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Advanced UHPLC-Based Analytical Framework for Concurrent Quantification of Amlodipine and Lisinopril: A Design of Experiments Optimization Strategy

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Abstract
Amlodipine and lisinopril combination treatment is frequently utilized in the management of hypertension. This research aimed to develop a novel, straightforward, rapid, precise, efficient, and reproducible UHPLC method for these medications in solid dosage forms, with validation conducted in line with ICH standards. Pharmaceutical analysis plays a pivotal role in identifying, quantifying, and characterizing pharmaceutical compounds. Chromatographic analysis was performed using the statistical software Design Expert 13. A stability indicator technique was established, developed, and fine-tuned for the simultaneous quantification of Amlodipine and Lisinopril. The CCD was prepared with three independent variables: wavelength (210-215 nm), flow rate (0.5-1mL/min), and runtime (2-10 minutes). The dependent variables selected were retention time, peak area, and theoretical plate. The effective separation of AMD and LSN was achieved on Luna C18 (4.6 mm × 150 mm; 5 µm particle size), which was used to aid in accomplishing chromatographic separation using Acetonitrile: Water: Phosphoric acid (pH 2.5) adjusted by phosphoric acid at 245:740:15 % volume with a 1.0 mL/min flow rate at 215 nm. The developed method was successful in achieving good retention times and peak shapes for amlodipine (1.694 min) and lisinopril (3.365 min). The suggested approach was confirmed under the ICH Q2(R1) guidelines, showing outstanding linearity, precision, accuracy, and robustness. This comprehensive analytical approach demonstrates UHPLC's relevance for routine pharmaceutical quality control.

Keywords: amlodipine, lisinopril, UHPLC, method validation, central composite design, experimental design.

1. Introduction

Amlodipine (AMD) is chemically known as 2-[(2-aminoethoxy) methyl]-4-(2-chlorophenyl)-1,4-dihydro-6-methyl-3,5-pyridine dicarboxylic acid (3-ethyl-5-methyl ester). It is a calcium channel blocker (dihydropyridine derivative) used as an antihypertensive agent and to treat angina.

Over 1 billion adults worldwide have hypertension, and its prevalence rises with age, making it a major health concern. Calcium channel blockers, ACE inhibitors, angiotensin receptor blockers, and diuretics are some of the medications used to treat hypertension. AMD is a beta-blocker that reduces blood pressure,

heart rate, and chest pain in patients with CAD (Babar et al., 2021; Budawari S. The Merck Index, 1996; Ghogare RC & Godge RK, 2023; Sweetman SC, 32 C.E.).

Lisinopril (S)-1-[N2-(1-Carboxy-3-phenylpropyl)-L-lysyl] is the chemical formula, and it is a strong, competitive blocker of angiotensin-converting enzyme (ACE). This enzyme converts angiotensin I (ATI) into angiotensin II (ATII). (Chauhan et al., 2011; Ghogare RC & Godge RK, 2023). Lisinopril (LSN) is an oral medication that inhibits angiotensin-converting enzyme, utilized for managing high blood pressure, heart failure, and complications related to diabetes (*Lisinopril Dihydrate*, 2024). Many analytical methods, like spectrophotometric, chromatographic, and electrochemical techniques, have been reported for both simultaneous and individual detection of AMD and LSN. However, most current methods either lack enough sensitivity, need extensive sample preparation, or show poor separation efficiency for complex pharmaceutical mixtures. (Babar et al. 2021). A technique utilizing ultra-high-performance liquid chromatography (UHPLC) for the simultaneous determination of AMD and LSN in a combination dose form has not yet been developed.

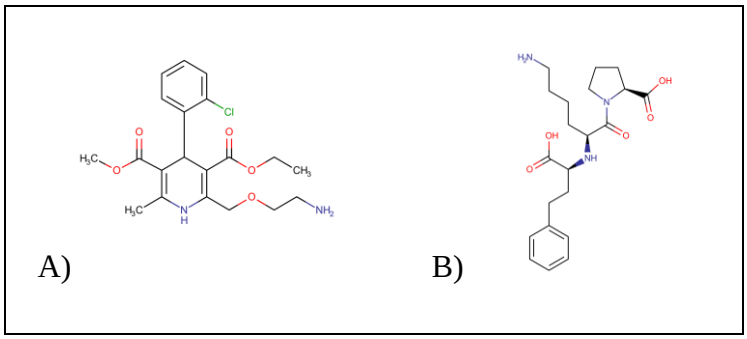

Figure. 1: Chemical structures of the analytes. Chemical structure of (A)Amlodipine and (B)Lisinopril.

Conventional HPLC methods for the contemporaneous determination of these compounds constantly suffer from long run times and high solvent consumption. Ultra High-Performance Liquid Chromatography is a powerful method for separating compound mixtures. It is utilized in analytical chemistry to determine, measure, and assess the purity of individual components within a mixture. In general, UHPLC is an effective analytical technique that provides greater efficiency, sensitivity, and throughput than conventional HPLC and UPLC(Popat Nagare et al., 2022). Thus, this research presents the establishment of a UHPLC method for the simultaneous determination of AMD and LSN via a Design of Experiment (DoE) approach for method validation.

The Implementation of the Design of Experiments (DOE) streamlines method development by statistically evaluating critical chromatographic factors and their interactions. Chemometric approaches offer various experimental design options, including Box–Behnken, Do Ehlert, and central composite designs (CCD), which are effectively employed for the optimization of UHPLC methods (Solanki et al., 2014). DOE utilizes a statistical approach to establish mathematical equations or models for outcomes based on the level of factors studied. This study assesses the strength of the UHPLC analytical method by using central composite design (CCD). Central Composite Design (CCD) is a popular experimental approach within response surface methodology (RSM), which is beneficial for creating second-order (quadratic) models aimed at optimizing processes that involve multiple variables (Shreya, 2025). Among the different types of experimental designs, the Central Composite Design (CCD) was chosen as a response surface design

for predicting nonlinear responses. Its flexibility regarding the number of experimental runs and the information it provides on main and interaction effects of factors also contributed to its preference (Bhutani et al., 2014). CCD that combines a three-level fractional factorial design that includes factorial points, axial points, and center points. Hence, a DOE-driven development and validation of a UHPLC method for the concurrent quantification of amlodipine and lisinopril in tablet formulations was conducted using a CCD design for robustness testing.

2. Materials and Methods

2.1. API and Reagents

Sample amlodipine (AMD) and lisinopril (LSN) were acquired from Synthia Labs. The method utilized Analytical grade reagents and chemicals and HPLC grade solvents were obtained from Rankem Chemicals.

2.2. Instrumentation and Chromatographic Conditions

An Agilent 1220 Infinity Ultra-high-performance liquid chromatography system was used to develop the technique. Chromatographic separation was performed using a Luna C18 stationary phase column (4.6 mm × 150 mm; 5 µm particle size) under isocratic elution conditions. The components of the mobile phase were acetonitrile, water, and phosphoric acid (which was used to bring the pH down to 2) in the following ratio: 245:740:15% v/v/v. The eluents were monitored with a detector set to 215 nm, and the flow rate was determined to be 1.0 ml/min. The system was controlled using a system controller and a personal computer with UHPLC (Agilent ChemStation**)** software installed on it. The mobile phase was degassed by an ultrasonicator, Newtown Sonics & Materials, Inc. Absorbance spectra were recorded using a double-beam UV spectrophotometer. Experimental design, data analysis, and desirability function calculations were performed using Design Expert 13.0.

2.3. Preparation of Mobile Phase

In a beaker, HPLC grade acetonitrile, water, and phosphoric acid were mixed according to the ratio. Dissolve 4.08 g of potassium phosphate monobasic in 800 mL of water, then adjust the pH to 2.5 using phosphoric acid and dilute with water to 1000 ml. The mobile phase was ready in ratio of 245:740:15 % v/v/v of {Acetonitrile: Water: Phosphoric acid}

2.4. Preparation of Standard stock and Sample solutions

Separate 100 mL and 50 mL volumetric flasks containing 50 mg of AMD and 25 mg of LSN from each of the reference standards. Both of the drugs were dissolved in 50 mL of phosphoric acid and sonicated until dissolved. Dissolve and adjust the volume with a diluent. Transfer 5 mL of the AMD standard stock solution and 5 mL of the LSN standard stock solution into a 50 mL volumetric flask, then dilute with the diluent.

2.5. Preparation of sample solution

Precisely measure the equivalent of a 5 mg powdered sample of amlodipine and place it into a 100 ml volumetric flask. The sample was dissolved in Acetonitrile. The solution was filtered. Then, 5 ml of this solution was diluted in a 50 ml volumetric flask. The volume was then filled with the mobile phase.

2.6. Selection of detection wavelength

The standard solutions of AMD and LSN were analyzed within the 200–400 nm range to identify the drug's maximum absorption wavelength. Detection wavelength at 215 nm was chosen as the peak wavelength for analysis.

2.7. Analytical Method Validation

Method validation is a written proof that offers a high level of assurance for a particular technique, and the procedure used to ensure that the analytical method is suitable for its intended use. In accordance with ICH Q2 (R1) recommendations, ICH HARMONISED TRIPARTITE GUIDELINE VALIDATION OF ANALYTICAL PROCEDURES (2005) the devised UHPLC technique for the concurrent quantification of both drugs was verified.

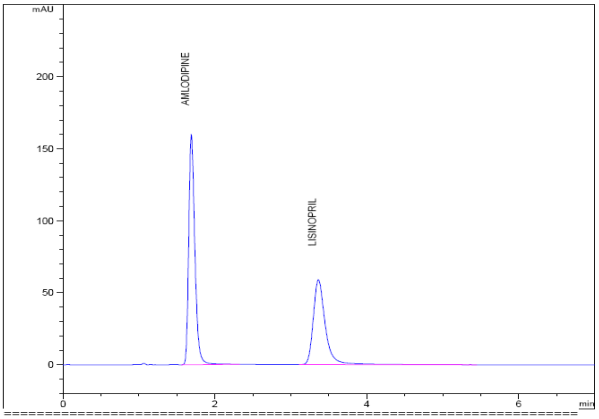

Figure 2. System suitability chromatogram

2.7.1. System suitability parameters

As per the standards in the ICH guidelines, various parameters, such as resolution, theoretical plates, and tailing factor, are checked to ensure that the developed method can be used for its intended purpose. The % RSD for the area of the five standard injection results should not be more than 2%, as shown in Figure 2.

2.7.2. Specificity:

Checking for any interference in the optimized method. No interfering peaks should appear in the blank and placebo at the retention times of these drugs, as per the developed method. Hence, this method is considered to be specific.

2.7.3. Precision

Here, the solutions were analyzed for six replicates to check the extent of deviation in the results. For inter-day, concentrations were analyzed, three times at short intervals on the same day, and intraday, three concentrations were analyzed for three different days. The results were accepted as per the guidelines if the RSD was less than 2%.

2.7.4. Linearity(Lopez Garcia et al., 2011.)

Concentration series of 50-100 µg/ml and 2.5-7.5 µg/ml of Amlodipine and Lisinopril were injected into UHPLC and studied at 215 nm wavelength. The data of the peak area versus concentration graph were plotted to obtain the calibration curve. Using Microsoft Excel tools, the equation of the regression line and the R^2 coefficient were determined.

2.7.5. LOD and LOQ

This can be estimated using the signal-to-noise ratio. It determines the smallest concentration of drug that can be detected, called LOD, and the smallest concentration of drug that can be quantified, called LOQ.

LOD = 3.3 × standard deviation ÷ slope of curve

LOQ = 10 × standard deviation ÷ slope of curve

2.7.6. Accuracy

To characterize the accuracy, the percent recovery was calculated using different sample weight methods. Triplicate injections were administered for each level of accuracy of the analytical solution at three levels: 80%, 100%, and 120%.

2.7.7. Robustness

Minor changes in parameters, such as flow rate and wavelength, were performed to determine whether any major effects were observed on the developed method. %RSD was within the limit.

2.7.8. Assay

About 5 mg of each powder of amlodipine and lisinopril was weighed into a 100 mL volumetric flask. The mobile phase (50 mL) was added, and ultrasonic treatment was performed for 10 min. The solution was passed through a 0.45 mm nylon syringe filter, and the volume was adjusted to the mark. Further dilution was done with mobile phase and mixed with standard stock solutions to form a mixed sample solution of 5mg of each drug. Injection into UHPLC was conducted, and the mean was determined for six replicates. The assay was carried out using the above-mentioned formulation.

3. UHPLC method development using the design of experiments

3.1. Optimization of design and analysis

Central Composite Design (CCD) is a popular experimental design in response surface methodology (RSM), especially for developing second-order (quadratic) models to optimize processes with multiple variables. Hence, CCD that combines the two-level factorial design with a star design and center points covers the factor space near the center with more points than at the periphery, and hence allows for a greater number of levels without necessarily experimenting with every combination of factor levels (Central Composite Design: Ultimate Guide, 2025). Chromatographic conditions were optimized using CCD (Design Expert 13.0). Before initiating an optimization process, one should explore the curvature term by employing a factorial design with center points. ANOVA produced a 2K factorial design. It showed that curvature was significant for all responses, as the p-value was less than 0.0500. This means the quadratic model should be seen as a separation process. (Krishnan et al., 2020)

To obtain the second-order predictive model, CCD under response methodology is employed. It was chosen because of the flexibility that will come with optimizing the UHPLC separation, allowing for a better understanding of the main and interaction effects of the factors. The selection of factors for optimization was based on a Preliminary experiment and prior knowledge from literature, as well as certain instrumental limitations. A rotatable response surface Central Composite Design (CCD) design by incorporating the CMVs and setting the goals for developing optimal conditions for increased peak areas, good resolution, and less time of analysis. The rotatable CCD was chosen because of its high consistency and relatively low variability.(Jonnalagadda et al., 2024) Dependent variables were chosen as wavelength, flow rate, and run time, and independent variables were chosen as retention time, peak area, and theoretical plates. Table 1 shows the levels of each factor studied for finding out the optimum values and responses.

Table 1: Layout of design matrix using CCD

Std	Run	Factor-1 Wavelength (nm)	Factor-2 Flow rate (ml)	Factor-3 Run time (min)	Response-1 Retention time (min)	Response-2 Peak area (mAU/ml)	Response-3 Theoretical plate (N)
9	1	210	0.5	2	3.1	250	3900
1	2	215	0.5	2	2.4	200	4100
3	3	210	1	2	4	275	3800
5	4	215	1	2	3	230	4000
7	5	210	0.5	10	2.8	270	4430
17	6	215	0.5	10	2.5	240	4490
14	7	210	1	10	3.5	305	4200
11	8	215	1	10	2.9	280	4280
13	9	208.3	0.75	6	3.6	260	4400
20	10	216.7	0.75	6	2.2	220	4290
16	11	212.5	0.33	6	3	240	4080
18	12	212.5	1	6	3.8	300	4550
12	13	212.5	0.75	2	2.8	230	3980
19	14	212.5	0.75	10	2.9	283	4700
15	15	212.5	0.75	6	3	285	4250
6	16	212.5	0.75	6	3.2	276	4260
4	17	212.5	0.75	6	2.9	278	4295
8	18	212.5	0.75	6	3	271	4350
2	19	212.5	0.75	6	3.1	284	4450
10	20	212.5	0.75	6	3.3	267	4540

4. Results and discussion

4.1. Statistical approach and profile of prediction with the help of quality by design experiments

Table 2: ANOVA Model for Quadratic Analysis

Response	Source	Sum of Squares	df	Mean Square	F-value	p-value	
Response 1-Retention time	**Model**	3.50	9	0.3890	18.59	< 0.0001	significant
	A-Wavelength	0.6870	1	0.6870	32.84	0.0002	
	B-Flow rate	0.3625	1	0.3625	17.33	0.0019	
	C-Run time	0.0040	1	0.0040	0.1912	0.6712	
	Residual	0.2092	10	0.0209			
	Lack of Fit	0.1009	5	0.0202	0.9312	0.5302	not significant
	Pure Error	0.1083	5	0.0217			
	Cor Total	3.71	19				
	Model	13782.63	9	1531.40	18.00	< 0.0001	significant

Response 2-Peak area	A-Wavelength	4308.56	1	4308.56	50.66	< 0.0001	
	B-Flow rate	1759.47	1	1759.47	20.69	0.0011	
	C-Run time	2624.40	1	2624.40	30.85	0.0002	
	Residual	850.57	10	85.06			
	Lack of Fit	599.73	5	119.95	2.39	0.1804	not significant
	Pure Error	250.83	5	50.17			
	Cor Total	14633.20	19				
Response 3 – Theoretical plate	**Model**	7.872E+05	9	87463.95	3.30	0.0383	significant
	A-Wavelength	1663.19	1	1663.19	0.0628	0.8072	
	B-Flow rate	3359.48	1	3359.48	0.1268	0.7291	
	C-Run time	1.769E+05	1	1.769E+05	6.68	0.0272	
	Residual	2.649E+05	10	26489.82			
	Lack of Fit	1.980E+05	5	39602.15	2.96	0.1294	not significant
	Pure Error	66887.50	5	13377.50			
	Cor Total	1.052E+06	19				

Based on design expert software analysis, a quadratic model was found suitable for the data. ANOVA outcomes in Table 2 indicate highly significant model F-values for Retention time (18.59), Peak area (18.0), and Theoretical plate (3.30). This indicates that the chosen quadratic model effectively explains the variation in each response. Furthermore, p-values less than 0.05 for all three models confirm the significance of the model terms. The predicted R^2 of 0.8929 matches well with the adjusted R^2 of 0.7301. Similarly, 0.8896 aligns with the adjusted R^2 of 0.5872, and 0.4306 corresponds with the adjusted R^2 of 0.2016. The suitable precision values that measure the signal-to-noise ratio are 17.028, 16.462, and 5.704. Each of these values is greater than 4, showing an adequate signal. In this study, the ratio was observed to be in the range of 5.704-17.028, which demonstrates an adequate signal and thus the model is significant for the separation procedure. The coefficient of variation (C.V.) measures how reproducible the model is. Generally, a model is considered fairly reproducible if the C.V. is less than 10%. In Figure 4a, the polynomial equation for Response 1 is as follows: Retention time = +3.00 + 0.28A - 0.32B + 0.05C + 0.08AB - 0.06AC + 0.04BC - 0.12A^2 + 0.09B^2 - 0.02C. In Figure 4b, the polynomial equation for Response 2 is: Peak area = +270 + 38A - 27B + 31C - 12AB + 10AC + 8BC - 18A^2 + 22B^2 - 15C. In Figure 4c, the polynomial equation for Response 3 is: Theoretical plate = +4300 + 22A + 18B + 85C - 10AB + 6AC + 12BC - 5A^2 - 9B^2 + 66C, which is presented in Table 3. When seen as a two-dimensional plane in a contour plot, the response surface is formed by joining all points that have the same response to construct counter lines of constant responses. A surface plot can make the reaction easier to observe by providing a three-dimensional image of it. Analyze the perturbation plots and response plots of optimization models revealed that factors A, B, and C had a significant impact on the separation of the analytes. Contour plots and Response Surface plots for Retention time, Peak area, and Theoretical plate between them are depicted in Figure 3 and Figure 4.

Table 3: Predicted response models and statistical parameters obtained from ANOVA for CCD

Response	Regression Mode	Adjusted R^2	Model p-value	%CV	Adequate Precision
Retention Time(R1)	$3.00 + 0.28A - 0.32B + 0.05C + 0.08AB - 0.06AC + 0.04BC - 0.12A^2 + 0.09B^2 - 0.02C^2$	0.7301	< 0.0001	~6.5%	17.028
Peak Area(R2)	$270 + 38A - 27B + 31C - 12AB + 10AC + 8BC - 18A^2 + 22B^2 - 15C^2$	0.5872	< 0.0001	~7.2%	16.462
Theoretical Plates(R3)	$4300 + 22A + 18B + 85C - 10AB + 6AC + 12BC - 5A^2 - 9B^2 + 66C^2$	0.2016	0.0383	~9.6%	5.704

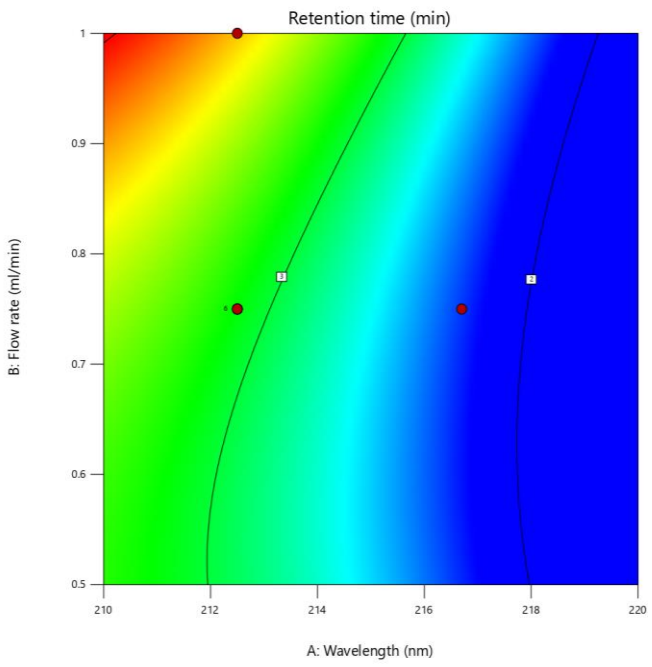

Figure 3(A)

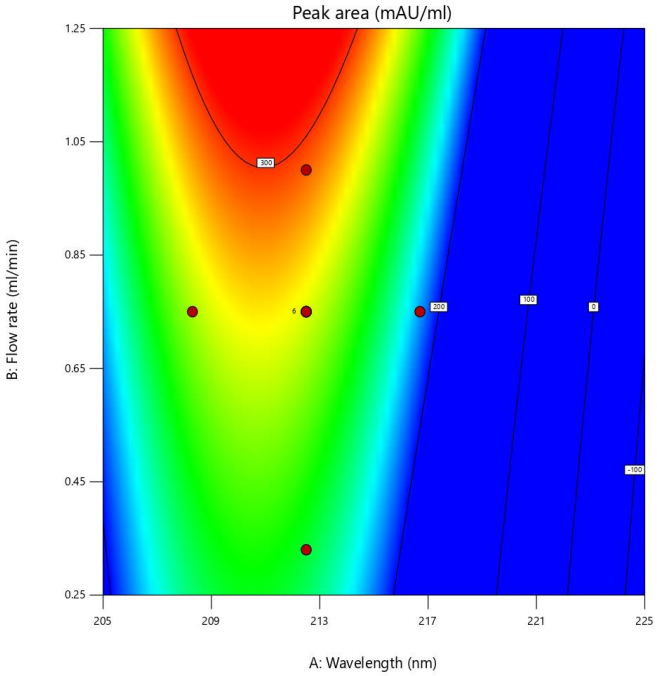

Figure 3(B)

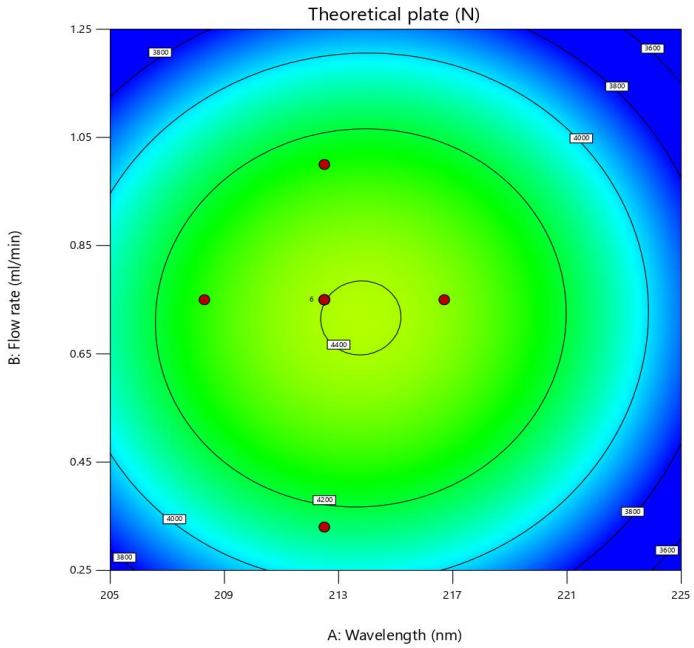

Figure 3(C)

Figure 3. Contour plots of (A) Retention time, (B) Peak area, (C) Theoretical plate

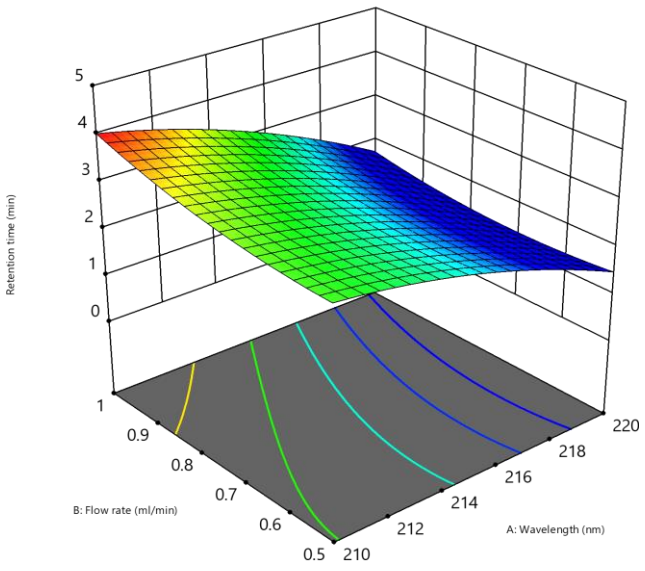

Figure 4 (A)

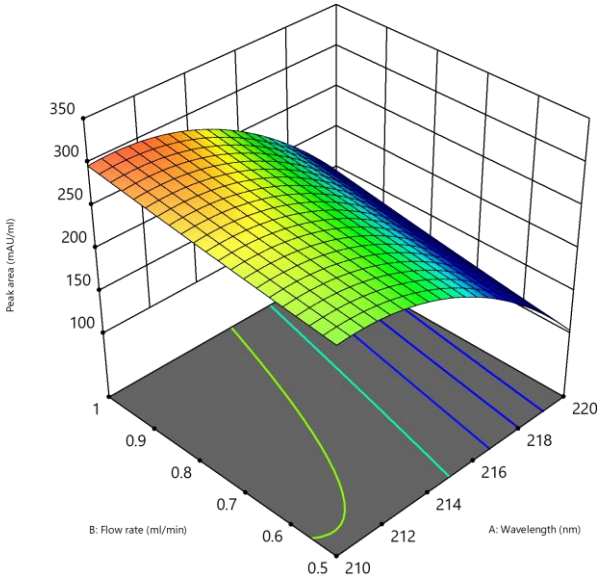

Figure 4 (B)

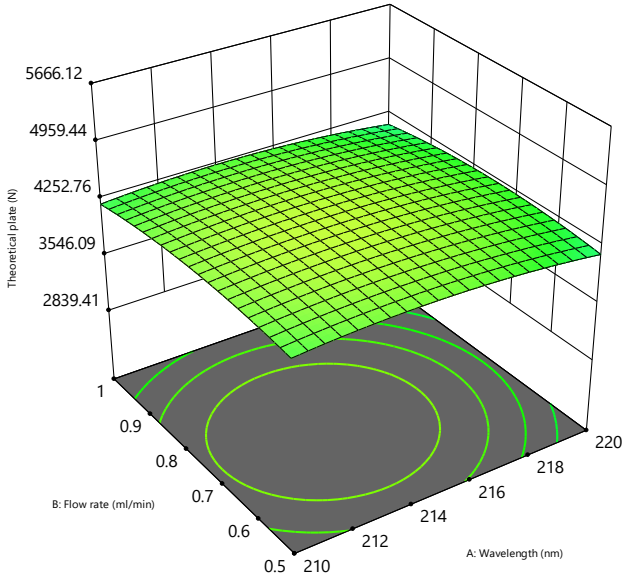

Figure 4 (C)

Figure 4. Response surface plots of (A) Retention time, (B) Peak area, (C) Theoretical plate

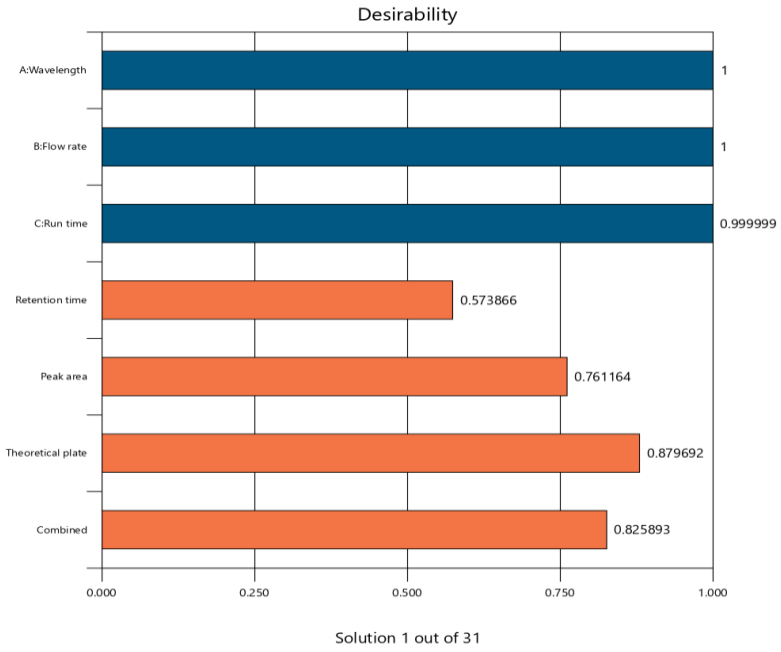

Figure 5. Graphical representation of the overall desirability function

From Figure 5, we can conclude that there was a set of coordinates producing a high desirability value D=0.825893.

4.2.Method Validation

Linearity, LOD, and LOQ

The data obtained for linearity in the range of 17.5-52.7µg/ml and 22.8-68.4 µg/ml of Amlodipine and Lisinopril by taking the mean of six replicates, and the lowest concentration for detection and quantitation limit is shown in Table 4. For linearity, R^2 was achieved to 1, which shows the relation is linear. LOD and LOQ were calculated with the help of slope and standard deviation.

Table 4. Linearity, LOD and LOQ

Parameters	AMD	LSN
Linearity concentration (µg/ml)	17.57-52.73	22.8-68.4
Linearity coefficient R^2	1.000	1.000
Linearity equation	y=66.6478x+2.0134	y=36.3321x+2.1847
LOD(µg/ml)	0.688	0.432
LOQ(µg/ml)	2.085	1.310

Specificity

Here, the placebo and standard solution are injected into the UHPLC. By comparing the peak, we check the developed method for any interference from excipients or degradants. Figure 6 shows that we did not find any interfering peaks in the blank and placebo at the retention times of these drugs. Therefore, we concluded that this method is specific.

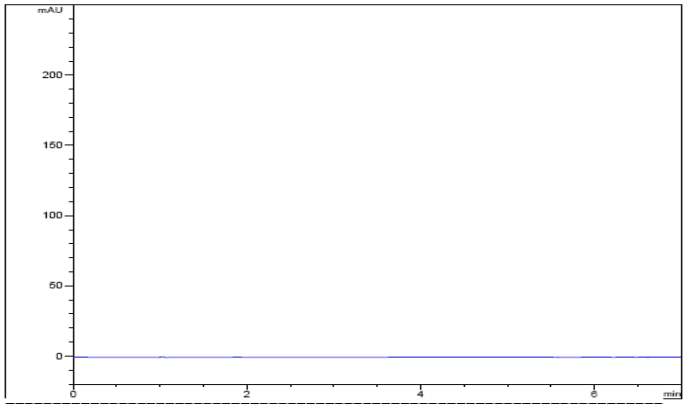

Figure 6. Blank chromatogram

Precision

The % RSD for Amlodipine and Lisinopril was calculated by measuring of same concentration for six replicates. Repeatability and intraday precisions were shown in Table 5

Table 5. Precision studies

Sample	Repeatability		Intraday-1		Intraday-2	
	% Assay found ± SD	RSD (%)	% Assay found ± SD	RSD (%)	% Assay found ± SD	RSD (%)
AMD	100.57%±0.013	0.26%	101.0%±0.009	0.17%	99.07%±0.007	0.13%
LSN	99.41%±0.034	0.69%	100.36%±0.053	1.06%	99.69%±0.014	0.27%

Robustness

In the proposed approach, we made slight changes to the parameters to check the performance of the unaffected method. We maintained robustness conditions like Flow minus (0.9 ml/min), Flow plus (1.1 ml/min), Wavelength minus (214 nm), and Wavelength plus (216 nm). We injected the samples in

duplicate. The system suitability parameters were mostly unaffected, and all of them passed. The percentage relative standard deviation (RSD) was within acceptable limits, as shown in Table 6. This demonstrates the method's robustness.

Table 6. Robustness Method

S. No	Condition	%RSD of Amlodipine Assay	%RSD of Lisinopril Assay
1	Flow rate (-) _0.9	0.26%	0.42%
2	Flow rate (+) _1.1	0.22%	0.19%
3	Wavelength (-) _214	0.38%	0.28%
4	Wavelength (+) _216	0.24%	1.01%

Accuracy

A recovery study was performed to study accuracy. Three different amounts of spiking were used to create the sample solutions: 80%, 100%, and 120%. Table 7 displays the percentage recovery results that were acquired using the suggested UHPLC procedure. The percentage of recovery within 98–102% supports the accuracy of the established approach in accordance with ICH Q2 (R1) recommendations.

Table 7. Accuracy study

Sample	Label claim	%Recovery (80-120%)	%RSD
AMD	5	99.93%-101.37%	0.39
LSN	5	99.95%-101.06%	0.35

Assay

By performing the assay method, the results are shown in Table 8. Drug content found was compared with the label claim, and % assay recovery was found to be 100.66% for Amlodipine and 99.92% for Lisinopril.

Table 8. Assay table

Samples	Standard conc	Test conc	%Assay recovery
AMD	5 µg/ml	5.033 µg/ml	100.66%
LSN	5 µg/ml	4.996 µg/ml	99.92%

5. Conclusion

In this study, the DOE strategy was adapted to develop a novel, robust, accurate, and precise method for the simultaneous estimation of Amlodipine and Lisinopril in solid dosage form. The CCD model plays a crucial role in response surface methodology. The most significant benefit of this kind of optimization model is that it is more precise, which eliminates the need for a three-level factorial experiment when developing a second-order quadratic model. (Bhattacharya et al. 2021) There is a lower possibility of

failure during method validation and transfer thanks to the improved understanding of method variables made possible by the QbD approach to method creation. The Design Expert software-based automated QbD method development technique has offered a more reliable, high-performing technique in less time than developing a manual approach. The retention time of Amlodipine was found to be 1.694 minutes, and Lisinopril was found to be 3.365 minutes. %RSD of the Amlodipine was found to be 0.65% & Lisinopril was found to be 0.53%. % Assay was obtained as 100.66% for Amlodipine & 99.92% Lisinopril. Regression equation of Amlodipine y = 66.6206 x – 2.0134 R^2= 1.0000 & Lisinopril is y = 36.3321x + 2.1847 R^2 = 1.0000. Retention times were decreased, and the run time was decreased, so the method developed was simple and economical. This analytical concurrent quantification of Ultra High-Performance Liquid Chromatography (UHPLC) may be implemented in routine Quality Control assessments within the pharmaceutical sector.

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7. Conflict of interest

The authors declare that they have no conflict of interest. The article does not contain any studies with animals or human participants performed by any of the authors**.**

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