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INVITED ARTICLE

DEVELOPMENT AND VALIDATION OF HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHIC METHOD FOR SIMULTANEOUS ESTIMATION OF CILNIDIPINE AND VALSARTAN ITS STANDARD MIXTURE USING BOX-BEHNKEN DESIGN

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Abstract

High performance thin layer chromatography (HPTLC) method has been developed for the separation of cilnidipine and valsartan using pre-coated silica gel aluminium plate 60F254, with UV detection at 300 nm. Box- Behnken design was applied for multivariate optimization of the experimental conditions of HPTLC method. Three independent factors: Ethyl acetate content in mobile phase composition, saturation time and migration distance whereas Rf was taken as response which was used to design mathematical models. The predicted optimum assay conditions consisted of toluene: methanol: ethyl acetate: GAA (6:2:2:0.1, v/v/v/v), respectively as the mobile phase. The method was validated according to ICH guidelines.

Keywords: *Cilnidipine, valsartan, HPTLC, Box- Behnken design, validation*

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Introduction

Cilnidipine (CIL) chemically is 3-(*E*)-3-Phenyl-2-propenyl 5-2-methoxyethyl 2,6-dimethyl-4-(*m*-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate (fig. 1a) and Valsartan (VAL) 3-methyl-2-[N-({4-[2-(2H-1, 2, 3, 4 tetrazol- 5yl phenyl] phenyl} methyl) pentanamido] butanoic acid (fig. 1b) both are commonly used to for the treatment of hypertension [1-3].

CIL is official in Japanese Pharmacopoeia (JP) and VAL is official in United States Pharmacopoeia (USP) and Indian Pharmacopoeia (IP) [4-6].

The extensive literature survey revealed that several methods are available such as UV-spectrometry [7-11], RP-HPLC [12-16], UPLC [17], LC-MS [18], HPTLC [19-22] etc. for estimation of CIL and VAL individually or in combination with other drugs. Based on literature survey few analytical methods such as UV spectroscopy (second order derivative and simultaneous estimation) [23, 24] and HPTLC (forced degradation study) [25] method have been reported so far for simultaneous estimation of these drugs in their combined dosage form. However, all reported method lacks systematic study of various factors affecting separation of these drugs and appropriate statistical treatment of obtained data using suitable design of experiment. Hence, it was thought of interest to develop and validate a chromatographic method (HPTLC) using Box- Behnken design.

Now-a-days regulatory authorities are promoting and requesting the application of experimental design approach to understand chromatographic selectivity and support better method control, including method transfer [26]. The main objective of the work to develop and validate (as per ICH guideline) analytical method for simultaneous estimation of afore mentioned drugs with experimental design approach in their standard mixture and provide information on the effect of various factors and their interaction effects on the separation characteristics. The optimization of chromatographic factors like ethyl acetate concentration in mobile phase, saturation time and migration distance have significant effect on chromatographic separation. All these independent factors can easily be optimized using the design of experiments (DOE) that is used to obtain the optimum conditions with good assurance of quality. Design space is generated through experimental design that shows the flexible region in which post approval changes are not required during any of changes in the parameters (ICH Q8 (R2). When one needs to optimize more than one response at a time the use of Derringer's desirability function was first used in chromatography by the scientist Deming; to get better resolution and shorter analysis time as objective functions to get better separation quality [27,28].

The present research was aimed at development and optimization of a new HPTLC method for the simultaneous estimation of CIL and VAL from standard mixture.

Materials and Methods

Materials

Standards of CIL and VAL were obtained from Torrent Research Centre, Gujarat as gift samples. AR grade toluene, methanol, ethyl Acetate, and Glacial acetic acid (GAA) were supplied by Finar chemicals Ltd, Ahmadabad. The formulation available in Japanese market had a label claim of 10 mg Cilnidipine and 80 mg Valsartan. Hence, as per the label claim the standard mixture was prepared using both drugs for their simultaneous analysis.

Instrumentation

Analytical HPTLC Camag Hamilton syringe (100 µL) on pre-coated silica gel aluminium plate 60F254, (10 ×10 cm; E. Merck, Darmstadt, Germany) using a Linomat V Camag (Muttenz, Switzerland) sample applicator. The plates were prewashed by methanol and activated at 60 °C for 2.5 min prior to chromatography. Before the application of sample it was filtered to 0.22 µm Nylon filter. Constant application rate, 0.1 µL/s was applied and the space between the two bands was 10 mm. The slit dimension was kept at 5 x 0.45 mm and 10 mm/s scanning speed was employed. The mobile phase composition of toluene: methanol: ethyl acetate: GAA (6:2:2:0.1, v/v/v/v). Linear ascending development was carried out in 10 x 10 cm twin – trough glass chamber saturated with the mobile phase to a distance of 80 mm. The optimized saturation time for the mobile phase was 30 min at room temperature (25 ± 2 °C) and at relative humidity of 55 ± 5 %. Subsequent to the development, TLC plates were dried in a current of air with the help of an air dryer. Densitometer scanning performed on Camag TLC scanner III in the absorbance mode was tired ait 300 nm to see if there was any difference in the absorptivity. The source of radiation utilizing was a deuterium lamp emitting a continuous UV spectrum in the range of 200- 300 nm. Evaluation was done using linear regression analysis via peak areas. Experimental design (Box- Behnken design), desirability function and data analysis calculations were performed by using Design-Expert ® version 7.0.0.

Preparation of standard stock solutions

Accurately weighed portions of CIL (50 mg) and VAL (50 mg) were transferred individually to amber colored volumetric flasks (50 mL), dissolved and diluted to the mark with methanol to obtain standard stock solutions having concentrations of CIL (1000 µg/mL) and VAL (1000 µg/mL) respectively.

Selection of wavelength for detection

Overlain spectra of CIL (20 µg/mL) and VAL (20 µg/mL) were recorded by scanning standard drug solutions in the range of 200-400 nm against methanol as a blank in UV-Visible spectrophotometer. The optimum wavelength for detection was set at 219 nm from overlain spectrum.

Preparation of standard mixture solution

Accurately weighed portions of CIL (10 mg) and VAL (80 mg) were transferred to

50 mL amber colored volumetric flask, and diluted to the mark with methanol to obtain standard mixture solution having concentration of CIL (200 µg/mL) and VAL (1600 µg/mL) respectively.

Preparation of test solution

Accurately weighed the portions of CIL (20 mg) and VAL (160 mg) and were transferred to 50 mL amber colored volumetric flasks and was sonicated for 10 min to get clear solution and diluted to the mark with methanol and then filtered by Whatman filter paper No. 41 to get the test solution having concentration of CIL (400 µg/mL) and VAL (3200 µg/mL) respectively.

Method optimization using design of experiment (DOE)

Response Surface Methodology (RSM) is a collection of mathematical and statistical techniques useful for the modelling and analysis of problems in which a response of interest is influenced by several variables and the goal is to optimize this response and to understand how the response changes in a given direction by adjusting the design variables. When there is more than one response then it is important to find the compromise optimum that does not optimize only one response. When there are constraints on the design data, then the experimental design has to meet requirements of the constraints.

The Box-Behnken design was specifically selected since it requires fewer runs than a central composite design while working with three or four variables. Box-Behnken statistical screening design was used to optimize the compositional parameters and to evaluate interaction effects and quadratic effects of the mobile phase composition, saturation time and migration distance on the retardation factor (R_f) of the drugs [29]. A 17-run, was set up to standardize the chromatographic conditions which are likely to be employed using Design Expert. Proportion of ethyl acetate in mobile phase (X_1), saturation time (X_2), and migration distance (X_3) were selected as factors. The higher and lower values of factors were selected as mentioned in (Table 1). Retardation factor (R_f) and area of the drug were taken as responses (Y).

The non-linear computer generated quadratic model is given as

$$Y=b_0 \text{''} b_1 X_1 + b_2 X_2 \text{''} b_3 X_3 \text{''} b_4 X_1 X_2 \text{''} b_5 X_1 X_3 + b_6 X_2 X_3 \text{''} b_7 X_1^2 + b_8 X_2^2 + b_9 X_3^2 \text{———-———————————————————(1)}$$

Where, b_0, b_1……. b_9 etc are coefficients.

Method validation [30]

Linearity and range

The aliquots of 1.0, 1.5, 2.0 2.5, 3.0 µL from the standard mixture solutions 200 µg/mL of CIL and 1600 µg/mL of VAL were spotted on TLC plate using spotter that gave 200-600 ng/band for CIL and 1600-4800 ng/band for VAL. The peak areas obtained were plotted against concentration and regression analysis was used to interpret the data. Range is the

interval between upper and lower concentration (amount) of analyte in sample for which it has been demonstrated that the analytical method has suitable level of precision accuracy and linearity.

Precision

Method precision

Method precision was performed by preparing the test solution for six times and 1 µL of each test solution was applied on same TLC plate (400 ng/band of CIL and 3200 ng/band of VAL). Plate was developed and analyzed. The areas of six replicate bands were measured and % RSD was calculated.

Intermediate precision (Reproducibility)

The intraday and interday precision of the proposed method was determined by analyzing mixed standard solution having 400 ng/band of CIL and 3200 ng/band of VAL on the same day and on different days. The results were reported in terms of relative standard deviation (%RSD).

Accuracy (% recovery study)

The accuracy of the methods was determined by calculating recoveries of CIL and VAL by the standard addition method. Known amounts of standard solution of CIL (400 ng/band) and VAL (3200 ng/band) with three different concentrations of standards (320, 400 and 480 ng/band for CIL and 2560, 3200 and 3840 ng/band of VAL) at 80%, 100% and 120% respectively were added to pre-quantified sample solutions.

Limit of detection (LOD) and limit of quantitation (LOQ)

The limits of detection and quantification of the developed method were calculated from the standard deviation of the intercepts and mean slope of the calibration curves of CIL and VAL using the formulae as given below.

$$LOD = 3.3 \times \sigma/S \quad (2)$$

$$LOQ = 10 \times \sigma/S \quad (3)$$

Where, σ = the standard deviation of the response

S = slope of the calibration curve

Robustness

The robustness of an analytical method is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage. Minor changes in mobile phase ratio, chamber saturation time and migration distance were evaluated during method robustness.

Analysis of standard mixture

Standard mixture was prepared because the formulation was not available in the Indian market as it is newly launched combination of drugs. So, the standards of CIL (20 mg) and VAL (160 mg) were taken in mortar and pestle; mixed thoroughly and transferred to 50 mL volumetric flask. It

was then sonicated for 10 min and volume was made up to mark with methanol and filtered with Whatman filter paper No. 41 to obtain the sample stock solution for the determination of 400 ng/spot CIL and 3200 ng/spot of VAL was evaluated using the proposed method and peak area was calculated. The amount of CIL and VAL were determined by fitting the peak area into the respective regression line equations.

Results and Discussion

Optimization of mobile phase using Box-Behnken design

Box–Behnken experimental design is an orthogonal design. Based on the previous trials with chosen solvents the factor levels were decided, which were evenly spaced and coded for low, medium and high settings, as "1, 0 and +1. The experimental parameters and its responses for all the 17 optimized runs are shown in the Table 2. The values of response Y_1 (R_f of Valsartan) and Y_2 (R_f of CIL) ranged from 0.43-0.60 and 0.73-0.81 respectively.

The selection of model for analysing the response was done after comparing several statistical parameters including Standard deviation (SD), R-square values and predicted residual sum of square (PRESS). The model having low SD, higher R-square value and lower

PRESS values were selected. The details of these significant parameters are mentioned in Table 3 which suggested quadratic model was best fit for analysing both the responses. The predicted R-Square of 0.7586 and 0.8627 are in reasonable agreement with the adjusted R-Square of 0.7488 and 0.9633 for Y_1 and Y_2 respectively. The higher value of correlation coefficients signifies an excellent correlation between the independent variables. All the above considerations indicate an excellent adequacy of the regression model.

For estimation of significance of the model, the analysis of variance (ANOVA) was applied. Using 5% significance level, a model is considered significant if the *p*-value (significance probability value) is less than 0.05. The Model F-values of 6.30 and 47.63 retardation factor (R_f) of VALand CIL, respectively, implies the model is significant. Values of "Prob > F" less than 0.05 indicate model terms are significant. Therefore, X_1, X_2, X_3 and X_3^2 are significant model terms for VAL and X_1 and X_2 are significant model terms for CIL.

The mathematical relationship in the form of a polynomial equation generated by Design-Expert® 7.0 software for the measured responses, Y_1 and Y_2, are shown below as equation 1 and 2, respectively.

$$Y_1 = +3.68750 + 0.143\ X_1 - 0.060\ X_2 - 0.066\ X_3 - 6.000\ X_1 X_2 - 5.000\ X_1 X_3 + 4.500\ X_2 X_3 + 0.030\ (X_1)^2 + 7.000\ (X_2)^2 + 3.500\ (X_3)^2 \quad (4)$$

$$Y_2 = +0.352 + 0.092\ X_1 + 2.850\ X_2 + 4.900\ X_3 + 0.000\ X_1 X_2 - 5.000\ X_1 X_3 - 5.000\ X_2 X_3 + 4.000\ (X_1)^2 + 4.000\ (X_2)\ 1.500\ (X_3)^2 \quad (5)$$

The above equations represent the quantitative effect of independent variables (X_1, X_2, and X_3) and their interactions on the responses (Y_1 and Y_2). A positive sign represents a synergistic effect, while a negative sign indicates an antagonistic effect. The theoretical values of Y_1 and Y_2 were obtained by substituting the values of X_1-X_3 into the above equation.

The relationship between the dependent and independent variables was further elucidated using perturbation and response surface plots. A perturbation graph was plotted to find those factors that affect the response most significantly. A steep slope or curvature in a factor shows that the response is sensitive to that factor. A relatively flat line shows insensitivity to change in that particular factor. In case of response Y_1, factors X_3 show a steep slope whereas X_1 and X_2 exhibit slight slope. Whereas in case of response Y_2, factor X_1 shows a steep slope and factor X_2 and X_3 exhibit slight slope. Figure 2 represents perturbation plot for responses Y_1 and Y_2.

Three-dimensional (3D) and contour response surface plots for the measured responses were formed, based on the model polynomial functions to assess the change of the response surface. Also the relationship between the dependent and independent variables can be further understood by these plots. Figure 3 (a) and (b) represents the effect of factors X_1, X_2, and X_3 on the response Y_1 and Y_2.

It could be seen in Figure 3 (a) that the factors X_1, X_2 and X_3 increases, there is no effect on the response Y_1 and in Figure 3 (b), the factors X_1, X_2 and X_3 increases; there is an increase on the response Y_2.

In order to get the best chromatographic performance, the multi-criteria methodology was employed by means of Derringer's desirability function [Figure 4(a)]. Individual desirability functions range from 0 (undesired response) to 1 (fully desired response). If any of the responses or factors falls outside their desirability range, the overall function becomes zero.

Validation of chosen model

After studying the effect of the independent variables on the responses, the levels of these variables that give the optimum response were determined. To perform the optimization of mobile phase that would yield a minimum value of VAL with maximum value of CIL, the three responses were over laid and software generates the overlay plot [Figure 4(b)] using the goals as shown in Table 4. Any point in the overlaid region will satisfy our desired criteria. To validate the model, three such points were chosen as check point 1, 2 and 3 for which the predicted values were: X_1 (1.84, 2.12 and .58), X_2 (29.92, 30.23 and 30.77), X_3 (80, 80 and 70.47) for CIL and VAL respectively. For confirmation, a fresh mixture in triplicate was prepared at the optimum levels of the independent variables, and the resultant mixture were evaluated for the responses. The experimental values obtained for estimation of CIL and VAL are given in the **Table 5**, which were in close agreement

with the predicted values. The % error was less than 10% indicating the good predictability of the chosen model.

Method validation

Linearity

Linear responses were observed in the concentration range of 200-600 ng/band for CIL and 1600- 4800 ng/band for Valsartan. Correlation co-efficient for calibration curve of CIL and VALwere found to be 0.9985 and 0.998 respectively. 3D chromatogram of standard CIL and VALin linearity range is depicted in Figure 5. The results for linearity study of CIL and VALis depicted in Table 6.

The regression line equations for CIL and VALare as following:

y = 4.9614x + 1160.3 for CIL

y = 0.5363x + 945.02 for Valsartan

Where, y= Peak area

x= Concentration in ng/band

Precision

Method precision

The % RSD of method precision of CIL and VAL were found to be 0.4390 and 1.105 respectively.

Intra-day and Inter-day precision

Mean % RSD for intra-day precision of CIL and VAL were found to be 0.423 and 1.213 respectively. The Mean RSD for inter day precision of CIL and VAL was found to be 0.404 and 1.282 respectively.

The % RSD values were found to be <2% indicating that the method is precise.

Accuracy

Accuracy of the method was confirmed by recovery of drugs from their standard mixture by spiking it at three levels. The % RSD of CIL and VAL were found to be 0.3512 and 0.2426, respectively. The data for accuracy of CIL and VAL are depicted in Table 7 and Table 8 respectively.

Limit of detection (LOD) and limit of quantitation (LOQ)

The LOD for CIL and VAL were found to be 2.406 ng/band and 21.04 ng/band respectively. The LOQ for CIL and VAL were found to be 7.292 ng/band and 63.76 ng/band respectively.

The data for LOD and LOQ of CIL and VAL are depicted in Table 9.

Robustness

For change in chamber saturation time by ± 5 min, % RSD for peak area was found to be 0.175 % and 0.478 % for CIL and VAL respectively. For change in mobile phase ratio by ± 0.5 mL, % RSD for peak area was found to be 0.224 % and 0.453% for CIL and VAL respectively. For change in migration distance by ± 5 mm, % RSD for peak area was found to be 0.314% and 0.506 % for CIL and VAL respectively.

Robustness data clearly shows that the proposed method is robust at small but deliberate changes that are shown in Table 10.

Analysis of standard mixture by proposed method

CIL (10 mg) and VAL (80 mg) were taken in mortar and pestle and mixed properly and transferred the powered mixture in to 50 mL volumetric flask. Sonicated for 10 min and made up the volume with methanol up to the mark and filtered. The assay results in **Table 11** which was obtained by using the proposed method for the analysis of a standard mixture were in good agreement with the labeled amounts of CIL and VAL.

Conclusion

The HPTLC method was developed and validated as per ICH guidelines wherein the mobile phase optimization was done using the Box- Behnken design. The optimization of mobile phase using experimental design helped us for better understanding of the effect of one or more factors at the same time on the desired parameters. So, this approach could be time saving and beneficial to study the interacting and most contributing factors affecting separation of CIL and VAL in standard mixture. Based on the results, obtained from the analysis using described method, it can be concluded that the method has linear response in the range of 200-600 ng/band for Cilnidipine and 1600-4800 ng/band for Valsartan. The method shows that the % RSD values of both the drugs from their standard mixtures for precision lies within its corresponding limit of 2. LOD and LOQ values were also low so, detection of drugs in very low concentration was possible using this method. So, it can be concluded that the proposed analytical methods have great promise for simultaneous determination of CIL and VAL in standard mixture.

Acknowledgement

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Declaration of conflict of interest

The authors report no conflict of interest.

Table 1: Variables selected in Box – Behnken design

Factors	Variables	Levels		
		Low (-)	Nominal (0)	High (+)
A	Change in amount of Ethyl acetate in mobile phase composition(mL)	1.5	2	2.5
B	Change in saturation time (min)	25	30	35
C	Change in migration time (mm)	70	80	90

Table 2: Box-Behnken design: Independent (X) and dependent variables (Y)

Sr. No.	X1	X2	X3	Y1	Y2
1	1.5	25	80	0.43	0.73
2	1.5	30	70	0.47	0.73
3	1.5	30	90	0.51	0.74
4	1.5	35	80	0.48	0.74
5	2	25	70	0.47	0.76
6	2	25	90	0.47	0.76
7	2	30	80	0.46	0.77
8	2	30	80	0.46	0.76
9	2	30	80	0.46	0.77
10	2	30	80	0.46	0.77
11	2	30	80	0.46	0.77
12	2	35	70	0.51	0.78
13	2	35	90	0.6	0.77
14	2.5	25	80	0.52	0.8
15	2.5	30	70	0.5	0.8
16	2.5	30	90	0.53	0.8
17	2.5	35	80	0.51	0.81

a) X1: Amount of ethyl acetate (mL), b) X2: Saturation time (min) and c) X3: Migration distance (mm) d) Y1: Retardation factor (Rf) of Valsartan, e) Y2: Retardation factor (Rf) of CIL

Table 3: Statistical analysis for measured responses

Model	Co-efficient	Y_1	Y_2
	b_1	+0.143	+0.092
	b_2	- 0.060	+2.850
	b_3	-0.066	+4.900
	$b_{12}(X_1X_2)$	-6.000	+0.000
	$b_{13}(X_1X_3)$	-5.000	- 5.000
	$b_{23}(X_2X_3)$	+4.500	-5.000
	$(X_1)^2$	+0.030	+4.000
	$(X_2)^2$	+7.000	+4.000
	$(X_3)^2$	+3.500	+1.500
Linear	R^2	0.4882	0.9770
	Adjusted R^2	0.3701	0.9717
	Predicted R^2	0.0809	0.9598
	PRESS	0.023	3.874
Quadratic	R^2	0.8901	0.9839
	Adjusted R^2	0.7488	0.9633
	Predicted R^2	0.7586	0.8627
	PRESS	0.044	1.325
Sp. Cubic	R^2	1.0000	0.9917
	Adjusted R^2	1.0000	0.9668
	Predicted R^2	-	-
	PRESS	-	-
2FI	R^2	0.4882	0.9822
	Adjusted R^2	0.3680	0.9715
	Predicted R^2	-0.396	0.9417
	PRESS	0.035	5.622

Table 4: Goals of multi-criteria optimization for each response

Factor and Response	Goal	Lower limit	Upper Limit
Amount of Ethyl acetate	In range	1.5	2.5
Saturation time	In range	25	35
Migration time	In range	70	90
R_f of CIL	In range	0.4	0.5
R_f of VAL	In range	0.7	0.8

Table 5: Validation of chosen model

Variables	Values	Response	Observed Values	Predicted Values	% Error
Check Point 1					
X1	1.84	Y1	0.72	0.75	4.16
X2	29.92	Y2	0.47	0.45	-4.25
X3	80				
Check Point 2					
X1	2.12	Y1	0.77	0.76	1.29
X2	30.23	Y2	0.45	0.46	2.22
X3	80				
Check Point 3					
X1	1.58	Y1	0.71	0.73	2.81
X2	30.77	Y2	0.47	0.46	-2.12
X3	70.47				

Table 6: Results of linearity for CIL and Valsartan

Parameters	CIL	Valsartan
Linearity range (ng/spot)	200 - 600	1600 - 4800
Regression line equation	y = 4.9614x + 1160.3	y = 0.5363x + 945.02
Slope ± S.D. (n= 3)	4.9614 ± 0.0023	0.5363 ± 0.00026
Y- intercept ± S.D. (n= 3)	1160.3 ± 3.619	945.02 ± 3.419
Correlation coefficient (R^2)	R^2 = 0.999	R^2 = 0.998

Table 7: Recovery data of CIL

Sr. No.	Amount taken (ng/band)	Amount added (ng/band)	Area	Amount Recovery (ng/band)	% Recovery	Mean % Recovery
1	400	320	4722.56	717.99	99.72	99.71
		320	4720.44	717.56	99.66	
		320	4724.35	718.35	99.77	
2	400	400	5143.29	802.79	100.34	100.35
		400	5149.4	804.27	100.50	
		400	5138.10	801.74	100.21	
3	400	480	5529.16	880.57	100.06	99.78
		480	5519.02	878.52	99.83	
		480	5502.91	875.27	99.46	

Mean= 99.94
Standard Deviation = 0.3510%
Relative Standard Deviation = 0.3512

Table 8: Recovery data of VAL

Sr. No.	Amount taken (ng/band)	Amount added (ng/band)	Area	Amount Recovery (ng/band)	% Recovery	Mean % Recovery
1	3200	2560	4025.98	5744.84	99.73	99.78
		2560	4037.05	5765.48	100.09	
		2560	4019.56	5732.87	99.52	
2	3200	3200	4375.05	6395.70	99.93	99.88
		3200	4362.49	6372.1	99.56	
		3200	4383.19	6410.90	100.17	
3	3200	3840	4694.55	6991.47	99.31	99.42
		3840	4713.69	7027.16	99.81	
		3840	4689.03	6981.18	99.16	

Mean = 99.69
Standard Deviation = 0.2419
% Relative Standard Deviation = 0.2426

Table 9: LOD and LOQ data

Parameters	CIL	VAL
Standard deviation of the Y- intercepts of the three calibration curves (6)	3.6198	3.4190
Mean slope of the three calibration curves (S)	4.9638	0.6362
LOD = 3.3 × (SD/Slope) (ng/band)	2.406	21.04
LOQ = 10 × (SD/Slope) (ng/band)	7.292	63.76

Table 10: Robustness parameters for CIL and VAL

Sr No.	CIL (400 ng/band)			VAL(3200 ng/band)		
1.	Change in chamber saturation time					
	Normal Condition (30 min)	Changed Condition (25 min)	Changed Condition (35 min)	Normal Condition (30 min)	Changed Condition (25 min)	Changed Condition (35 min)
Area	3129.53	3132.72	3140.26	2590.26	2599.32	2614.86
Mean	3134.17			2601.48		
SD	5.51			12.4414		
% RSD	0.1758			0.4782		
R_f	0.52	0.51	0.53	0.73	0.74	0.77
2.	Change in amount of Ethyl acetate					
	Normal Condition (2 mL)	Changed Condition (1.5 mL)	Changed Condition (2.5 mL)	Normal Condition (2 mL)	Changed Condition (1.5 mL)	Changed Condition (2.5 mL)
Area	3129.82	3132.01	3118.90	2650.38	2662.09	2674.54
Mean	3126.91			2662.337		
SD	7.022			12.081		
% RSD	0.2245			0.4538		
R_f	0.51	0.51	0.52	0.74	0.73	0.74
3.	Change in migration distance					
	Normal Condition (90 mm)	Changed Condition (70 mm)	Changed Condition (80 mm)	Normal Condition (90 mm)	Changed Condition (70 mm)	Changed Condition (80 mm)
Area	3128.44	3130.45	3146.42	2655.48	2653.99	2678.04
Mean	3135.10			2662.503		
SD	9.8519			13.4757		
% RSD	0.314			0.5061		
R_f	0.52	0.50	0.51	0.72	0.74	0.73

Table 11: Estimation of CIL and VAL in standard mixture

Drug	Label claim (mg)	Amount found (mg)	% Label claim
CIL	10	9.90	99.02
VAL	80	79.01	98.77

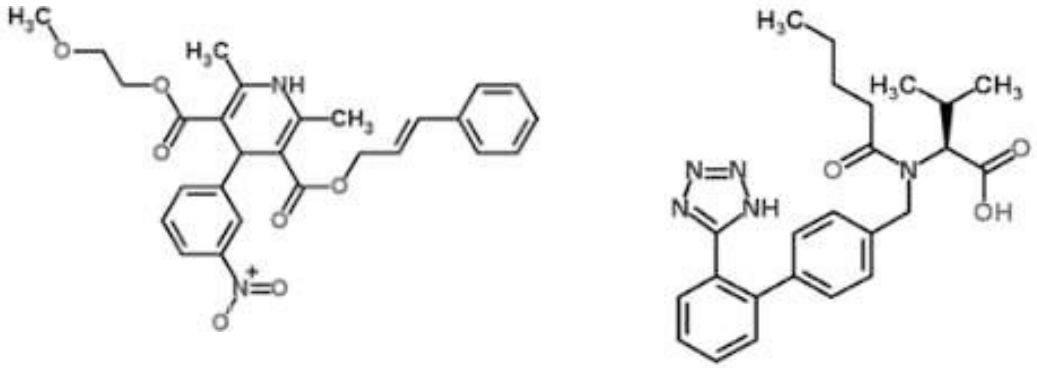


Figure 1: Chemical structure of (a) CIL and (b) VAL

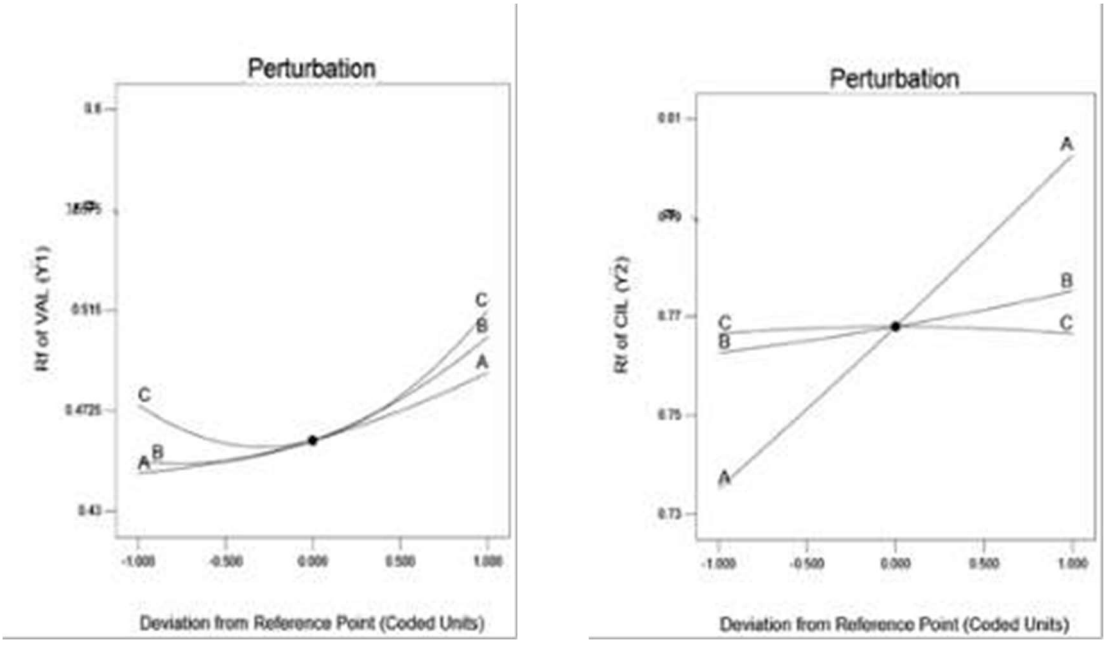
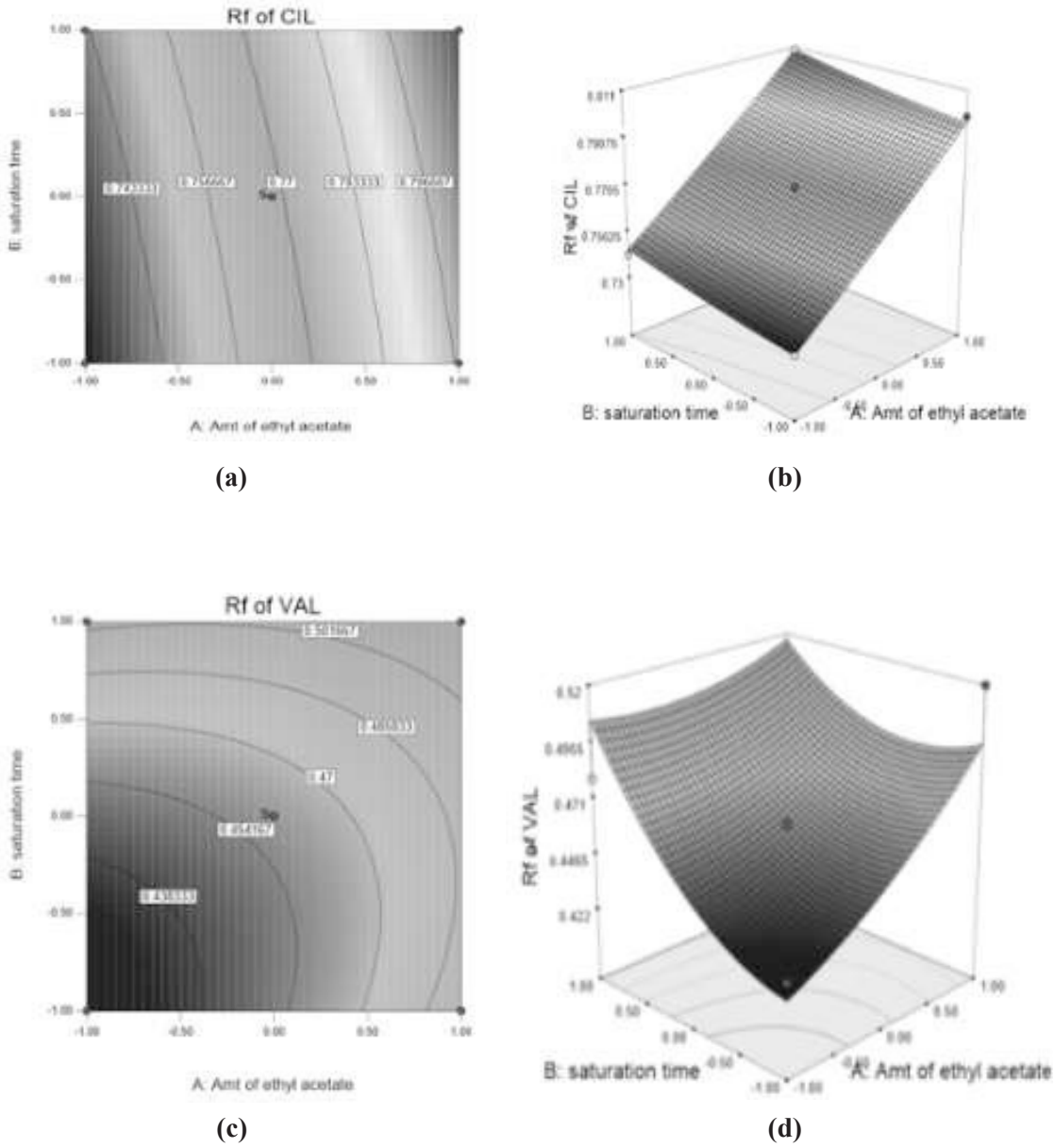


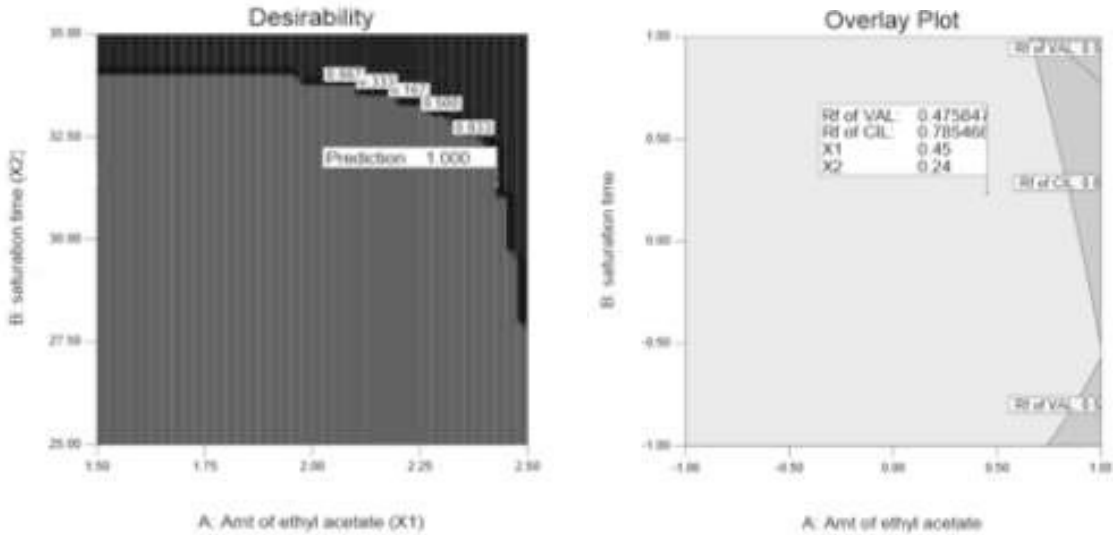
Figure 2: Perturbation graph for effect of individual factor on response (a) Y1 retardation factor of VAL and (b) Y2 retardation factor of CIL



(a) **(b)**

(c) **(d)**

Figure 3: The effect of mobile phase, saturation time, and migration distance on retardation factor in (a) Contour plot and (b) 3D Response surface plot for CIL (c) Contour plot and (d) 3D Response surface plot for Valsartan



(a) **(b)**

Figure 4: (a) Desirability Plot and (b) Overlay Plot of Experimental Design

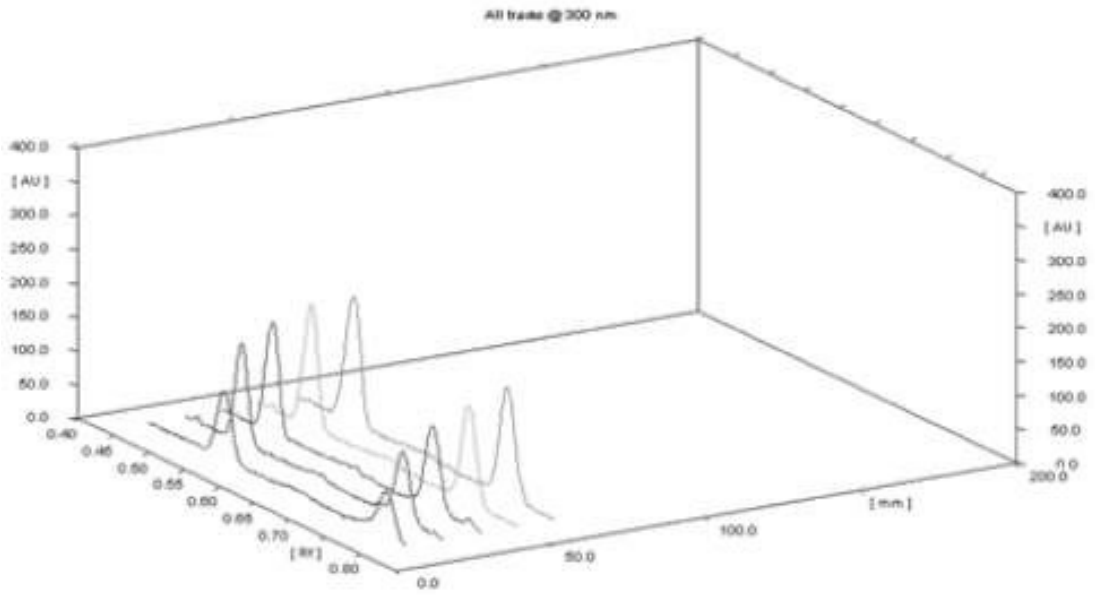


Figure 5: 3D chromatogram of CIL (200-600 ng/band) and VAL (1600- 4800 ng/band)

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