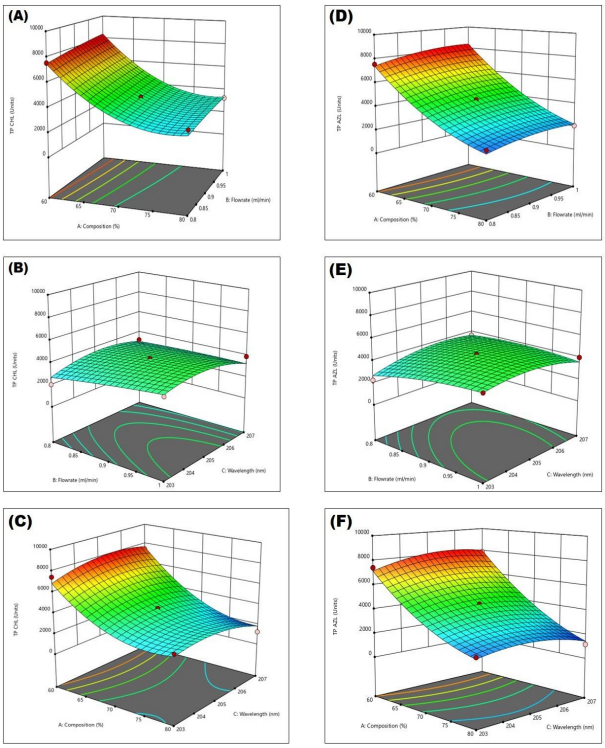

Figure 6 3D response surface plots for effect of various Control Method Parameters (CMPs) on the Theoretical Plates (TP) of the Azelnidipine and Chlorthalidone. Where, Factor A: Mobile Phase Composition Methanol (%) Vs Factor B: Flow Rate (ml/min) Factor C: Detection Wavelength Z axis: Retention Time for CTD (6A, 6B, 6C), Retention Time for AZE (6D, 6E, 6F); XY axis: Figure 6 A) AB 6 B) BC 6 C) AC for Chlorthalidone, Figure 6 D) AB 6 E) BC 6 F) AC for Azelnidipine.

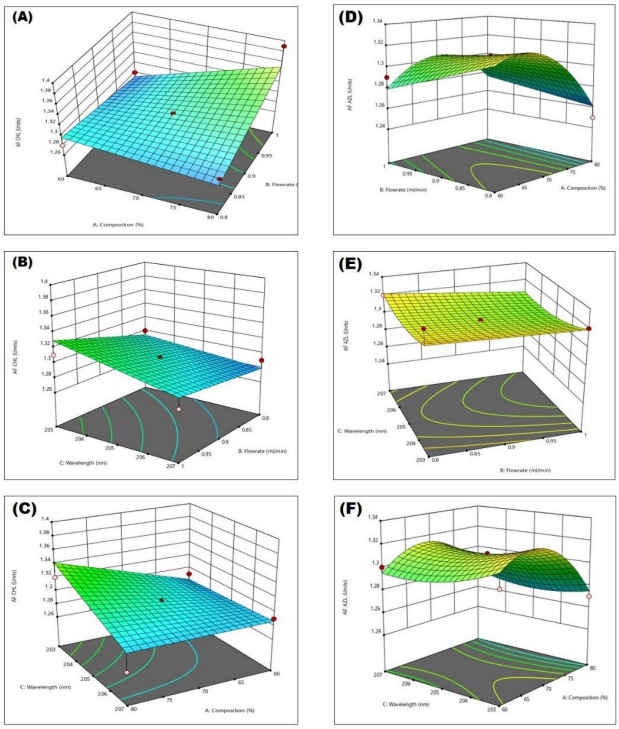

Figure 7 3D response surface plots for effect of various Control Method Parameters (CMPs) on the Asymmetry Factor (AF) for the peak of Azelnidipine and Chlorthalidone. Where, Factor A: Mobile Phase Composition Methanol (%) Vs Factor B: Flow Rate (ml/min) Factor C: Detection wavelength Z axis: Retention Time for CTD (7A, 7B, 7C), Retention Time for AZE (7D, 7E, 7F) ; XY axis: Figure 7 A) AB 7 B) BC 7 C) AC for Chlorthalidone, Figure 7 D) AB 7 E) BC 7 F) AC for Azelnidipine

Numerical optimization was carried out to find out method condition with desirability near one. The criteria for numerical optimization is shown in Table 6.

Table 6 Criteria for numerical optimization; Where, RT: Retention Time, TP: number of theoretical plates, AF: Assymetry factor

Parameter	Criteria	Limit

Composition (%)	In Range	60-80
Flow rate (ml/min)	In Range	0.8-1.0
Detection Wave length (nm)	Target	205
RTCHL(min)	In Range	5-6
RTAZL(min)	In Range	7-8
Resolution	Maximize	LowerLimit2
TP CHL	Maximize	LowerLimit2000
TP AZL	Maximize	LowerLimit2000
Asymmetry Factor(CHL)	Minimize	UpperLimit2
Asymmetry Factor (AZL)	Minimize	UpperLimit2

Based on the numerical optimization, 3D surface plots, mobile phase ratio of Methanol:Water (60:40 % v/v), pH 3.0 of mobile phase and flow rate of 1.0 ml/min and 40°C column oven temperature was found to be optimum method condition with the desirability of 0.987. Desirability contour plot is shown in Figure 8 and optimized condition chromatogram is shown in Figure 9.

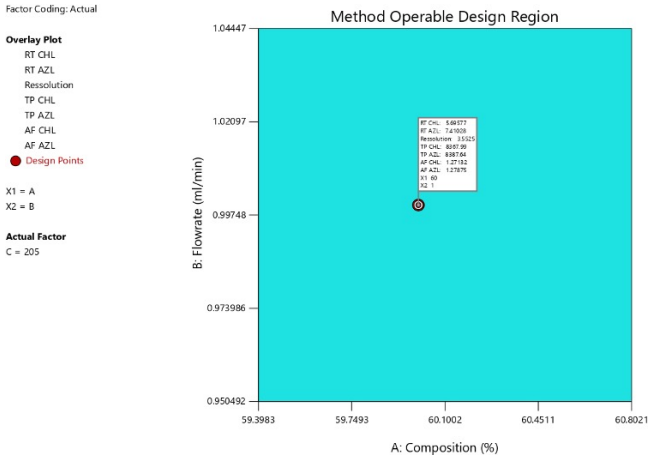

Figure 8 Contour Plot of chromatographic condition of desirability 0.987 (near 1) Where, Factor A: % methanol in mobile phase Factor B: flow rate (ml/min) at constant factor C: Detection wavelength (205nm);

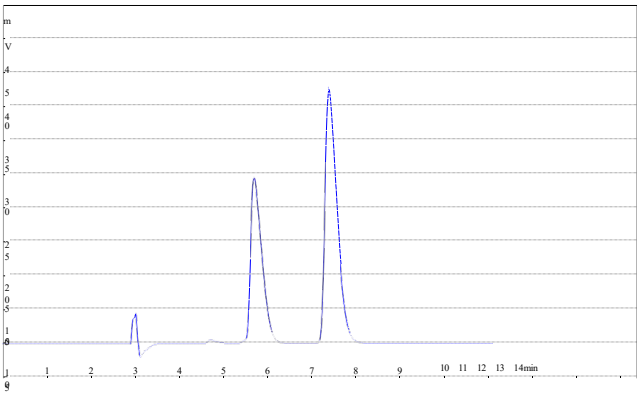

Figure 9 Optimized Condition Chromatogram of Chlorhtalidone and Azelnidipine (Retention Time (min) Vs Response (mV)); Retention Time of CTD: 5.684 min, Retention Time of AZE: 7.379 min, Total Run Time:11.10 min.

Method Operable Design Region:

Method operable design region was established using the graphical optimization. Moving within established MODR do not require regulatory reporting and method performance characteristics remain within the acceptance limits to maintain overall analytical method performance adhered to ATP. The criteria for MODR establishment is mentioned in Table 7 and established MODR is shown in Figure 10. The blue region in the overlain contour plot is the method operable design region.

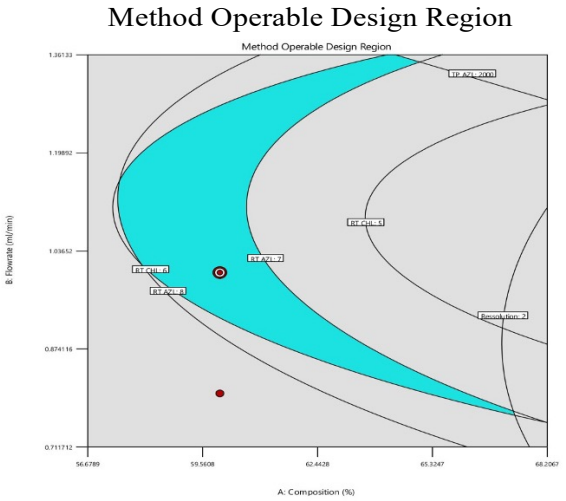

Figure 10 Method operable design region (MODR) obtained from overlain contour plots of retention time of CTD and AZE, Number of theoretical plates of CTD and AZE, Resolution between peaks, and Assymetry factor of CTD and AZE peaks according to the selected criteria for MODR.

Table 7 Criteria for method operable design region (MODR); Where, RT: Retention Time, TP: number of theoretical plates, AF: Assymetry factor

Parameter	Criteria/Limit
RT CHL	5-6
RT AZE	7-8
TP CHL	Lowerlimit:2000
TP AZE	LowerLimit:2000
Resolution	LowerLimit:2000
AFCHL	1-2
AFAZE	1-2

System suitability:

The system suitability was checked for each validation stage. The five different concentration was checked for the system suitability parameters and they were found within the acceptance limits and results are shown in Table 8.

Table 8 System suitability testing

Standard	Concentration (CTD +AZE)	Peak Area CTD	Peak Area AZE	Resolution	No. Of Theoretical Plate	No. Of Theoretical Plate	Tailing Factor	Tailing Factor

		(AU)	(AU)		s CTD	sAZE	CTD	AZE
1	25+32	403565	767291	3.18	8116	8237	1.29	1.30
2	50+64	858068	1435281	3.36	8429	8632	1.28	1.29
3	75+96	1350667	2059323	3.36	8574	8396	1.26	1.27
4	100+128	1900010	2713699	3.35	8512	8449	1.28	1.27
5	125+160	2386139	3358378	3.48	8422	8346	1.27	1.26

Analytical method validation:

Linearity:

The regression analysis of the working standard solutions (25 µg/ml- 125 µg/ml of CTD and 32 µg/ml-160 µg/ml of AZE) was carried out. The correlation coefficient was found to be 0.9995 for CTD and 0.9991 for AZE. The linearity graph of CTD and AZE along with the regression equation is shown in the Figure 11 and Figure 12.

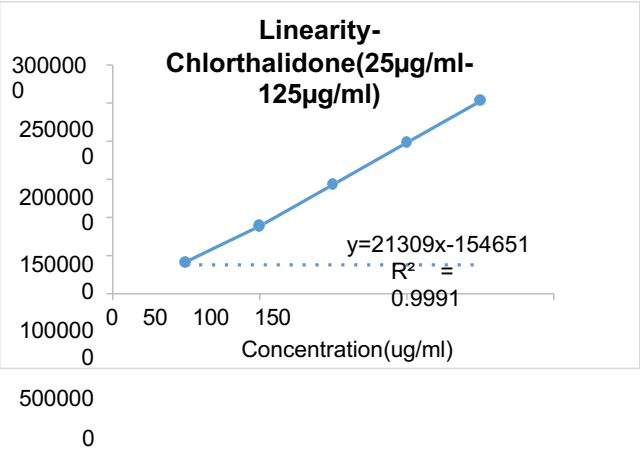

Figure 11 Linearity Plot of Concentration Vs Peak Area of Chlorthalidone in the range of 25- 125 ug/ml

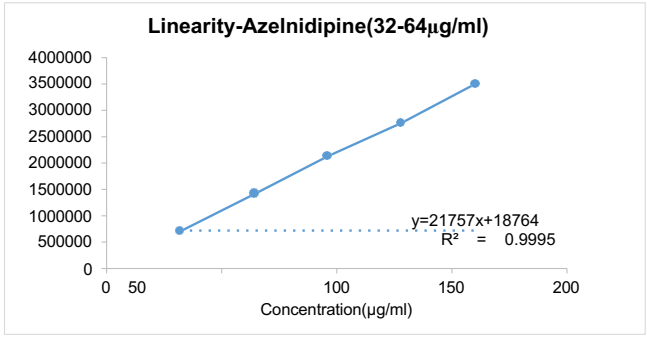

Figure 12 Linearity Plot of Concentration Vs Peak Area of Azelnidipine in the range of 25-125 ug/ml

Specificity

Percentage interference was calculated, and it was should be found less than 0.5 %. Thus the method is specific. The blank, standard and sample chromatogram are shown in Figure 13, Figure 14, Figure 15.

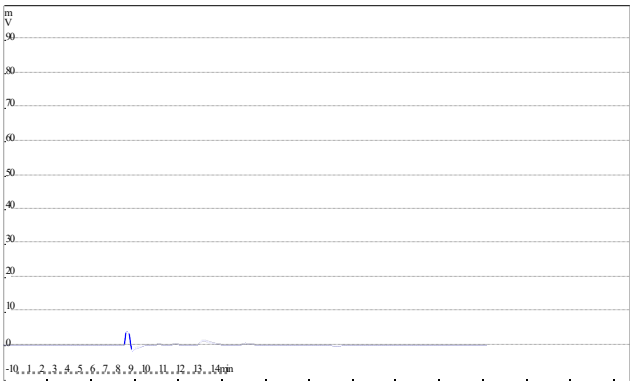

Figure 13 Chromatogram of blank (Retention Time (min) Vs Response (mV))

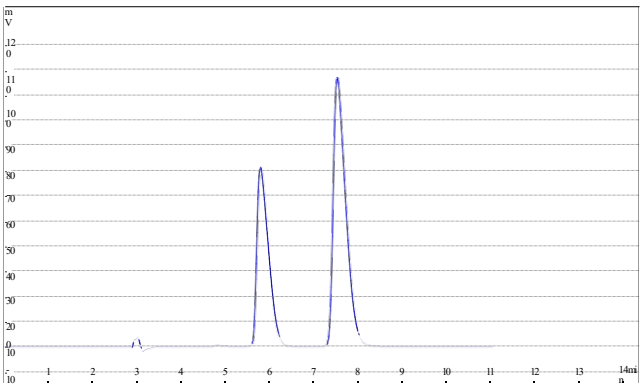

Figure 14 Chromatogram of Standard (Retention Time (min) Vs Response (mV))

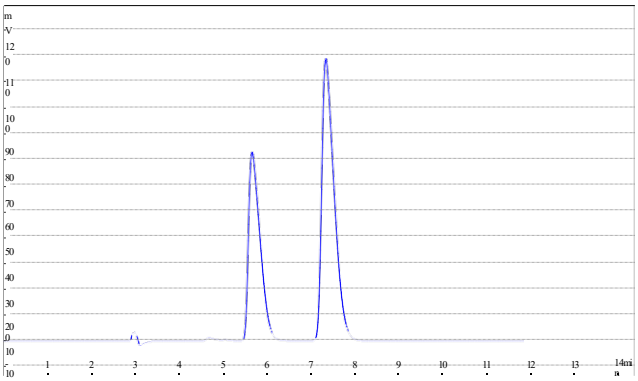

Figure 15 Chromatogram of sample (Retention Time (min) Vs Response (mV))

Accuracy

Percentage recovery was found in the range of 99.17-101.50% and 99.66-101.72% for CTL and AZL, respectively. The recovery is within the acceptance limits. The results for accuracy study is shown in Table 9.

Precision

The repeatability and intermediate precision was carried out and expressed in the terms fo the RSD. The RSD obtained was within the acceptance limit. The results for accuracy and precision are shown in Table 9.

LOD and LOQ

LOD and LOQ of CTD and AZE were computed and found 0.157606994 µg/ml and 0.477597 µg/ml and 0.245047313 µg/ml and 0.7425676 µg/ml respectively.

Robustness:

The robustness of method was evaluated by changing the values of pH of the mobile phase and the detection wavelength. The system suitability parameters were within the acceptance limits and obtained responses %RSD was less than 2 for all parameters.

Analysis of the dosage form

Percentage assay of the test solution was calculated, and the result was found to be within acceptance limits. Results are mentioned in Table 9.

Table 9 Validation parameters summary; Where, n=number of samples, SD: Standard deviation

Drug	**Accuracy**	**Precision(%RSD) Repeatability**		**Intermediate Precision (Ruggedness)**	**Assay**	
		Inter day (n=6)	**Intra day (n=6)**	(n=6)	**Label Claim**	**%Assay (n-=3) ±SD**
CTD	99.17-101.50 %	1.4463	0.7128	0.11477	8 mg	99.35 ± 0.042
AZL	99.66-101.72 %	0.8043	0.9689	0.30032	6.25 mg	99.86 ± 0.185

Greenness Assessment:

AGREE:

The AGREE assessment results are shown in Figure 16. In current analytical method, the AGREE score was 0.66. The red, yellow and orange colored areas in the AGREE image are the weak points as per green chemistry principles. The section 1 and 3 is about Sample treatment and device positioning, which is off line in case of the HPLC. The section 7 is about the waste generated for sample analysis, which is 11.1 ml per sample in current analytical method. The method uses methanol, which is inflammable and can lead to occupational hazard. However, the analysis is performed in a safe environment. Overall the method can be considered as green as per AGREE assessment.

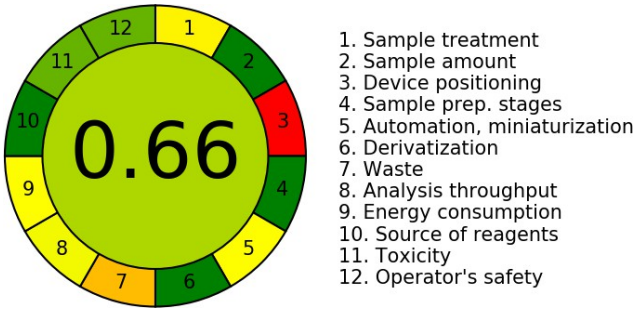

Figure 16 AGREE analysis result for developed and validated analytical procedure

The analytical eco scale assessment (analytical ESA):

For the proposed analytical procedure, the results for analytical eco scale assessment is given in Table 10. The analytical eco scale score obtained was 78. The score ranks the current analytical procedure on analytical eco scale of excellent green analysis.

Table 10 Analytical Eco Scale Assessment

Parameters	**Penalty Points(PP)**
Reagents	
Methanol	6
O-Phosphoric Acid	4
Energy Consumed	
HPLC	1
Ultra-sonicator	0

Occupational Hazard	3
Waste	8
Total Penalty Points	∑PP=22
Eco-Scale Score	Score:100-22=78

CONCLUSION:

Based on the results, it can be concluded that a robust, regulatory flexible, green and economic method was developed for simultaneous estimation of Azelnidipine and Chlorthalidone with improved method understanding using the analytical quality by design approach and validated

successfully using ICH Q2(R2) guideline. The developed method can be easily transferred and used into the routine analysis. The method remains flexible throughout the lifecycle, as MODR is established, within which the method maintains its intended performance. The established MODR reduces the burden of regulatory approval for the developed analytical method as moving within the MODR do not require regulatory reporting. The developed RP-HPLC method for simultaneous estimation of the Azelnidipine and Chlorthalidone satisfied all the selected validation parameters as per ICH Q2 (R2). The developed analytical method was used for the analysis of the dosage form, and % recovery of fixed dose combination as compared to the standard was found to be 99.35 ± 0.042 % for Chlorthalidone and 99.86 ± 0.185 % for the Azelnidipine. The greenness estimation by AGREE and ESA shown the developed analytical method as excellent green analysis. This shows that method is accurate, precise and robust along with additional advantage of the regulatory flexibility, ease of method transfer and greeness.

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