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Research Article

Response Surface Methodology Based Development and Quantification of Celecoxib and Amlodipine Using RP-HPLC.

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Abstract: A novel RP-HPLC method for estimation of Amlodipine Besylate and Celecoxib using an experimental design approach employing response surface technique was developed and validated using a C18 column and its application in marketed formulation. A multivariate optimization of experimental conditions was carried out using experimental design employing organic content in the mobile phase, pH, and flow rate of the mobile phase as three independent variables. The aim of the study was to apply response surface methodology and to study the effect of the independent variables on separation and estimation of both drugs by RP-HPLC method using faced central composite (FCC) experimental design which is novel in this area of research. Optimization of retention time of the last eluting peak and peak symmetry was performed using Derringer's desirability function in which potassium dihydrogen phosphate buffer with pH 4. 45, 25 mM, 0.5% Triethylamine and acetonitrile in an isocratic proportion of 70:30% v/v and 1.2 ml/min flow rate was found to be the predicted optimum condition. DoE approach based RP-HPLC method development using response surface methodology led to efficient separation with a lower retention time of eluted peaks below 7 min indicating novelty of research. A linear response was observed over the concentration range of 40-240 µg/mL for Celecoxib and 1-6 µg/ mL for Amlodipine Besylate. Limit of detection (LOD) and limit of quantitation (LOQ) for Celecoxib were found to be 0.02 µg/mL and 0.08µg/mL, and for Amlodipine Besylate were 0.0053 µg/mL and 0.0162 µg/mL, respectively. The method was successfully validated in accordance with ICH guideline acceptance criteria for linearity, accuracy, precision, specificity, robustness.

Keywords: Response Surface Methodology, Celecoxib, Amlodipine Besylate, RP-HPLC, Validation

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1. INTRODUCTION

In the last few years, there has been a tremendous increase in the need for quality standards improvement in drug analysis followed by stringent regulations laid down by global regulatory authorities. To meet the constant amending regulatory requirements by the regulatory authorities, a strong change in the traditional analytical procedures are necessary. RP-HPLC method of analysis has been a traditional method employed by the majority of the global pharmaceutical industry since the last two decades based on the one factor at a time (OFAT) approach, which is an exhaustive process involving a large number of experimental runs. As per the amendments in drug regulations pharmaceutical companies are constantly striving to have continuous improvement in quality and purity of drugs in different dosage forms in a short frame of time. As a consequence, there has been a void in the area of improvement in analytical methods which can be filled by employing design of experiment (DoE) approach wherein statistical based design of the experiment is developed to determine the design space. Hence, the study aims to quantify amlodipine besylate and celecoxib in pharmaceutical formulation by RP-HPLC technique employing DoE approach. The objective of the study is to optimize various chromatographic parameters for better separation and quantification of both the drugs in combined formulation, simultaneously evaluating the effects of individual experimental parameters on separation and estimation of them using faced central composite (FCC) experimental design. Amlodipine (AML), chemically is 3-O-ethyl 5-O-methyl 2-(2-aminoethoxymethyl)-4-(2-chlorophenyl)-6-methyl-1, 4-dihydropyridine-3, 5-dicarboxylate (Fig. 1a) oral dihydropyridine calcium channel blocker used in the treatment of hypertension, chronic sf angina, vasospastic angina and angiographically documented coronary artery disease. The therapeutic efficacy of a calcium channel blocker depends upon its ability to block the voltage-dependent L-type calcium channels and thereby inhibits the calcium influx. A reduced level of intracellular calcium prevents its binding to calmodulin thereby, preventing activation of myosin light chain kinase responsible for vasoconstriction. Amlodipine also improves vascular endothelial function and reduces afterload leading to lowering myocardial oxygen demand. [1, 2] Celecoxib chemically is 4-[5-(4-methylphenyl)-3-(trifluoromethyl) pyrazol-1-yl] benzene sulfonamide a nonsteroidal anti-inflammatory drug (NSAIDS) used in multiple indications like rheumatoid arthritis, osteoarthritis, acute pain and ankylosing spondylitis. It is also indicated for the treatment of acute pain primary dysmenorrhea. Celecoxib prevents synthesis of prostaglandins through selective inhibition of cyclooxygenase-2 (COX-2) which is an integral part of the inflammation and pain pathway.[3] The release of arachidonic acid from cellular phospholipid is the primary step involved in prostaglandin synthesis followed by catalysis of prostanoids by the help of COX-1 and COX-2 isoenzymes. Celecoxib possesses a higher quantum of inhibitory activity against COX-2 with potential cancer chemo-preventive activities against various malignancies. [4] The advantages of this single formulation over sequential administration includes increased compliance, possibly reduced cost, and less likelihood of dosage-related issues. Moreover, this single tablet formulation combines the anti-inflammatory activity of the celecoxib with the systemic vasodilation induced by the amlodipine. It is a promising treatment for patients with osteoarthritis and hypertension.[5] The basis of the novelty of the present research work lie in recent development in QbD, which involves optimization of separation parameters for better understanding of the effect of various parameters on the quality of separation using experimental design separation. Response surface methodology is an important novel optimization tool used in pharmaceutical analysis to identify the most imperative parameters, applying minimum number of trials. The aim of the study is to develop a robust method using statistical Design of Experiments (DoE) based on Quality by Design (QbD) approach. Our objective is to study variations in experimental parameters and their implications on the results of the experiment in an experimental region defined by the design space. [6, 7] The study would be helpful to major pharmaceutical organizations as FDA (Food and Drug Administration) is actively implementing the concept of QbD to improve the quality of products and processes in a systemic risk based manner rather than determining the test quality into product retrospectively, for which ICH guidelines have been issued for implementation of QbD in analytical field. [8,9] Extensive literature survey reveals that, few traditional RP-HPLC methods based on the one factor at a time (OFAT) approach have been developed and reported for quantification of Amlodipine besylate and Celecoxib individually and in combination dosage form. But all the reported methods were based on quantification of both the drugs using single factor-single response mode study which lacks interactive study approach, whereby effect of individual parameters on separation and quantification of drugs was a limiting factor.[10-17] Few spectrophotometric methods have been reported for estimation of Celecoxib and Amlodipine in combined dosage form which also had similar limitations as that present in chromatographic techniques.[18-21] Identification of a particular goal of the proposed analytical method for routine quality control process and assay of the pharmaceutical formulation is the first stage to be accomplished in the QbD approach. Determination of a specific goal of an analytical method is followed by evaluation of critical factors affecting critical quality attributes (CQA) such as peak resolution, peak asymmetry, and run time during the method development. A relationship is established among the critical factors and their effect on experimental responses by creating a suitable experimental design and a mathematical model which is one of the most important stages of QbD approach wherein critical factors are optimized using a central composite design experiment.[9] Present research work is found to be novel in terms of lack of literature, pertaining to the application of experimental design approach based on optimization of chromatographic conditions simultaneously, evaluating the impact of experimental parameters on results of the study. This QbD approach improves selectivity and provides better method control parameters as well as method transfer. Various methods related to the adoption of QbD approach in HPLC have been reported for other drugs [22-25] which prompted the authors to select the present research task as there are no analytical methods based on RP-HPLC, developed and reported for the estimation of Amlodipine and Celecoxib in combined pharmaceutical dosage form implementing QbD approach.

2. MATERIALS AND METHODS

2.1 Materials

Amlodipine and Celecoxib were obtained as gift samples from Dr Reddy's Laboratories Ltd, Hyderabad, and Zydus Cadila, Ahmedabad India respectively. Different HPLC Grade solvents like methanol, acetonitrile (METHANOL), HPLC grade water,

potassium dihydrogen phosphate and phosphoric acid were obtained from Ricca Chemical Company. HPLC grade water was used to prepare all solutions. The formulation was prepared in a laboratory which has 500 mg of Celecoxib (CEL) and 50 mg of Amlodipine (AML). The formulation contains Mannitol, Croscarmellose Sodium, Polyvinylpyrollidone as excipients.

3. STATISTICAL ANALYSIS

Design-Expert version 8.0.4.1 of Stat-Ease was used for Experimental design (Faced central composite), calculations of desirability function, and data analysis. The standard deviation and relative standard deviation of validation data were calculated using Microsoft Excel 13.

3.1 Preparation of Standard Stock Solution

A quantity of 100 mg of AML and CEL API (Active pharmaceutical ingredient) were weighed and transferred into a suitable volumetric flask and dissolved in sufficient quantity of mobile phase and made to volume to obtain 1 mg/ml of both AML and CEL. Standard stock solutions were diluted using the mobile phase to obtain working standard solutions (40-240 µg/mL and 1-6 µg/mL) and both were protected from light during analysis.

3.2 Chromatographic Procedure

Kromasil C18 column (150 mm × 4.6 mm, 5 µm) was used for Chromatographic separations. The mobile phase employed in chromatographic separation was a mixture of methanol and potassium dihydrogen phosphate (25 mM KH_2PO_4) buffer and its pH was adjusted with phosphoric acid (H_3PO_4). 0.5% triethylamine (TEA) was used as an ion pairing agent in the mobile phase to decrease the tailing of the AML peak. The flow rate of 1 ml/min, injection volume of 10 µl, and maximum wavelength of 253 nm was used for analysis.

3.3 Experimental Design and Response Surface Methodology

A faced Central composite design (FCCD) was provided for three independent variables using a partial factorial design combined with five replicates including center points and five axial points taken at extreme levels to optimize optimum pH of mobile phase, flow rate and % of organic solvent of mobile phase for effective separation. An R2 coefficient of determination was used to evaluate the quality to of the fitted polynomial models and Derringer's desirability function was applied to recognize the position of true optimum condition. Response surface methodology (RSM) was employed to accurately optimize the variable parameters for the best possible outcome of responses in the experimental region.

4. RESULTS & DISCUSSION

4.1 Method Development and Optimization

RP-HPLC modes of chromatography were used for separation and analysis of both the compounds due to their intermediate polarity [26, 27]. The C18 column was considered for analytical separation of both the drugs due to better resolution and peak symmetry. Based on overlain UV spectra of both the drugs 260 nm was selected as optimum detection wavelength with good detector sensitivity and response with minimum noise. Optimization of pH of the mobile phase has always been a critical parameter for better selectivity of the analytical method. Various mobile phase compositions with pH from 3.0 to 5.5 were tried for better separation and peak symmetry of the analyte based on pKa of molecules and prior research literature, wherein mobile phase with pH 4.7 was finally optimized. [28, 29] Excess tailing (>2) was observed for both the analyte with the lower composition of the organic phase modifier. Tailing was improved with an increase in the composition of organic phase. [29-31] Best resolution and minimum interference were observed with a mobile phase composed of methanol and phosphate buffer at 70:30, v/v ratio at pH 4.45, and a flow rate of 1.2 ml/min.

4.2 Design of Experiment and Design Space

Central composite design concept has emerged in the process of optimization wherein, the best possible outcome can be determined by employing many response variables. [33,34] CCD model also known as Box-Wilson Central Composite Design where augmentation of center points is carried out using a group of star points which helps in the estimation of curvature.[35, 36] The α value is to be determined for each axial point and if the value of alpha is less than 1, it indicates that the axial point lies in the cube. While, if the alpha value is more than 1, it indicates that the axial point lies outside the cube.[37-39]Faced Central Composite Design matrix consisting of 15 optimized experiments was developed and shown in table 1. A two-level factorial design consisting of center points within the experimental region is developed and implied in the method development. Optimization of acetonitrile concentration and flow rate of the mobile phase was done based on the responses obtained and was finalized between 60% v/v and 80% v/v of organic component modifier and 0.8 ml/min to 1.2 ml/min of flow rate respectively to obtain better peak symmetry for both the drugs and accurate quantification of drugs with minimum run time. The pH range was optimized in the range of 4.2 to 4.7 due to improvement in peak symmetry, as higher peak tailing was occurring at pH more than 4.7 and shelf life of the column was decreasing at pH less than 4.0. (Table I & II) The adjusted R^2 value was obtained well within the acceptable limits with probability $P<0.05$ indicating best fit and significant models. The value of % CV of reproducibility was lower than 10 with signal to noise ratio greater than four.

Table I: Dependant variables in HPLC for Central Composite Design

Factor	Name	Level (-)	Level (0)	Level (+)
A	Buffer pH	4.2	4.45	4.7
B	Flow rate (ml/min)	0.8	1	1.2
C	Methanol (%v/v)	60	65	70

Table II: Experimental Conditions and Responses for Central Composite Design

Design Points	Factor Levels			Responses	
	pH	Flow rate (ml/min)	Methanol (%)	Mean Rt (Amlodipine)	Mean T (Celecoxib)
1	4.2	0.8	60	16.642	1.137
2	4.7	0.8	70	9.035	1.528
3	4.45	1.2	65	8.493	1.312
4	4.45	1	65	9.165	1.316
5	4.2	0.8	70	9.035	1.372
6	4.7	1.2	70	5.812	1.412
7	4.7	1	65	9.213	1.335
8	4.45	0.8	65	11.276	1.317
9	4.7	0.8	60	13.268	1.339
10	4.2	1.2	70	4.756	1.290
11	4.2	1.2	60	12.831	1.125
12	4.2	1	65	10.224	1.229
13	4.45	1	60	14.246	1.206
14	4.7	1.2	60	10.572	1.282
15	4.45	1	70	5.773	1.315

Table III: ANOVA based Statistical Parameter and regression Model

Response	Regression Model	Adjusted R^2	Model P- Value	% C.V.	Adequate Precision
R_t	10.02-0.5588A-1.68B-3.31C+0.27AB+0.83AC-0.1244AC	0.96	<0.0001	6.78	26.31
T	1.3-0.074A-0.027B+0.083C-0.009AB-0.0104AC-0.0159BC	0.90	<0.0001	2.70	15.28

P value <0.05 Significant (n=3)

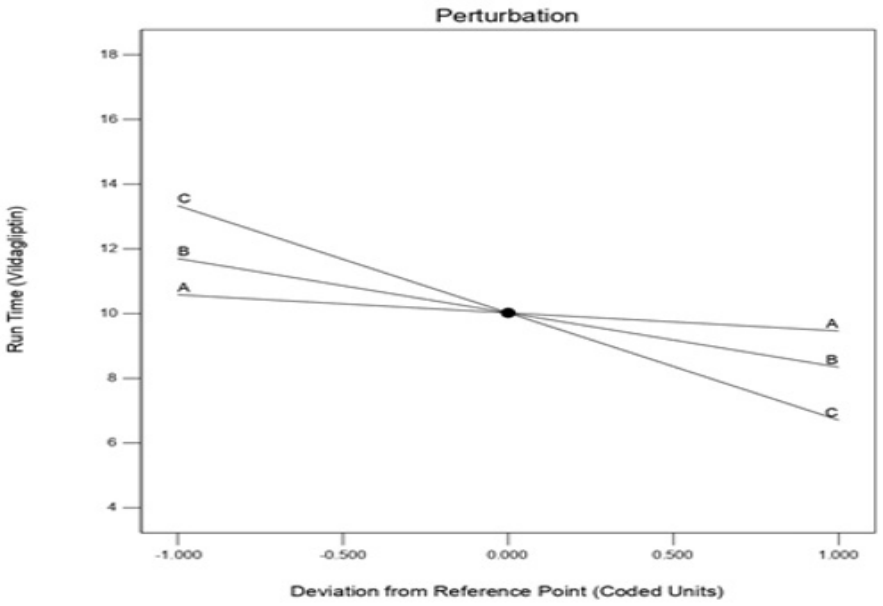


Fig I: (a) Perturbation plot showing effect of each factor on retention time of Amlodipine

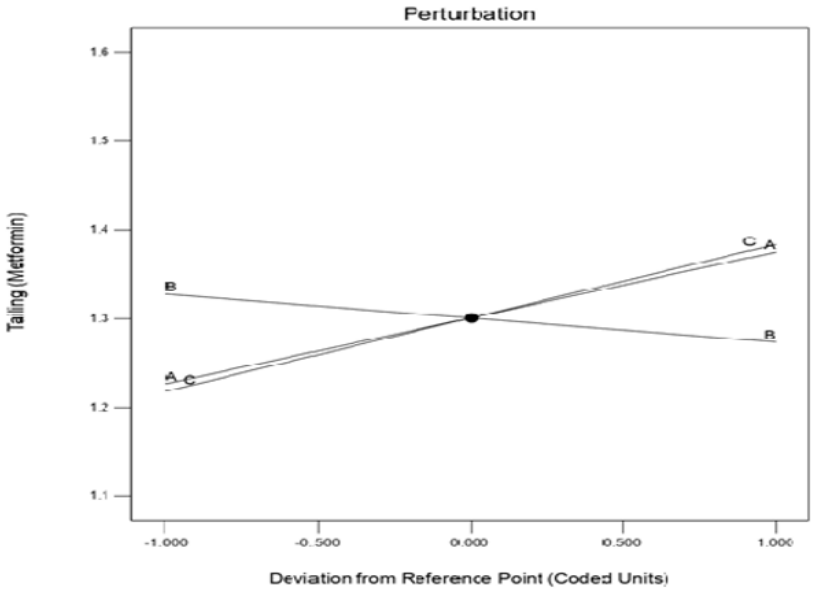
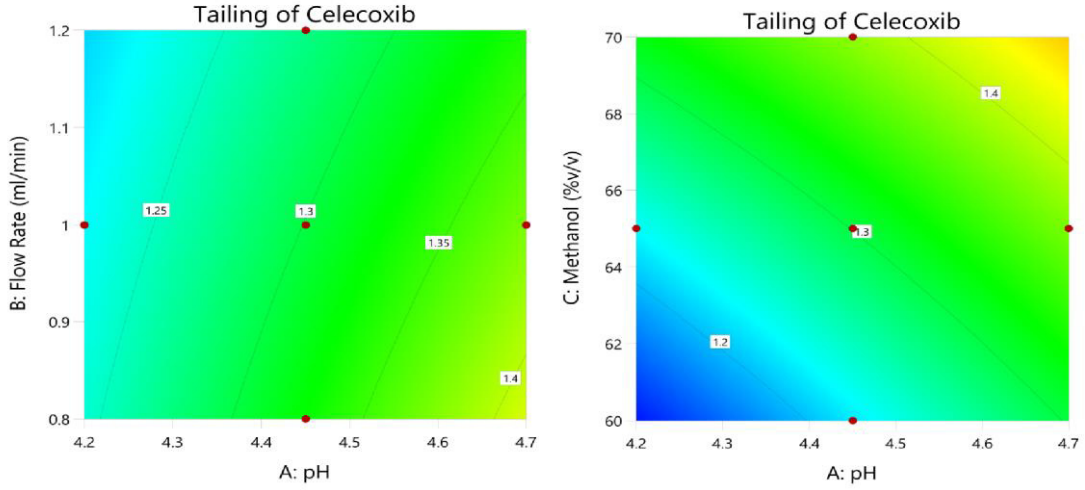


Fig I: (b) Perturbation plot showing effect of each factor on tailing of Celecoxib

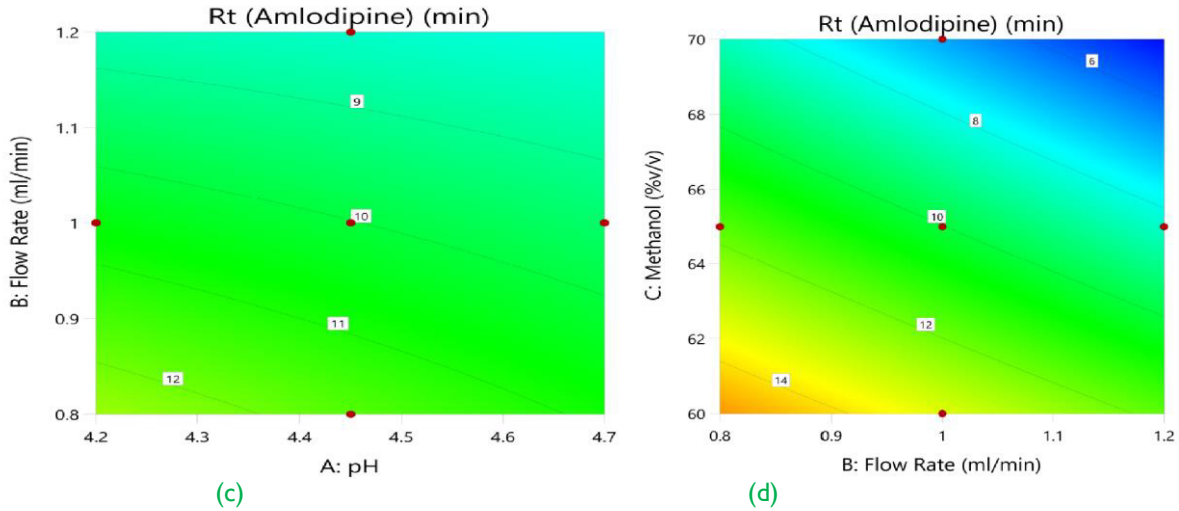
Table III illustrates the interaction term with the largest absolute coefficient among the fitted models is 0.83AC of Rt model. By applying a regression model , the interaction between A and C was found statistically significant (P =0.001) for Rt. By performing various trials, it was identified that by increasing Methanol concentration, there was a rapid decrease in retention time at any pH. It was also observed that, at a lower level of factor A, with a minor raise in the pH of buffer, pH leads to a marginal reduction in the retention time of AML (Rt). This interaction was found to be synergistic because it led to a decrease in run time of analysis. All factors have a moderate effect on the tailing of CEL which was evidently demonstrated by the second response model T.

4.3 Interpretation of Pertubation Plots

The perturbation plots presented in Fig. I. provides a better understanding of the results. Design space was generated after processing all data using the modelling software Design Expert. Two dimensional colour maps are represented in Fig. II that shows high retention time and tailing with warm "red" and low retention and tailing of peaks with cold "blue" colours. From the constructed Design Space, the working point was selected by visual examination looking for the least retention time of AML and tailing of peak of CEL. Fig. II and III shows that retention time of AML increased toward the pH 4.2, flow rate 0.8 ml/min and % of methanol 60%. At the same time, tailing of CEL peak was decreased toward acidic pH and low methanol content. As per our method's goal, at pH 4.45, % of methanol 70% v/v and flow rate of 1.2 ml/ min satisfy faster separation (<7.0 min) with good resolution and optimum tailing of CEL peak (T =1.22).

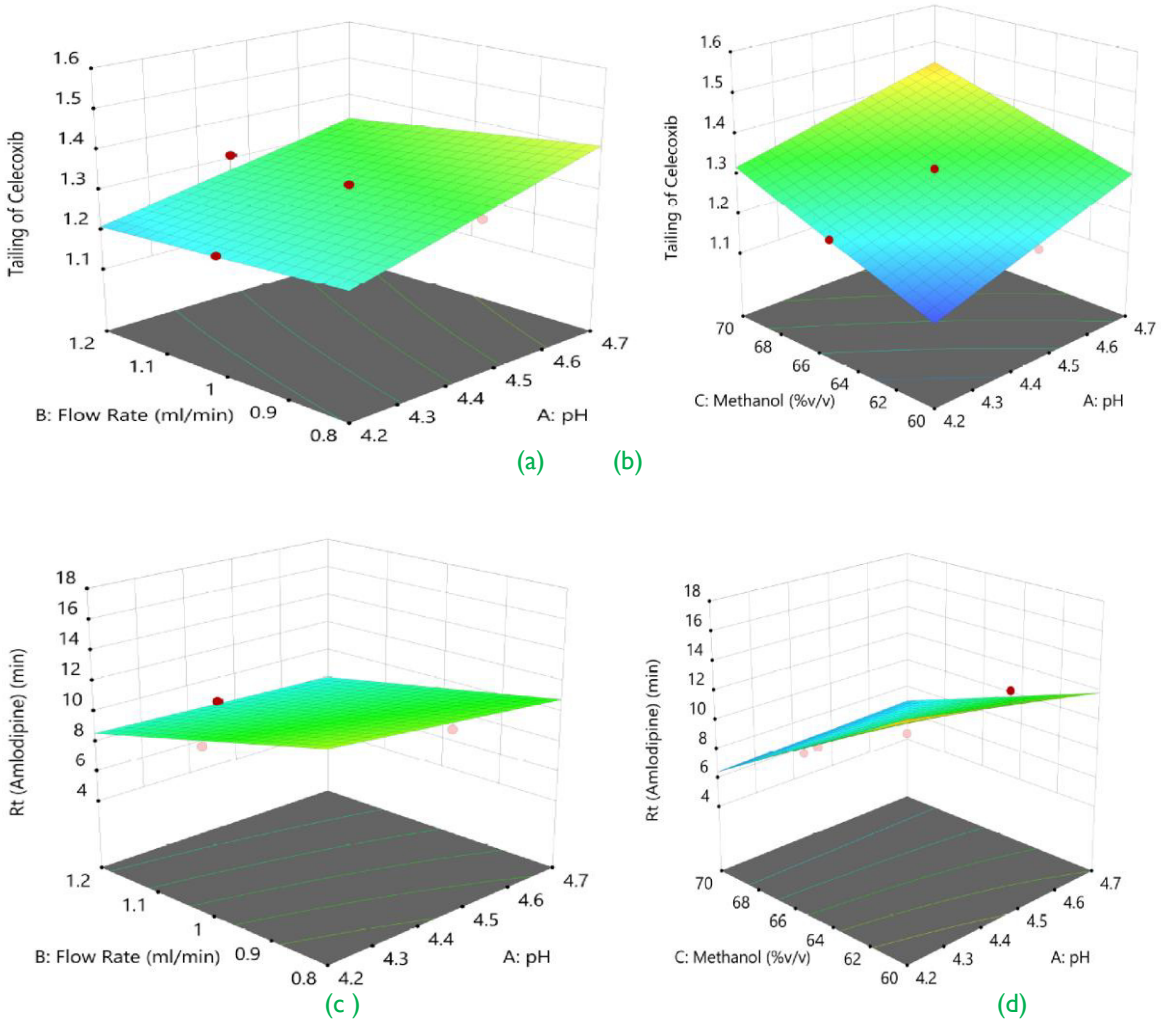


(a) (b)



(c) (d)

Fig II: (a) 2-D model shows design space for tailing factor of Celecoxib pH_Flow rate (b) 2-D model shows design space for tailing factor of Celecoxib pH_%METHANOL (c) 2-D model shows design space for retention time of Amlodipine in pH_Flow rate model (d) 2-D model shows design space for retention time of Amlodipine in pH_% METHANOL model



(a) (b)

(c) (d)

Fig III (a): Response Surface Methodology for Tailing of Celecoxib pH_Flow rate (b): Response Surface Methodology of Tailing of Celecoxib pH_%Methanol (c): Response Surface Methodology for retention time of Amlodipine pH_Flow rate (d): Response Surface Methodology of Retention time of Amlodipine pH_%Methanol

4.4 *Derringer's Desirability Function*

The Derringer desirability (D) function is defined as the geometric mean, weighted, or otherwise, of the individual desirability functions [40] which is the most suitable technique in situations where multiple responses need to be optimized with different targets. Table IV indicates criteria to optimize individual response which are proposed to select optimum experimental conditions. Optimization of criteria was carried out using Design Expert@ with high value was assigned to retention time as an important criterion in method development. Maximum desirability value (D=0.894) was obtained with Methanol 70% v/v, buffer pH 4.45, and flow rate of 1.2 ml/min as optimized coordinates for the proposed method.

Table IV: The comparison of experimental and predictive value of different objective functions under optimal condition

METHANOL (%v/v)	Flow Rate (ml/min)	Buffer pH		R_t (min)	T	Total Desirability
			Experimental Value	5.23	1.22	
70	1.2	4.45	Predicted Value	5.9	1.34	0.894

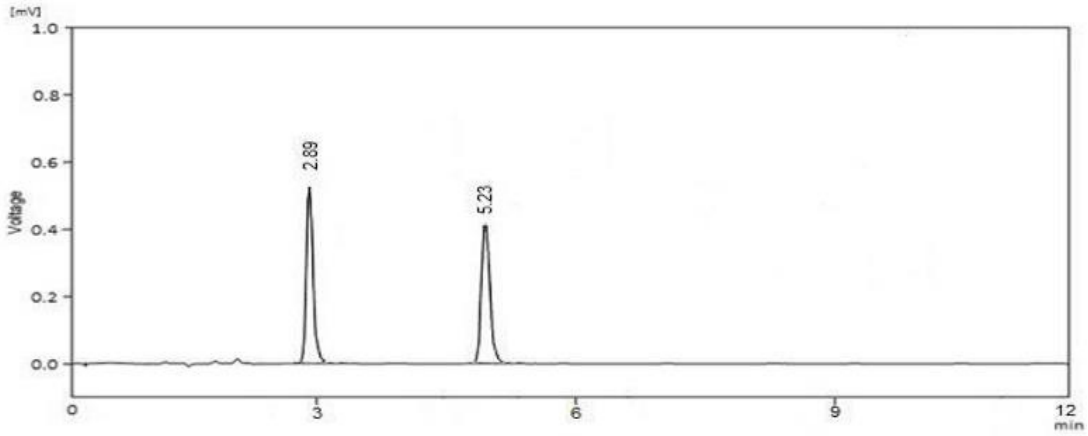


Fig IV: Optimized Chromatogram of Celecoxib and Amlodipine

4.5 *Method Validation* [41-45]

Method validation is the process of "establishing documented evidence" which provides high degree of assurance that the developed analytical method will meet the specified requirements for the intended analytical applications. The validation of the developed DoE based analytical method was performed as per the recommendations by ICH and FDA regulations wherein linearity, specificity, precision, accuracy, system suitability, limit of detection and quantification, robustness and ruggedness were taken into account to evaluate the effectiveness of the developed analytical method.

4.6 *Specificity*

Specificity of the HPLC method was performed to confirm no interference of excipient on the retention time of Celecoxib and Amlodipine. The mixture of excipients were prepared and injected to check the interference on the retention time of Amlodipine and Celecoxib. No interference was observed due to excipients on retention time of Celecoxib and Amlodipine. [41]

4.7 *Linearity*[43, 44]

Linearity of the proposed HPLC method was studied at the concentration range of 10-60 µg/mL for Celecoxib and 1-6 µg/mL for Amlodipine. The standard stock 1000 µg/mL for Celecoxib was diluted with mobile phase to obtain 40, 80, 120, 160, 200, 240 µg/mL concentration of the Celecoxib and 100 µg/mL of Amlodipine was diluted with mobile phase to obtain 1, 2, 3, 4, 5, 6 µg/ml concentration of Amlodipine. The six replicates of each injection were performed. Table 5 shows the results of the linearity of both drugs. The calibration curve of mean area under curves versus concentration was plotted and regression coefficient was established. The results were R^2=0.999 for Celecoxib and R^2=0.999 for Amlodipine. Based on regulatory requirements laid by International conference on Harmonization and Food and Drug Administration, a regression coefficient, R^2 > 0.999 is necessary for compliance.[43]

Table V: Linearity Data of Celecoxib and Amlodipine

Celecoxib		Amlodipine		Celecoxib	Amlodipine
Concentration (mcg/ml)	Mean Area±SD	Concenteration (µg/ml)	Mean Area±SD	%RSD	%RSD
40	1438922 ±3678	1	325392 ±1364	0.26	0.42
80	2869013 ±2937	2	649025 ±2246	0.10	0.35
120	4325579 ±10236	3	975538 ±9254	0.24	0.95
160	5790153 ±21462	4	131273±11268	0.37	0.86
200	7153890 ±15243	5	1633409±17589	0.21	1.08
240	8632874 ±34242	6	1942094 ±11365	0.40	0.59

Mean Area ±SD (n=6)

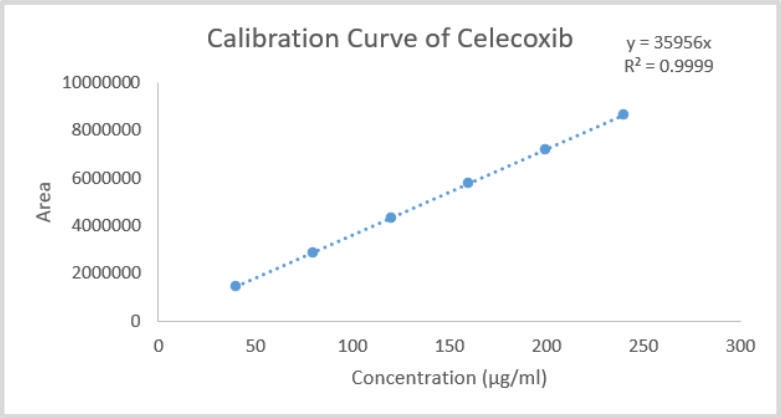


Fig VI (a): Calibration Curve of Celecoxib

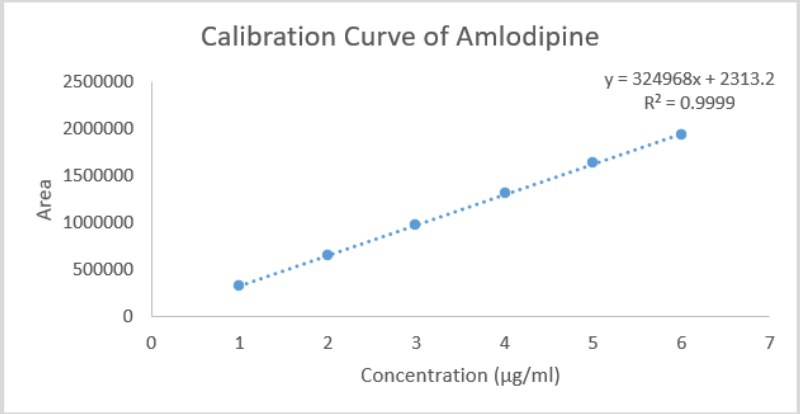


Fig VI (b): Calibration curve of Amlodipine

4.8 Accuracy

Accuracy was studied by performing recovery experiments.[42-46] The accuracy of the proposed HPLC method was established at 80%, 100% and 120% level. The placebo was added to the standard solution and % recovery was calculated. The results of the accuracy study were mentioned in Table VI. The mean recovery of both the drugs were found in the range of 98-102% with RSD<15% as imposed by regulatory guidelines indicates that the proposed method was accurate for both the drugs.

Table VI: Accuracy data of Celecoxib and Amlodipine

Drug	Level	Amount of sample taken (µg/ml)	Amount of Standard spiked (µg/ml)	Total Amt. of Drug	Amount of Standard Recovery Mean	% Recovery	SD* (n=3)
Amlodipine Besylate	50	2	1	3	2.95	98.33	0.127
	100		2	4	3.92	98.00	0.472
	150		3	5	4.96	99.20	0.278
Celecoxib	50	80	40	120	118.35	98.62	0.8834
	100		80	160	156.94	98.08	0.9269
	150		120	200	202.34	101.17	1.2427

SD: Standard deviation

4.9 Precision

The precision of the proposed HPLC method was studied by performing repeatability study and intraday precision for both the drugs. [43-46] The repeatability study was performed by injecting 40 mcg/ml of Celecoxib and 4 mcg/ml for Amlodipine (n=6). The intraday precision was studied by injecting 20, 30 and 40 mcg/ml for Celecoxib and 2, 3 and 4 µg/mL for Amlodipine (n=3) and % RSD was calculated. The results were shown in Table no. VII. The results were within targeted criteria (<2%) as recommended by regulatory guidelines.

Table VII: Precision Data of Celecoxib and Amlodipine			
Repeatability (n=6)			
	Concentration	**Mean Area ±SD**	**%RSD**
Celecoxib	40	5792146±11246	0.19
Amlodipine	4	1302257±4672	0.36
Intra-Day Precision (n=3)			
	Concentration	Area± SD	%RSD
Celecoxib	30	4315349±11247	0.26
	40	5791245±13489	0.23
	50	7102278±32587	0.46
Amlodipine	3	962237±2246	0.23
	4	1283374±2389	0.19
	5	1623378±2726	0.17

SD: Standard Deviation
RSD: Relative standard deviation

4.10. Limit of Detection and Limit of Quantification

Limit of detection (LOD) and Limit of quatification (LOQ) were determined for Celecoxib and Amlodipine according to ICH guidelines Q2 (R1)[41, 44-46]. LOD is the smallest concentration of analyte which gives measurable response (signal to noise ratio for LOD is 3.3) while LOQ is the smallest concentration of analyte which can be quantified. (Signal to noise ratio is 10). LOD value for Celecoxib is 0.0287 and 0.0053 for Amlodipine and LOQ value for Celecoxib is 0.0868 and 0.0162 for Amlodipine. (Table VIII)

Table VIII: LOD and LOQ data of Celecoxib and Amlodipine (n=3)		
Parameter	**Celecoxib**	**Amlodipine**
Mean Standard Deviation	1247.5	526.43
Mean Slope	143683	324968
LOD (µg/mL)	0.0287	0.0053
LOQ (µg/mL)	0.0868	0.0162

4.11 Assay of Marketed Formulation

The proposed HPLC method was applied to three batches of the marketed formulation. Stock solution of 1000 mcg/ml for Celecoxib and 100 µg/mL for Amlodipine was prepared by dissolving the equivalent amount of tablet powder in the mobile phase. 0.4 ml of the solution was transferred to 10 ml of volumetric flask and made up the volume up to 10 ml with mobile phase. The sample was injected to the HPLC system and Area under curve was compared with standard solutions. The percentage of drug was calculated and standard deviation was determined. The result shows good acceptance with label claim of formulation. (Table IX)

Table IX: Assay result of Marketed formulation				
Assay (n=3)				
Formulation	Drug Name	Label claim	Amount found	% Label Claim ±SD (n=3)
Synthetic Mixture	Celecoxib	40	39.76	99.4±0.3863
	Amlodipine	4	3.99	99.75±0.9123

(n=Number of Replications)

5. CONCLUSION

FCCD design of Central Composite design and response surface methodology of DoE approach was used to optimize significant parameters for estimation of Amlodipine and Celecoxib HCl in combined dosage form using RP-HPLC. Derringer's desirability function was used to optimize independent variables affecting retention time and tailing factor as method response. The validation of the analytical method demonstrated good linearity, accuracy, precision, specificity and robustness as per ICH Guidelines. Further, the experimentally observed values of LOD and LOQ of both drugs were also lower hence demonstrating a high degree of practical utility for estimation of combination drugs in pharmaceutical dosage forms.

6. AUTHOR'S CONTRIBUTION STATEMENT

Darshil B. Shah has designed the concept of the study, literature review and data collection and analysis. Bhavesh H. Patel has contributed in study concept design and manuscript reviewing. Jignesh S. Shah has contributed in data analysis and manuscript writing.

7. CONFLICT OF INTEREST

Conflict of interest declared none.

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