



# QbD-based RP-HPLC method development for quantitative computation of phase III composition comprising apixaban and clopidogrel

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clopidogrel.

## ABSTRACT

The presented work highlights the use of the quality by design methodology in accordance with International Council on Harmonization (ICH) recommendations for developing an reverse phase high performance liquid chromatographic (RP-HPLC) method for apixaban (APX) and clopidogrel (CLP). An efficient approach based on a factorial design incorporating the essential method parameters of the RP-HPLC method such as flow rate, pH, and proportion of organic phase, is presented. The optimum conditions for analysis were derived by using Design Expert software 10.0 Version, i.e., Hypersil ODS C<sub>18</sub> column (5.0 μm, 25 cm × 4.6 mm), methanol and 0.05M potassium dihydrogen phosphate buffer (63.5:36.5%v/v, pH 7.0) as mobile phase, and 0.8 ml/minute as the flow rate. The derived condition gave excellent resolution between APX and CLP with Rt of 4.89 and 14.35 minutes, respectively, with the best possible system suitability parameters. The developed method presented excellent linearity in the range of 1.25–3.75 μg/ml for APX and 37.5–112.5 μg/ml for CLP at 215 nm. The optimized method was further validated in accordance with ICH guidelines on analytical method validation. Finally, the approach was successful in determining APX and CLP from a binary combination.

## INTRODUCTION

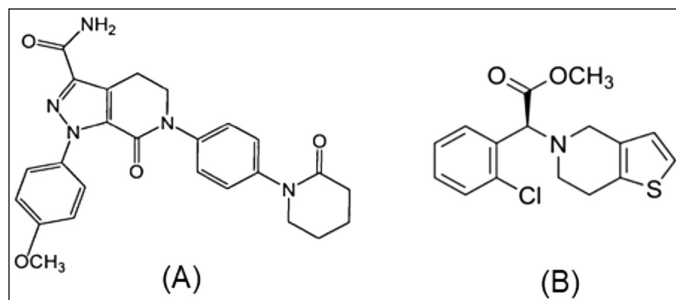
The application of quality by design (QbD) concepts in the creation of analytical methods is becoming more and more prevalent as the validation guidelines as per suggested International Council on Harmonization (ICH) criteria do not give significant dependability, especially with method variability reduction [1]. The disparity can be attributed to a variety of factors, including the use of solvents with different polarity, and buffer mixes, as well as careful monitoring of chromatographic parameters including composition of mobile phase, flow rate, injection volume, pH, and so on [2]. The AQbD approach promotes a scientific and risk-based knowledge of the primary causes of fluctuation, which is then followed by the recognition of CMPs via risk evaluation and factor examination

studies to locate vulnerable parameters with a major effect on analytical efficiency, and then optimize them using appropriate designs for experiments [3].

Apixaban (APX) is a derivative of pyrazole and a strong factor Xa inhibitor that is both direct and selective and works by directly and selectively blocking free and clot-bound factor Xa. Inhibiting factor Xa causes a reduction in the conversion of factor II (prothrombin) to factor IIa, resulting in a decrease in thrombin production [4]. Clopidogrel (CLP) is one of the most often used P2Y<sub>12</sub> antiplatelet medications in the world (Fig. 1). It has a widely recognized function as a platelet antagonist, resulting in a lower incidence of recurrent ischemic episodes. In the realm of cardiology, it is critical in preventing coronary heart disease [5]. The present combination of APX and CLP has been suggested by the AUGUSTUS clinical trial (NCT02415400) formally executed by Bristol-Mayers Squibb and Pfizer to reduce bleeding and hospitalization events after arterial fibrillation without considerably increasing the number of events such as heart attacks, strokes, and blood clots. The

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**Figure 1.** Chemical structures of A. APX and B. Clopidogrel.

clinical trial was conducted at the dose level of 2.5 mg of APX and 75 mg of CLP [6].

A thorough literature review on APX focuses mostly on UV spectroscopy [7], HPLC [8–11], LC-MS [12], and HPTLC [13–14] as analytical techniques for evaluation from bulk and pharmaceutical formulations. Similarly, a thorough examination of the literature on CLP indicated UV spectrophotometric [15–16], HPLC [17–20], LC-MS [21–22], and HPTLC [23–27] methods for quantitative determination either from bulk, pharmaceutical dosage form, or plasma.

A conclusion can be drawn that there is currently no HPLC technique accessible for the assessment of APX and CLP from their binary mixtures which is one of the most potential and promising regimens in upcoming months. Adoption of the QbD approach also fulfills the newer regulatory approach in method development for reducing risk to the quality and hence it was aimed at developing a QbD-based reverse phase high performance liquid chromatographic (RP-HPLC) method for determining APX and CLP from their binary mixture.

## MATERIALS AND METHODS

### Instruments and chemicals

The samples were processed using a SHIMADZU (Series 2010) HPLC equipment equipped with a UV-VIS detector with the ability of binary gradient operation, and the chromatogram was processed and integrated using LC solution software. The column used was a HYPERSIL ODS C<sub>18</sub> column with dimensions of 250 mm in length and 4.6 mm in internal diameter. A Mettler Toledo weighing scale with a sensitivity of 0.1 mg was used to determine the precise quantities of materials. The pH meter Labman LMPH 10 was used to adjust the pH.

Alembic Pharmaceutical Pvt. Ltd., Vadodara, Gujarat, India, and Sunij Pharma Pvt. Ltd., Vatva, Ahmedabad, Gujarat, India, provided both active ingredients as free samples. Other chemicals, such as methyl alcohol, KH<sub>2</sub>PO<sub>4</sub> buffer, O-phosphoric

acid, and water, were of HPLC quality and were obtained from Merck Life Sciences Private Limited (Mumbai, India).

### Factor screening studies

For factor screening investigations, certain critical method parameters (CMPs) such as pH, the proportion of Organic phase, and flow rate were considered based on a primary review of the literature and were transformed as a matrix to study their combined effect on critical method attributes (CMAs) (retention duration, resolution, and peak tailing). Table 1 illustrates the outline of CMPs and their assigned level that affects the CMA.

### Preparation of standard solution

For preparing a standard solution, an accurate amount of 0.01 g of APX and 0.3 g of CLP was weighed. After being transferred to a 100 ml volumetric flask 20 ml of methanol was added and sonicated, and the volume was increased to 100 ml using methanol. 5 ml of the previous solution was transferred to a 50 ml volumetric flask and the volume was again increased to the mark with methanol. 2.5 ml of the previous solution was diluted to 10 ml with methanol to get a working solution containing 2.5 g/ml of APX and 75 g/ml of CLP.

### Experimental design for method development

For selecting the analytical wavelength for method development, the solution containing 2.5 g/ml of APX and 75 g/ml of CLP was scanned individually between 200 and 400 nm. It is not only the identification of CMPs that is important as, always there is a combined effect of all the CMPs that we observed as a chromatographic separation and for the same. Three factorial designs were used to study the combined effect of each CMPs on the cited CMAs. For creating a three-factorial design, Design Expert 10.0 was used to create the study. Table 2 presents the design matrix with 27 trial runs generated from Design Expert 10 software. All experimental runs that were examined for CMAs (retention duration, resolution, and peak tailing) employed a standard concentration of 2.5 g/ml APX and 75 g/ml CLP.

### Optimizing and analyzing data

Multiple linear regression analysis and quadratic design model were used for analyzing and optimizing data using Design Expert. The polynomial equation was built with model coefficients that had a statistical significance of 0.05. Finally, the model's suitability for use was evaluated using a variety of metrics, including coefficient of correlation, projected error sum of squares, and lack of fit analysis. The 2D-contour and 3D-response surface plots were subjected to response surface analysis to determine the factor-response connection and possible interaction effect(s). The optimum chromatographic condition was finalized optimizing numerical functions and desirability.

### Simulation of desired conditions to chromatographic output

Based on the desirability data obtained from the design expert software, the conditions were mimicked and a solution containing 2.5 µg/ml of APX and 75 µg/ml of CLP was chromatographed. Once the chromatogram was obtained,

**Table 1.** Factorial design variables.

Factors	Factors level		
	-1	0	+1
Amount of organic phase (X1)	60%	70%	80%
pH (X2)	3	3.5	4
Flow rate (X3)	0.8	1	1.2

the system suitability parameters were checked three times, and a comparison between predicted values and mean actual values was made.

### Validation of optimized method

The improved approach was validated in compliance with the ICH Q2R2 requirements. For the linearity, a standard calibration curve was created by injecting five different levels of APX ranging from 1.25–3.75 µg/ml and CLP ranging from 37.5–112.5 µg/ml. Between peak area and drug concentration, a linear calibration curve was established. Linear regression analysis was used to test the linearity. The repeatability of the method was ensured by injecting a 100% concentration (2.5 µg/ml of APX and 75 µg/ml of CLP) for a total of six times and its relative standard deviation (RSD) was monitored. LOD and LOQ were statistically determined. A mixture representing the whole range was examined for intraday precision for a total of three times, and the same concentration was also analyzed on separate days for monitoring the interday precision (APX + CLP = 1.25 + 37.5, 2.5 + 75 and 3.75 + 112.5 µg/ml). RSD was monitored to adjudge the method's precision. Accuracy of the method was assessed by spiking of placebo with standard. The target concentration was APX + CLP = 2.5 + 75 µg/ml. The placebo was spiked at 50%, 100%, and 150% of the intended concentration. Placebo composition was selected based on generalized use for the mentioned category which comprises microcrystalline cellulose (MCC) (190 mg), magnesium stearate (4 mg), hydroxy propyl methyl cellulose (HPMC) (4 mg), and talc (2 mg). HPMC was introduced as a film-forming agent and MCC had the role of being a directly compressible material. Magnesium stearate was used as a gliding agent and talc has the role of lubricating agent. Again three replicates at each concentration were analysed and % recovery was monitored.

### Quantification of APX and CLP form synthetic mixture

The proposed active binary mixture comprised 200 mg placebo as mentioned before, 2.5 mg APX, and 75 mg CLP (277.5 mg). The constituents were diluted in 10 ml of methanol to produce a mixture comprising 250 µg/ml of APX and 7,500 µg/ml of CLP, respectively. The solution described above was filtered via a 0.45 µm Whatman filter disk. A total of 100 µl of filtrate was diluted to 10 ml with mobile phase to get a solution with 2.5 µg/ml of APX and 75 µg/ml of CLP. The combination was examined in triplicates to determine the percentage assay.

## RESULTS AND DISCUSSION

### Effect of independent variables on responses

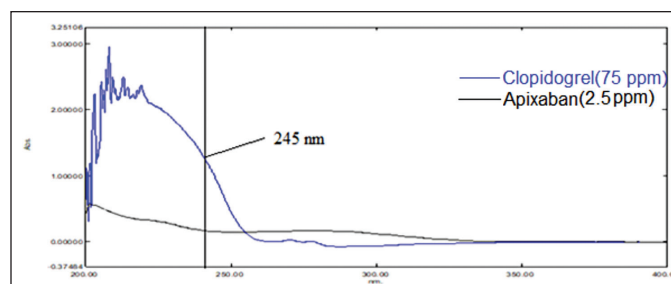
The only parameter, that is analytical wavelength was kept constant, as there is a wide difference in the dose and absorptivity, the selection of analytical wavelength was a tedious one; however, at 245 nm, the signal of APX and CLP in the chromatogram were adequate and hence was selected as analytical wavelength (Fig. 2)

After the application of factorial design, when the system was applied, the mentioned responses were noted which are shown in Table 3. When statistical treatment was applied to the obtained responses in Design Expert software, the following

observations were noted. The applicability of the model was determined based on agreement between Adj R-squared and Pred R-squared, the difference between the same should be less than 0.2 which was the case for all the responses. Another term, Adqe precision was observed whose ration should be greater than 4, which again was the case for all responses

**Table 2.** Executing three factorial designs (coded values).

Run	Actual value			Coded value		
	Factor 1 Proportion of Methanol	Factor 2 Flow rate	Factor 3 pH of mobile phase	Factor 1	Factor 2	Factor 3
1	80	1	3	1	0	-1
2	60	1.2	3.5	-1	1	0
3	60	1	3	-1	0	-1
4	70	1	3.5	0	0	0
5	60	1	4	-1	0	1
6	80	0.8	3.5	1	-1	0
7	60	0.8	3.5	-1	-1	0
8	60	0.8	4	-1	-1	1
9	70	0.8	3	0	-1	-1
10	60	1	3.5	1	0	0
11	80	0.8	4	1	-1	1
12	60	1	3.5	-1	0	0
13	70	1.2	4	0	1	1
14	70	1.2	3	0	1	-1
15	70	1.2	3.5	0	1	0
16	60	1.2	4	-1	1	1
17	60	0.8	3	-1	-1	-1
18	80	1.2	3.5	1	1	0
19	80	1.2	3	1	1	-1
20	80	1	4	1	0	1
21	70	1	4	0	0	1
22	70	0.8	4	0	-1	1
23	60	1.2	3	-1	1	-1
24	70	0.8	3.5	0	-1	0
25	70	1	3	0	0	-1
26	80	1.2	4	1	1	1
27	80	0.8	3	1	-1	-1



**Figure 2.** Overlain UV spectrum of APX (2.5 µg/ml) and CLP (75 µg/ml).

For response 1, A and B were identified as significant model terms. Adeq precision value 54.162 indicated a strong signal. Hence, the presented design was employed to explore the design space which is highlighted as the surface and counter plot (Fig. 3). The polynomial equation can be described as an RT APX = +4.46 – 0.48\*A – 0.13\*B (Reduced model, as C is nonsignificant term). For response 2 (Rt of CLP), there were significant model terms identified as A, B, AB, and A2. Adeq precision value 24.347 indicated an adequate signal. The same model is highlighted as a surface and counter plot (Fig. 4). The polynomial equation can be described as an RT CLP = + 12.34 – 1.83\*A – 0.80\*B + 0.64\*AB – 0.88\*A2 (Reduced Model). For Response 3 (Tf of APX), A, B, AC, A<sup>2</sup>, and B<sup>2</sup> were identified as significant model terms. Adeq precision value 35.73 indicated an adequate signal. The same model is highlighted as a surface and counter plot (Fig. 5). The polynomial equation can be described as a TF APX = + 1.50 + 0.18\*A + 0.067\*B + 0.020\*AC – 0.024\*A2 + 0.024B2 (Reduced Model). For response 4 (tailing factor of CLP), A, B, AB, AC, and A2 were significant model terms. Adeq precision value 32.39 indicated an adequate signal.

The same model is highlighted as a surface and counter plot (Fig. 6). The polynomial equation can be described as a TF APX = + 1.40 + 0.16\*A + 0.046\*B – 0.021\*AB + 0.020\*AC – 0.051\*A2 (Reduced model). For response 5, A, B, AB, and A<sup>2</sup> were significant model terms. Adeq precision value 22.78 indicated an adequate signal. The same model is highlighted as a surface and counter plot (Fig. 7). The polynomial equation can be described as a Resolution = + 1.40 – 0.78\*A – 0.36\*B + 0.40\*AB + 0.39\*A2.

From the above data, a desirability surface plot was generated (Fig. 8), which provided the outline of predicted conditions that can provide the best possible response for dependent variables. The proportion of organic phase was predicted as 63.52 volumes, the flow rate was predicted as 0.8 ml/minute and the pH was predicted as 3.0. The desirability for a given condition was identified as 0.683, which gave an idea regarding perfect suitability. The proposed conditions by design expert software predicted the best possible response. When the conditions obtained from the software were mimicked, the perfect separation was observed (Fig. 9). The actual value of responses

**Table 3.** Matrixes of factorial experimental design with responses.

Run	Factor A Proportion of Organic Phase	Factor B Flow rate	Factor C pH of mobile phase	R1 (Rt of APX)	R2 (Rt of CLP)	R3 (Tf of APX)	R4 (Tf of CLP)	R5 (Resolution)
1	80	1	3	3.724	9.746	1.621	1.503	7.822
2	60	1.2	3.5	4.832	11.855	1.371	1.262	8.439
3	60	1	3	4.899	13.015	1.319	1.208	9.513
4	70	1	3.5	4.531	12.512	1.501	1.389	8.269
5	60	1	4	4.919	13.013	1.299	1.168	9.481
6	80	0.8	3.5	4.084	10.023	1.611	1.473	7.886
7	60	0.8	3.5	5.027	15.159	1.234	1.116	10.127
8	60	0.8	4	5.019	15.172	1.222	1.086	10.522
9	70	0.8	3	4.687	12.713	1.411	1.364	8.346
10	80	1	3.5	3.916	9.758	1.652	1.502	7.819
11	80	0.8	4	4.092	10.028	1.642	1.492	7.874
12	60	1	3.5	4.914	13.026	1.302	1.199	9.492
13	70	1.2	4	4.281	11.985	1.621	1.435	8.015
14	70	1.2	3	4.276	11.994	1.601	1.411	8.027
15	70	1.2	3.5	4.269	11.896	1.599	1.427	8.018
16	60	1.2	4	4.827	11.862	1.352	1.244	8.406
17	60	0.8	3	5.014	15.163	1.262	1.122	9.874
18	80	1.2	3.5	3.876	9.294	1.733	1.536	7.746
19	80	1.2	3	3.884	9.278	1.721	1.517	7.789
20	80	1	4	3.921	9.777	1.693	1.514	7.799
21	70	1	4	4.529	12.510	1.511	1.399	8.252
22	70	0.8	4	4.688	12.711	1.483	1.386	8.309
23	60	1.2	3	4.853	11.864	1.396	1.294	8.463
24	70	0.8	3.5	4.679	12.719	1.459	1.392	8.331
25	70	1	3	4.533	12.511	1.421	1.359	8.281
26	80	1.2	4	3.892	9.282	1.743	1.555	7.714
27	80	0.8	3	4.098	10.014	1.605	1.421	7.892

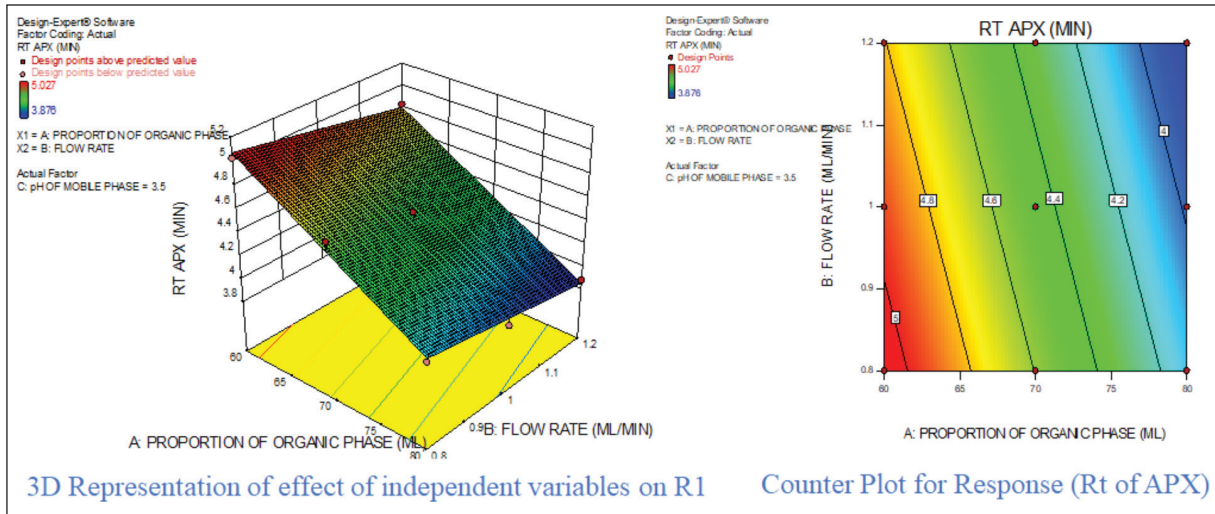


Figure 3. Effect of independent variable on retention of APX.

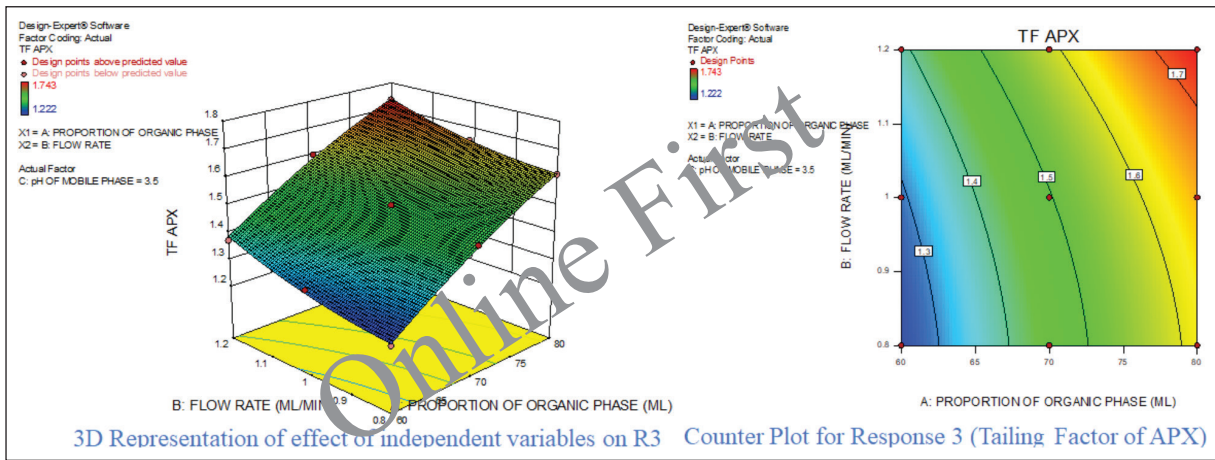


Figure 4. Effect of independent variable on tailing factor of APX.

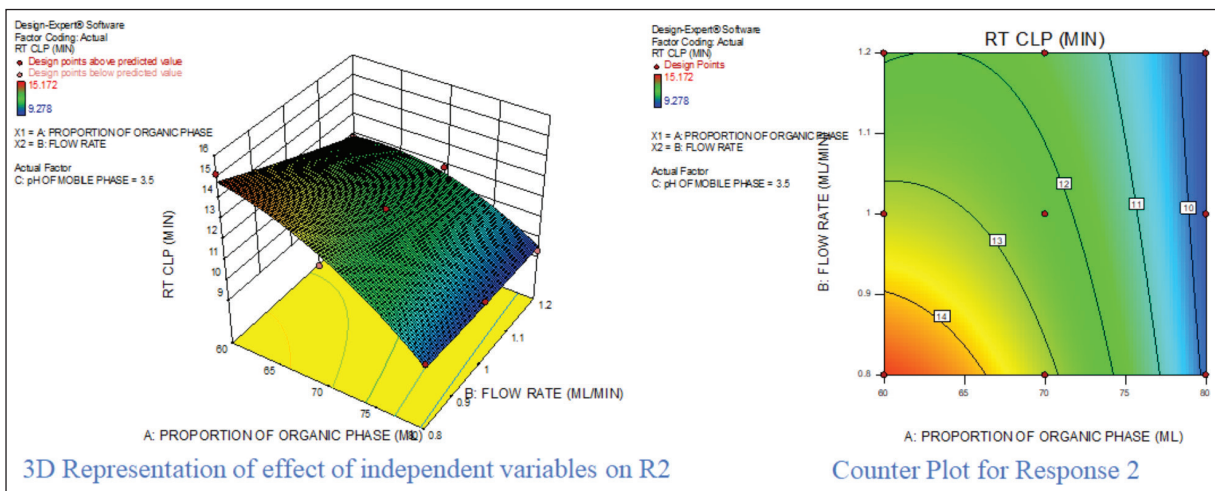


Figure 5. Effect of independent variable on retention of CLP.

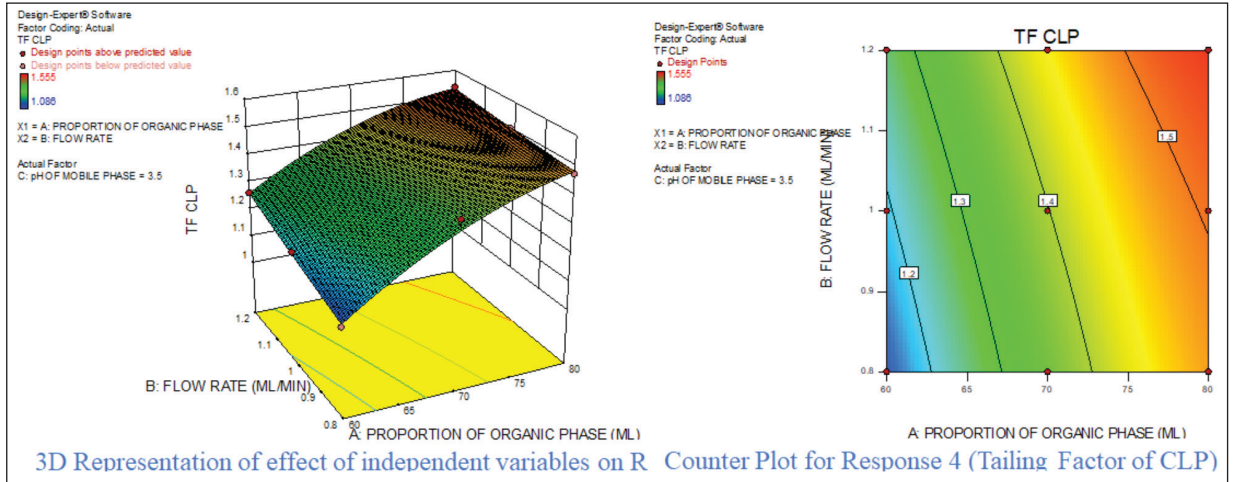


Figure 6. Effect of independent variable on tailing factor of CLP.

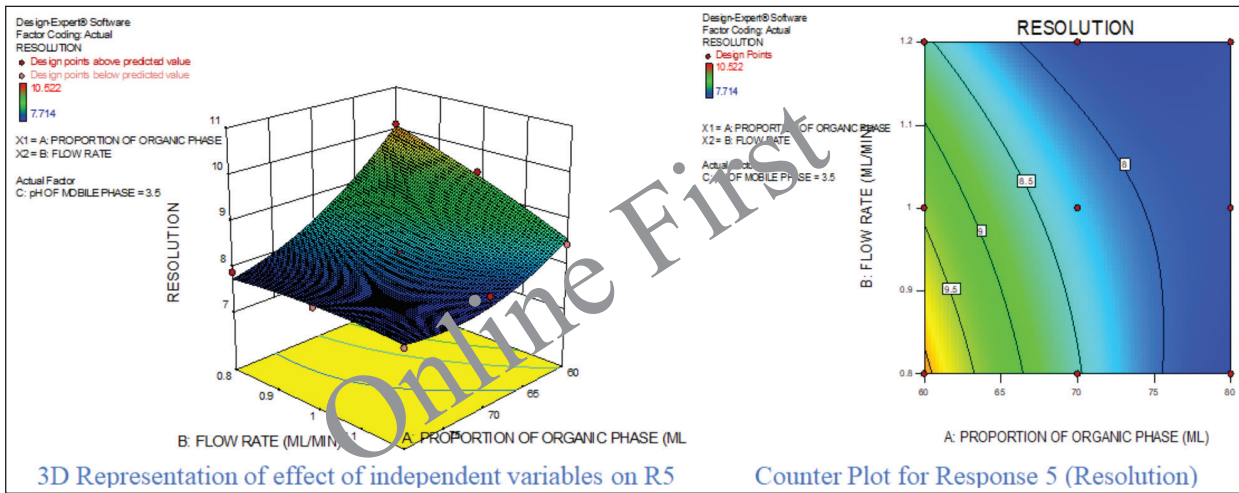


Figure 7. Effect of independent variable on resolution.

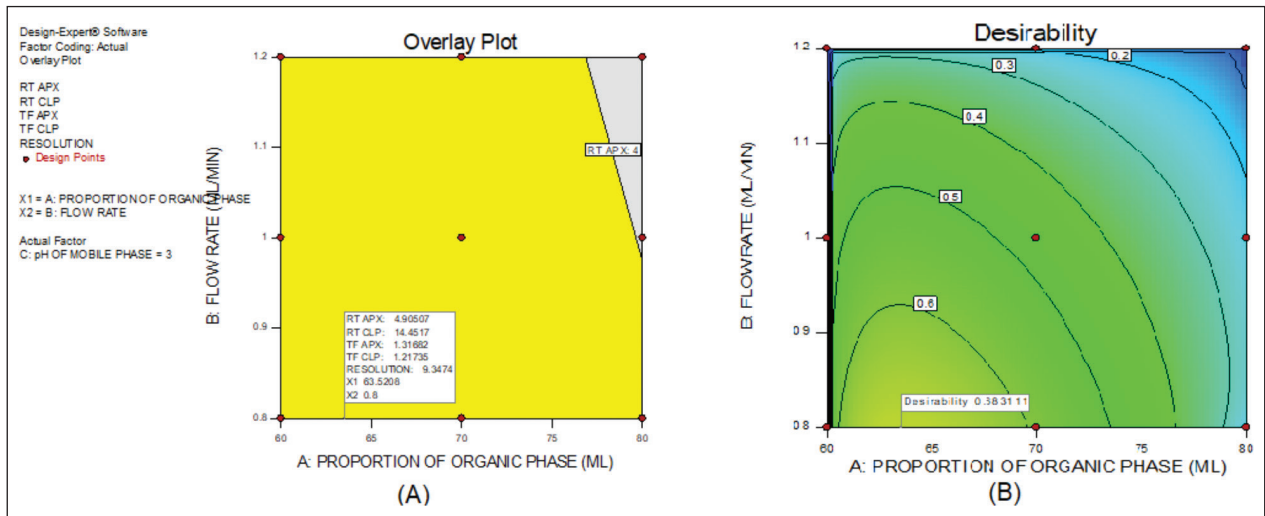


Figure 8. Desirability plot.

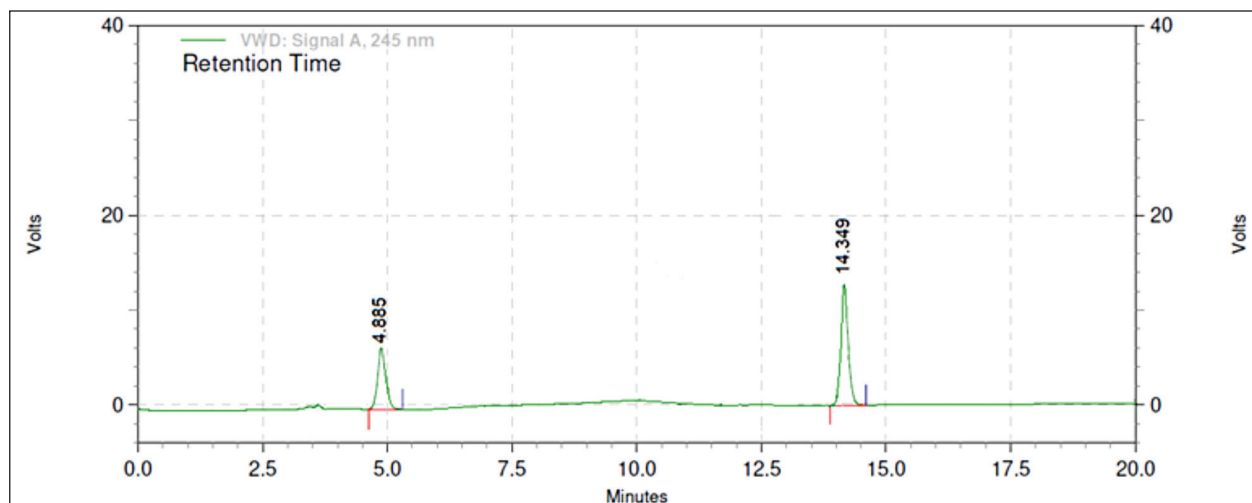


Figure 9. Chromatogram under optimized conditions.

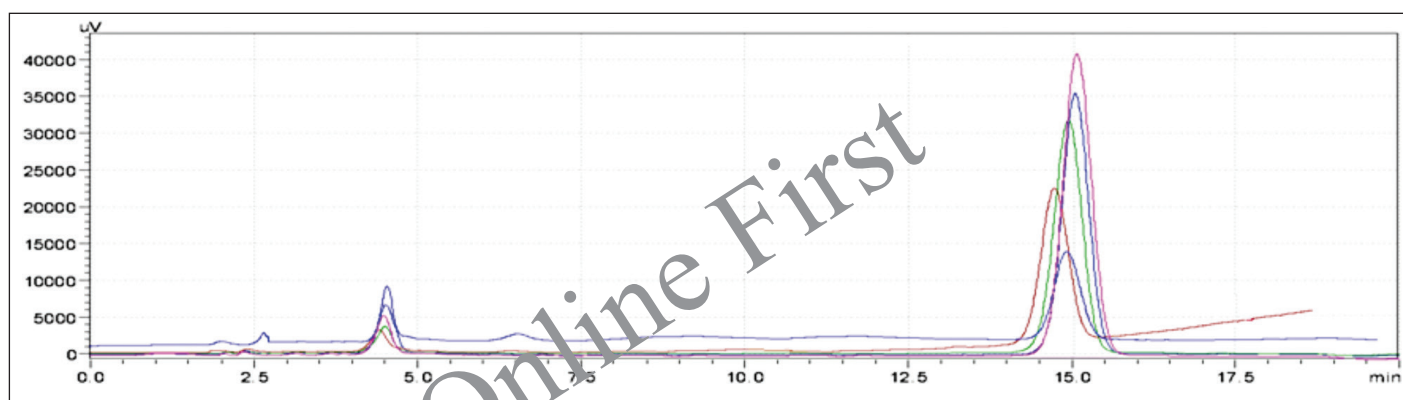


Figure 10. Overlain chromatogram of linearity.

Table 4. Comparison between predicted and actual values of dependent variables.

Factor	Predicted value	Actual value
Retention time of APX (R1)	4.90	4.89
Retention time of CLP (R2)	14.45	14.35
Tailing factor of APX (R3)	1.31	1.29
Tailing factor of CLP (R4)	1.21	1.23
Resolution (R5)	9.34	10.17
Desirability	0.683	

Table 5. System suitability parameters under optimized chromatographic conditions.

Parameter	APX	CLP
Retention time ( $R_t$ ) [minute.]	4.88 ± 0.02	14.34 ± 0.01
Tailing factor	1.28 ± 0.01	1.23 ± 0.01
Number of theoretical plates [plates/meter]	7,346.67 ± 23.16	50,741.67 ± 97.09
Resolution [ $R_s$ ]	10.16 ± 0.02	

( $n = 5$  determinations)

was in perfect correlation with the predicted values, which are cited in Table 4. Under these conditions, all the system suitability parameters are in accordance with the limits as shown in Table 5.

#### Method validation

The devised approach was linear throughout the concentration range of APX (1.25–3.75  $\mu\text{g/ml}$ ) and CLP (37.5–112.5  $\mu\text{g/ml}$ ), with correlation coefficients ( $r^2$ ) of 0.9989 and 0.9988, respectively (Fig. 10). This demonstrated that all the responses were within the required acceptable limit, demonstrating a good degree of similarity between the predicted

and observed data for the accuracy experiments at 50%, 100%, and 150% levels, with the % recovery being within 98–102. Intermediate accuracy and repeatability tests were performed, and the percentage RSD values were determined to be less than 2%. Table 6 provides an overview of the approach validation parameters.

#### Quantification from binary mixture

When the newly developed and validated procedure was applied to a mixture containing 2.5  $\mu\text{g/ml}$  of APX and 75  $\mu\text{g/ml}$  of CLP, no interference from the placebo components

**Table 6.** HPLC validation summary for quantification of APX and clopidogrel.

Parameter	Limit	Result		Inference
		APX	CLP	
Linearity and range	R <sup>2</sup> >0.999	0.9989	0.9988	Method is linear
Repeatability	%RSD <2	1.40	1.06	Method is repeatable
Inter-day precision	%RSD <20.99–1.34		0.72–1.13	Method is precise
Intraday precision	%RSD <2	0.82–1.01	0.81–0.94	Method is precise
% Recovery	98%–102%	99.73–100.27	99.52–100.01	Method is accurate
Assay	-	99.47	99.92	-

was observed and the amount found for APX was  $2.49 + 0.02$  µg/ml ( $99.47 + 0.83$  %w/w) and for CLP it was  $74.94 + 0.39$  µg/ml ( $99.92 + 0.52$  %w/w)

## CONCLUSION

A Robust and reliable RP-HPLC technique for APX and CLP was successfully developed using Design Expert 10 software with a desirability of 0.683, which indicates the best possible separation. The predicted conditions indicated a mobile phase containing 63.5% methanol having a 3.0 pH and flow rate of 0.8 ml/minute. When the method was operated with predicted conditions, all the responses were in perfect accordance with the predicted values. The method was finally successfully validated as per ICH Q2R2 guidelines and applied for quantification of APX and CLP from the binary mixture with % assay of 99.47 and 99.92%, respectively. As there is no HPLC method available to analyze this novel combination, based on method development expertise and risk assessment results, the QbD approach demonstrated outstanding linearity, accuracy, precision, and robustness for determination. However, the present developed method does not justify its applicability for the study of the stability of the mentioned components in the mixture as it has not been studied which is also the future plan of the current research.

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## AUTHORS' CONTRIBUTIONS

Rashmi Shukla oversaw research execution, including idea creation, design, data collecting, analysis, and interpretation. Pinak Patel oversaw statistical analysis of the data and critical revision of the text. Krunal Detholia oversaw funding and material acquisition. Ankit Chaudhary helped by overseeing the whole research process and correspondence.

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## CONFLICTS OF INTEREST

The authors have no financial or other conflicts of interest to disclose.

## ETHICAL APPROVALS

The present research work does not involve any experimental animal, animal parts, or human subjects.

## DATA AVAILABILITY

All data generated and analyzed are included in this research article.

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