EUROPEAN JOURNAL OF BIOMEDICAL AND PHARMACEUTICAL SCIENCES
http://www.ejbps.com

ISSN 2349-8870
Volume: 12
Issue: 5
Year: 2025

QBD BASED NOVEL ANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF CLOPIDOGREL AND ROSUVASTATIN IN BULK AND PHARMACEUTICAL DOSAGE FORMS BY RP-HPLC

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Article Received on 12/03/2025 **Article Revised on 02/04/2025** **Article Accepted on 21/04/2025**

ABSTRACT
A Quality design approach to method development involves method goal identification, method scouting and evaluation, method selection, and risk assessment. The present study describes the risk-based HPLC method development and validation of Clopidogrel and Rosuvastatin in bulk form and marketed pharmaceutical dosage form. The chromatographic conditions were optimized with the Design Expert software 11.0 version i.e., Phenomenex Gemini (250mm x 4.6mm) 5µm Particle size and Mobile phase used was Methanol and KH2PO4 buffer with pH 4.2 in the proportion of 20:80 % v/v, flow rate was found to be 1.0 ml/min with retention times of Clopidogrel and Rosuvastatin was found to be 2.474 min and 4.356 min respectively. The developed method was found to be linear in the range of Clopidogrel is 20-100 µg/ml and Rosuvastatin is 40-120 µg/ml with a correlation coefficient of 0.999 for both drugs. The % RSD of intraday and inter-day precision for Clopidogrel is 0.086 & 0.231 and Rosuvastatin is 0.59 & 0.274%. The robustness values were less than 2%. The assay was found to be 99.98% & 99.56% for Clopidogrel and Rosuvastatin respectively. The method validation parameters were in the prescribed limit as per ICH guidelines. Stress studies reveal that both drugs were degraded more in alkaline, thermal conditions than in acidic, oxidative and photolytic conditions. Hence, the proposed method was stability indicating, using QbD approach all the method parameters were better understood that, reduces the time and cost of the analysis.

KEYWORDS: Clopidogrel and Rosuvastatin, RP-HPLC, Quality by Design, Accuracy.

INTRODUCTION
Clopidogrel Bisulfate is a thienopyridine with antiplatelet activity. Clopidogrel bisulfate irreversibly alters the platelet receptor for adenosine diphosphate (ADP), thereby blocking the binding of ADP to its receptor, inhibiting ADP-mediated activation of the glycoprotein complex GPIIb/IIIa, and inhibiting fibrinogen binding to platelets and platelet adhesion and aggregation.[1] Clopidogrel is indicated to reduce the risk of myocardial infarction for patients with non-ST elevated acute coronary syndrome (ACS), patients with ST-elevated myocardial infarction, and in recent MI, stroke, or established peripheral arterial disease.[2] Clopidogrel is a prodrug of a platelet inhibitor used to reduce the risk of myocardial infarction and stroke. It has a long duration of action as it is taken once daily and a large therapeutic window as it is given in doses of 75-300mg daily.[3] The IUPAC Name of Clopidogrel is methyl (2S)-2-(2-chloro phenyl)-2-(6, 7-dihydro-4H-thieno [3, 2-c] pyridin-5-yl) acetate; sulfuric acid. The Chemical Structure of Clopidogrel is shown in following figure-1.

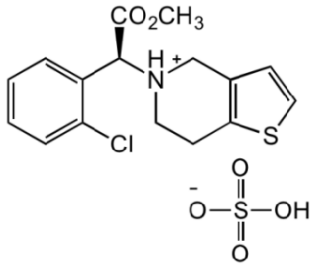

Fig. 1: Chemical Structure of Clopidogrel.

Rosuvastatin Calcium is a statin with antilipidemic and potential antineoplastic activities. Rosuvastatin selectively and competitively binds to and inhibits hepatic hydroxy methyl-glutaryl coenzyme A (HMG-CoA) reductase, the enzyme which catalyzes the conversion of HMG-CoA to mevalonate, a precursor of cholesterol.[4] This leads to a decrease in hepatic

cholesterol levels and increase in uptake of Low-Density Lipoprotein (LDL) cholesterol.[5] In addition, Rosuvastatin, like other statins, exhibits pro-apoptotic, growth inhibitory and pro-differentiation activities in a variety of tumor cell types; these antineoplastic activities may be due, in part, to inhibition of the isoprenylation of Ras and Rho GTPases and related signaling cascades. The FDA monograph states that Rosuvastatin is indicated as an adjunct to diet in the treatment of triglyceridemia, Primary Dysbetalipoproteinemia (Type III Hyperlipoproteinemia), and Homozygous Familial Hypercholesterolemia. Rosuvastatin is used together with diet, weight-loss, and exercise to reduce the risk of heart attack and stroke and to decrease the chance that heart surgery will be needed in people who have heart disease or who are at risk of developing heart disease.[6] The IUPAC name of Rosuvastatin is Calcium; (E, 3R, 5S)-7-[4-(4-fluoro phenyl)-2-[methyl (methyl sulfonyl) amino]-6-propan-2-yl pyrimidin-5-yl]-3, 5-dihydroxyhept-6-enoate. The Chemical Structure of Rosuvastatin is as follows.

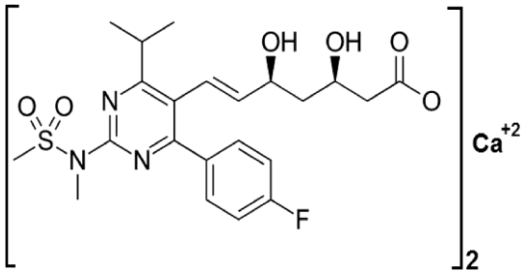

Fig. 2: Chemical Structure of Rosuvastatin.

MATERIALS AND METHODS

Glassware, Instruments, and Chemicals

All the chemicals, glassware and instruments are listed out below, which were used in the experimental work.

Glassware

Glassware used in the experimental work were of Borosil made and instruments used for the work were subjected through routine calibration procedure.

Instruments

UV Spectroscopy

Shimadzu 1800, a double beam UV-visible Spectrophotometer having two matched cells with 1cm light path, Shimadzu, Japan.

Hot air oven: - Lab line, India.

Analytical Balance: Shimadzu AUX220

Sonicator: Shimadzu, Japan, Life care Equipments Pvt. Ltd.

HPLC for Method Development of Clopidogrel and Rosuvastatin

Model: Agilent Technologies 1200 Series HPLC
Stationary Phase: Inertsil ODS (250x 4.6) mm; 5µm
Software: ChemStation

Miscellaneous Requirements

Ultrasonic bath: Frontline FS4 ultrasonic cleaner, Mumbai.
pH meter: Digital pH meter, Systronic, Mumbai
Electronic balance: Shimadzu analytical balance
Micro Pipette: Capacity: 10-200µl, Chromatopak
0.45 µ HPLC Filter, Merck
Whatmann Filter paper No 41

Reagents and Chemicals

Chemicals used were of analytical grade for UV and HPLC grade for HPLC method Development[7].

- Methanol-HPLC grade (S.D. Fine Chemicals, Mumbai)
- Acetonitrile-HPLC grade (S.D. Fine Chemicals, Mumbai)
- Water-HPLC grade (Finar Reagents, Ahmedabad)
- Potassium Hydrogen phosphate (Finar Reagents, Ahmedabad)
- Ortho Phosphoric acid (Finar Reagents, Ahmedabad)

Procurement of API and its Formulation:

Selected Drug APIs were obtained as a gift sample and dosage form of drugs was purchased from local market as given in Table 1.

Table 1: Procurement of API and Formulations.

API Name	**Source**	
Clopidogrel	Synpharma Research Lab, Hyderabad (Sun Pharma)	
Rosuvastatin	Synpharma Research Lab, Hyderabad (Sun Pharma)	
Formulation (Tablet)	**Brand Name**	**Label Claim**
Clopidogrel Bisulfate and Rosuvastatin Calcium	Rosuvas CV 10 Tablet	Clopidogrel 75mg Rosuvastatin 10mg

METHODOLOGY

Preparation of Clopidogrel Stock Solution

The standard stock solution of Clopidogrel was prepared by dissolving 100 mg drug in 100 ml methanol which gives 1000µg/mL concentration. From that 100 µg/mL solution of Clopidogrel was prepared by taking 1ml of above solution into 10 ml volumetric flask and was diluted up to the mark with Methanol.

Preparation of Rosuvastatin Stock Solution

The standard stock solution of Rosuvastatin was prepared by dissolving 100 mg drug in 100 ml methanol which gives 1000µg/mL concentration. From that 100

µg/mL solution of Rosuvastatin was prepared by taking 1ml of above solution into two separate 10 ml volumetric flask and were diluted up to the mark with Methanol.

Factor Screening Studies

A 2-factor nine -run fractional factorial design was employed for factor screening studies to identify the CMPs/CPPs (Critical Method Parameters/Critical Process Parameters) critically affecting the method CAAs (Critical Analytical Attributes) (i.e., Flow rate and pH). A total of nine experimental runs were performed, and the design was analyzed for Under the influence of studied factors on the CAAs (Critical Analytical Attributes).[8] Mathematical data analysis was carried out by fitting the obtained experimental data to the linear polynomial model by obviating the interaction term(s).

Selection of CAA (Critical Analytical Attributes) and Response Monitored Using CCD (Central Composite Design) Model

Mobile Phase composition and pH has selected as Critical Analytical Attributes and Resolution, Tailing of Clopidogrel and Retention time of Rosuvastatin were selected as responses monitored as per table 2.

Table 2: Selection of CAA and Response.

CAA	Response monitored
Mobile phase composition	Resolution
pH	Tailing of 1st drug
	Retention time of 2nd drug

Design Matrix as per the CCD for Optimization of the HPLC Method

Table 3: Design Matrix for Clopidogrel and Rosuvastatin.

Run	pH	Mobile phase Composition (methanol)
1	+1	+1
2	+1	-1
3	+1	0
4	0	+1
5	0	-1
6	0	0
7	-1	+1
8	-1	0
9	-1	-1

pH: 0 coded for 4.5, +1 coded for 5, -1 coded for 4.3

Mobile Phase: 0 coded for 40%v/v methanol, +1 coded for 42%v/v methanol, -1 coded for 38 %v/v methanol.

Optimization Data Analysis and Model Validation

The optimization data analysis was carried out by multiple linear regression analysis (MLRA) using Design Expert® ver. 13.0.1 software (M/s Stat-Ease Inc., MN, USA) for fitting the experimental data to the second-order quadratic polynomial model for estimating both the main effects and interaction effects. The model coefficients with statistical significance <0.05 were considered in framing the polynomial equation. The model aptness was finally ratified by analyzing various parameters like coefficient of correlation (r2), predicted error sum of squares (PRESS) and lack of fit analysis. Response surface analysis was carried out from the 2D-contour and 3D-response surface plots to discern the factor–response relationship and plausible interaction effect.[9]

Method Validation

As per ICH guidelines Various Validation Parameters have been performed for Clopidogrel and Rosuvastatin.

Method Validation Parameters[10-14]

System Suitability

Accurately weigh and transfer 10 mg of Clopidogrel and 10mg of Rosuvastatin working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.6ml of Clopidogrel and 0.8ml of Rosuvastatin from the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents. (60µg/ml-Clopidogrel & 80 µg/ml-Rosuvastatin).

Procedure

The standard solution was injected for five times and measured the area for all five injections in HPLC. The %RSD for the area of five replicate injections was found to be within the specified limits.[15]

Specificity

Preparation of Standard Solution

Accurately weigh and transfer 10 mg of Clopidogrel and 10mg of Rosuvastatin working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents

and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.6ml of Clopidogrel and 0.8ml of Rosuvastatin from the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents.

Preparation of Sample Solution

Take average weight of Tablets and crush in a mortar by using pestle and weight 10 mg equivalent weight of Clopidogrel and Rosuvastatin sample into a 10mL clean dry volumetric flask and add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.

Further pipette both 0.6ml and 0.8ml of Sample solution from the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents.

Procedure

Inject the three replicate injections of standard and sample solutions and calculate the assay by using formula[16]:

$$\%ASSAY = \frac{\text{Sample area}}{\text{Standard area}} \times \frac{\text{Weight of standard}}{\text{Dilution of standard}} \times \frac{\text{Dilution of sample}}{\text{Weight of sample}} \times \text{Purity} \times \frac{\text{Weight of tablet}}{100} \times \text{Label claim}$$

Preparation of Drug Solutions for Linearity

Accurately weigh and transfer 10 mg of Clopidogrel and 10mg of Rosuvastatin working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Preparation of Level – I (20ppm of Clopidogrel & 40ppm of Rosuvastatin)

Pipette out 0.2ml of Clopidogrel and 0.4ml of Rosuvastatin stock solutions was take in a 10ml of volumetric flask dilute up to the mark with diluent.

Preparation of Level – II (40ppm of Clopidogrel & 60ppm of Rosuvastatin)

Pipette out 0.4ml of Clopidogrel and 0.6ml of Rosuvastatin stock solutions was take in a 10ml of volumetric flask dilute up to the mark with diluent.

Preparation of Level – III (60ppm of Clopidogrel & 80ppm of Rosuvastatin)

Pipette out 0.6ml of Clopidogrel and 0.8ml of Rosuvastatin stock solutions was take in a 10ml of volumetric flask dilute up to the mark with diluent.

Preparation of Level – IV (80ppm of Clopidogrel & 100ppm of Rosuvastatin)

Pipette out 0.8ml of Clopidogrel and 1.0ml of Rosuvastatin stock solutions was take in a 10ml of volumetric flask dilute up to the mark with diluent.

Preparation of Level – V (100ppm of Clopidogrel & 120ppm of Rosuvastatin)

Pipette out 1.0ml of Clopidogrel and 1.2ml of Rosuvastatin stock solutions was take in a 10ml of volumetric flask dilute up to the mark with diluent.

Procedure

Inject each level into the chromatographic system and measure the peak area.

Plot a graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) and calculate the correlation coefficient.[17]

Precision

Repeatability

Preparation of Clopidogrel and Rosuvastatin Product Solution for Precision

Accurately weigh and transfer 10 mg of Clopidogrel and 10mg of Rosuvastatin working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.6ml of Clopidogrel and 0.8ml of Rosuvastatin from the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents. (60 µg/ml-Clopidogrel & 80 µg/ml-Rosuvastatin).

Procedure: The standard solution was injected for five times and measured the area for all five injections in HPLC.[18] The %RSD for the area of five replicate injections was found to be within the specified limits.

Intermediate Precision

To evaluate the intermediate precision (also known as Ruggedness) of the method, Precision was performed on different days by maintaining same conditions.

Procedure

Day 1

The standard solution was injected for six times and measured the area for all six injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits.

Day 2

The standard solution was injected for six times and measured the area for all six injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits.[19]

Accuracy

For Preparation of 50% Standard Stock Solution

Accurately weigh and transfer 10 mg of Clopidogrel and 10mg of Rosuvastatin working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.3ml of Clopidogrel and 0.4ml of Rosuvastatin from the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents.

For Preparation of 100% Standard Stock Solution

Accurately weigh and transfer 10 mg of Clopidogrel and 10mg of Rosuvastatin working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.6ml of Clopidogrel and 0.8ml of Rosuvastatin from the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents.

For Preparation of 150% Standard Stock Solution

Accurately weigh and transfer 10 mg of Clopidogrel and 10mg of Rosuvastatin working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.9ml of Clopidogrel and 1.2ml of Rosuvastatin from the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents.

Procedure

Inject the Three replicate injections of individual concentrations (50%, 100%, 150%) were made under the optimized conditions. Recorded the chromatograms and measured the peak responses. Calculate the Amount found and Amount added for Clopidogrel and Rosuvastatin and calculate the individual recovery and mean recovery values.[20]

Robustness

The analysis was performed in different conditions to find the variability of test results. The following conditions are checked for variation of results.[21]

Effect of Variation of Flow Conditions

The sample was analyzed at 0.9 ml/min and 1.1 ml/min instead of 1ml/min, remaining conditions are same. 20µl of the above sample was injected twice and chromatograms were recorded

Effect of Variation of Mobile Phase Organic Composition

The sample was analyzed by variation of mobile phase i.e. Methanol: Phosphate Buffer was taken in the ratio and 35:65, 25:75 instead (30:70), remaining conditions are same. 20µl of the above sample was injected twice and chromatograms were recorded.

RESULT AND DISCUSSION

Method Development

Trials taken for Clopidogrel and Rosuvastatin in RP-HPLC

Various RP-HPLC trials taken for Clopidogrel and Rosuvastatin by changing Mobile phase composition and ratio of mobile phase.[22] Trials are mentioned in Table 4.

Table 4: Trials taken on RP-HPLC.

Sr. No.	Mobile Phase	Ratio (%v/v)	Retention time (min)	Conclusion
1	Methanol: Water	60:40	3.119min (Clopi), 8.077 min (Rosu)	Poor separation & Peak Splitting
2	Acetonitrile: Water	70:30	3.274 min (Clopi), 4.565 min (Rosu)	Good separation was not obtained
3	Methanol: Acetonitrile	80:20	1.299 min (Clopi), 4.705 min (Rosu)	Broad Peak and Good separation was not obtained
4	Methanol: KH_2PO_4 Buffer pH 2.8	60:40	6.409 min (Clopi), 7.848 min (Rosu)	Retention time was too long
5	Acetonitrile: KH_2PO_4 Buffer pH 3.5	45:55	4.342 min (Clopi), 5.332 min (Rosu)	Good separation was not obtained
6	Acetonitrile: KH_2PO_4 Buffer pH 4.8	35:65	4.505 min (Clopi), 5.588 min (Rosu)	Good separation was not obtained
7	Methanol: Phosphate Buffer pH-4.2	30:70	2.474 min (Clopi), 4.356 min (Rosu)	Good separation was obtained

Optimization of RP-HPLC for Clopidogrel Bisulfate and Rosuvastatin Calcium by QbD Approach

QBD Approach for Development of RP-HPLC Method

QBD approach was applied for method development of Clopidogrel Bisulfate and Rosuvastatin Calcium using Central Composite Design. Critical Quality Attributes were identified as per Table 5 for RP- HPLC method Development.[23]

Critical Analytical Parameters Attributes

Table 5: Critical Analytical Parameters Attributes.

Chromatographic Method Parameters							
CAA	**Mobile phase pH**	**Mobile Phase Composition**	**Γ**	**Injection Volume**	**Flow rate**	**Column Dimension**	**Column Temp.**
Area	+	+	0	-	+	0	-
Rt	+	+	0	-	+	0	0
Tailing	+	+	0	-	+	0	-

(+) High risk Parameters, (-) Medium risk Parameters, (0) Low risk Parameters

Selection of CAA and Response Monitored using CCD model

Critical Analytical Attributes and response monitored are mentioned in Table 6.

Table 6: Selection of CAA and Response Monitored using CCD model.

CAA	Response Monitored
Mobile Phase Composition	Resolution
pH	Tailing of 1st drug
	Retention time of 2nd drug

ANOVA for Quadratic Model, Response 1, Resolution

ANOVA was applied for resolution, Tailing and Retention time for Clopidogrel and Rosuvastatin as per Table 7, 8 and 9 and 10.

Design Matrix as per the CCD for Optimization of the HPLC Method

Table-7: Design Matrix as per the CCD for Optimization of the HPLC Method.

Run	pH	Mobile phase Composition (methanol)
1	+1	+1
2	+1	-1
3	+1	0
4	0	+1
5	0	-1
6	0	0
7	-1	+1
8	-1	0
9	-1	-1

pH: 0 coded for 4.5, +1 coded for 5, -1 coded for 4.3

Mobile Phase: 0 coded 30%v/v methanol +1 coded 32%v/v methanol, -1 coded for 28 %v/v methanol

ANOVA for Quadratic Model

Response 1: resolution

Table 8: ANOVA for Quadratic Model.

Source	Sum of Squares	Df	Mean Square	F-value	p-value	
Model	2.62	5	0.5232	22.78	0.0003	Significant
A- Methanol	1.39	1	1.39	60.47	0.0001	
B-pH	0.4179	1	0.4179	18.20	0.0037	
AB	0.0004	1	0.0004	0.0171	0.8997	
A^2	0.8075	1	0.8075	35.16	0.0006	
B^2	0.316	1	0.3156	13.74	0.0076	

The Model F-value of 22.78 implies the model is significant. There is only a 0.03% chance that an F-value this large could occur due to noise.

P-values less than 0.0500 indicate model terms are significant. In this case A, B, A^2, B^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.[24]

Fit Statistics

Table 9: Fit Statistics.

Std. Dev.	0.1515	R^2	0.9421
Mean	4.82	Adjusted R^2	0.9008
C.V. %	3.14	Predicted R^2	0.7471
		Adeq Precision	15.7549

The Predicted R^2 of 0.7471 is in reasonable agreement with the Adjusted R^2 of 0.9008; i.e. the difference is less than 0.2.

Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 15.755 indicates an adequate signal. This model can be used to navigate the design space.[25]

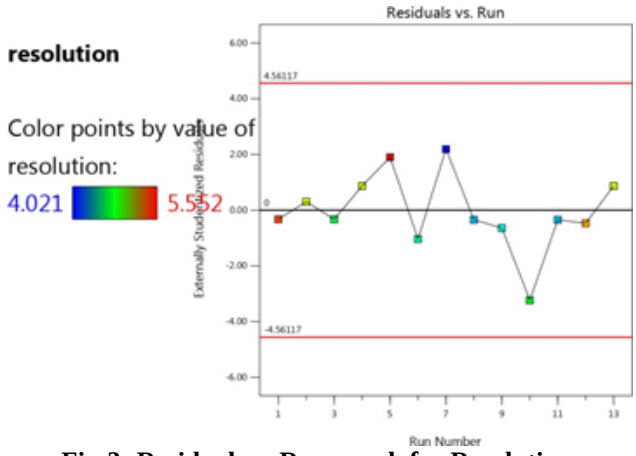

Fig-3: Residual vs. Run graph for Resolution.

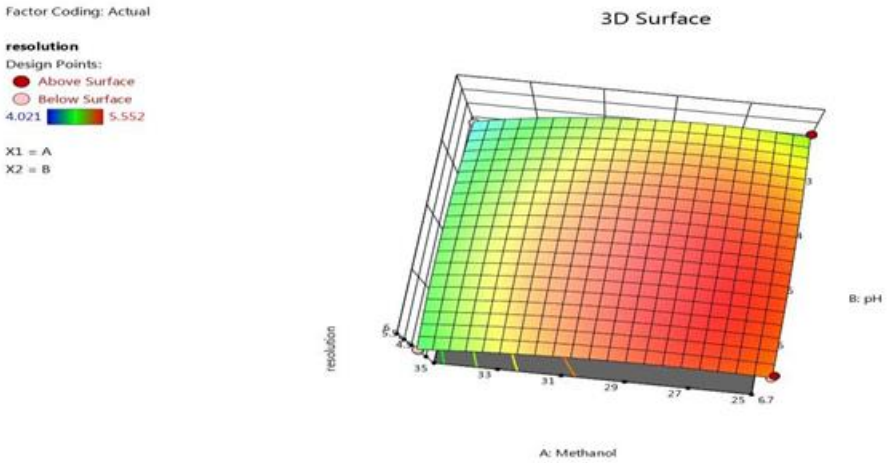

Fig. 4: 3D graph of Response Resolution.

The quadratic Polynomial equation for the measured response (Resolution), Y1 is given below:

Y1 = + 5.46 + 0.0784* A – 0.0229* B + 0.0370*AB-0.1056 A2 -0.0226* B2

The above polynomial equation represents the quantitative effect of predictor variables (Independent variables) on the measured response (Y1).

A positive sign in front of terms indicates Synergistic effect while a negative sign indicates antagonistic effect upon responses. Methanol concentration has positive effect. pH has negative effect but pH and Methanol Concentration both combined have Positive effect on Resolution.[26]

ANOVA for Quadratic Model
Response 2: Retention time of 2nd drug
Table-10: ANOVA for Quadratic Model.

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	1.90	5	0.3796	21.80	0.0004	**Significant**
A-Methanol	0.6053	1	0.6053	34.76	0.0006	
B-pH	0.0940	1	0.0940	5.40	0.0531	
AB	0.3144	1	0.3144	18.06	0.0038	
A^2	0.4934	1	0.4934	28.34	0.0011	
B^2	0.0001	1	0.0001	0.0051	0.9453	

The Model F-value of 21.80 implies the model is significant. There is only a 0.04% chance that an F-value this large could occur due to noise.

P-values less than 0.0500 indicate model terms are significant. In this case A, AB, A^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.[27]

Fit Statistics

Table-11: Fit Statistics.

Std. Dev.	**0.1320**	**R^2**	**0.9397**
Mean	2.80	**Adjusted R^2**	0.8966
C.V. %	4.72	**Predicted R^2**	0.7287
		Adeq Precision	15.0998

Predicted R^2 of 0.7287 is in reasonable agreement with the Adjusted R^2 of 0.8966; i.e. the difference is less than 0.2.

Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 15.100 indicates an adequate signal.[28] This model can be used to navigate the design space.

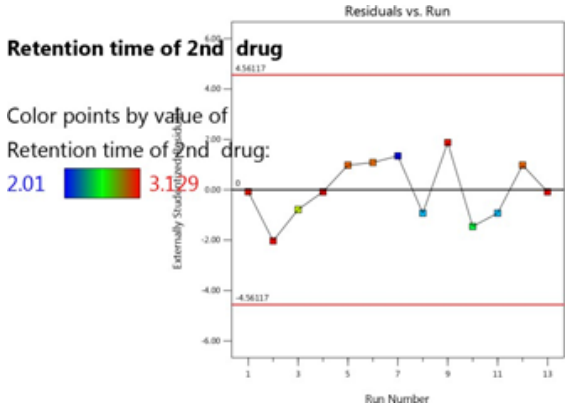

Fig-5: Residual vs. Run graph for Retention time

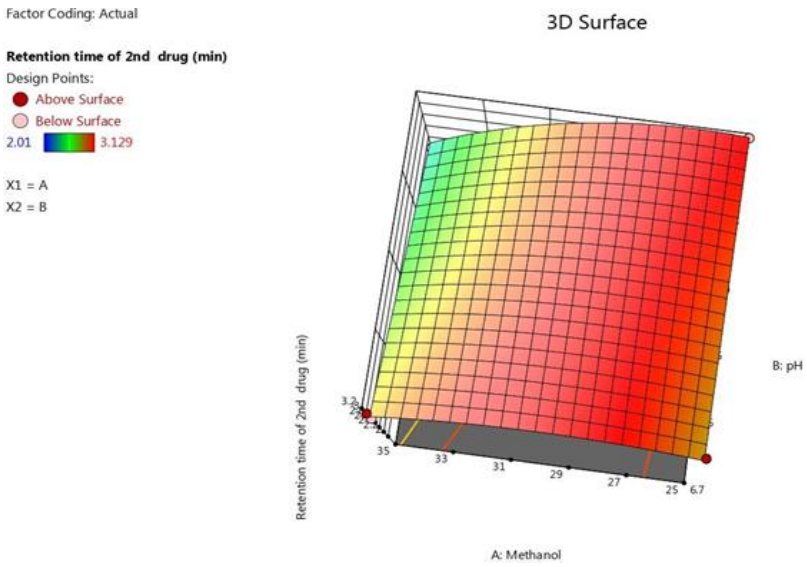

Fig. 6: 3D Graph for Retention Time.

The quadratic Polynomial equation for the measured response (Retention time of Clopidogrel), Y2 is given below:

Y2 = + 3.01 - 0.0250* A + 0.0060* B + 0.0035*AB-0.0285 A2 -0.0195* B2

The above polynomial equation represents the quantitative effect of predictor variables (Independent variables) on the measured response (Y2).

A positive sign in front of terms indicates Synergistic effect while a negative sign indicates antagonistic effect upon responses. Methanol concentration has negative effect. pH has Positive effect and pH and Methanol Concentration both combined have Positive effect on Retention time of Clopidogrel.[29]

□ ANOVA for Quadratic Model
Response 3: Tailing of Drug
Table-12: ANOVA for Quadratic Model.

Source	Sum of Squares	Df	Mean Square	F-value	p-value	
Model	0.3134	5	0.0627	32.60	0.0001	Significant
A-Methanol	0.1001	1	0.1001	52.06	0.0002	
B-pH	0.0587	1	0.0587	30.55	0.0009	
AB	0.0093	1	0.0093	4.84	0.0636	
A^2	0.0389	1	0.0389	20.21	0.0028	
B^2	0.0135	1	0.0135	7.01	0.0331	

The Model F-value of 32.60 implies the model is significant. There is only a 0.04% chance that an F-value this large could occur due to noise.

P-values less than 0.0500 indicate model terms are significant. In this case A, AB, A^2, B^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.[30]

Fit Statistics
Table-13: Fit Statistics

Std. Dev.	**0.0439**	**R^2**	**0.9588**
Mean	1.22	**Adjusted R^2**	0.9294
C.V. %	3.58	**Predicted R^2**	0.8149
		Adeq Precision	21.2401

Predicted R^2 of 0.8149 is in reasonable agreement with the Adjusted R^2 of 0.9294; i.e. the difference is less than 0.2.

Adeq Precision measures the signal to noise ratio.[31] A ratio greater than 4 is desirable. Your ratio of 21.240 indicates an adequate signal. This model can be used to navigate the design space.

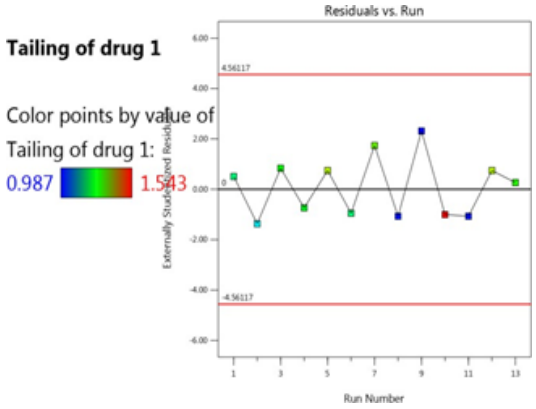

Fig. 7: Residual vs Run Graph for Tailing.

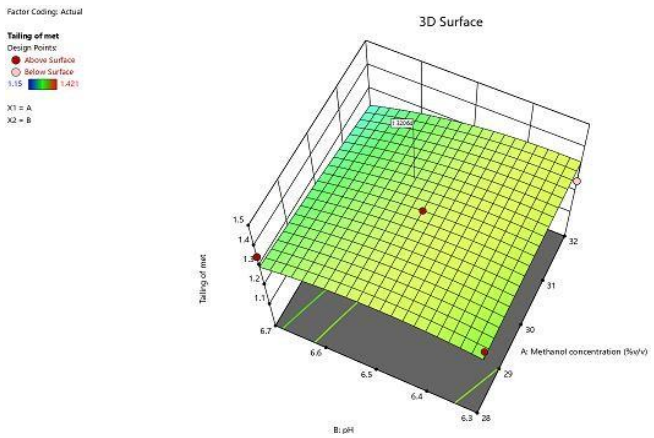

Fig. 8: 3D graph of Clopidogrel and Rosuvastatin for Tailing.

The quadratic Polynomial equation for the measured response (Tailing of Rosuvastatin), Y3 is given below:
Y3 = + 1.37 - 0.0143* A + 0.0033* B - 0.0018*AB +0.0171 A2 + 0.0195* B2

The above polynomial equation represents the quantitative effect of predictor variables (Independent variables) on the measured response (Y2).

A positive sign in front of terms indicates Synergistic effect while a negative sign indicates antagonistic effect upon responses. Methanol concentration has negative effect. pH has Positive effect and pH and Methanol Concentration both combined have negative effect on Tailing of Rosuvastatin.[32]

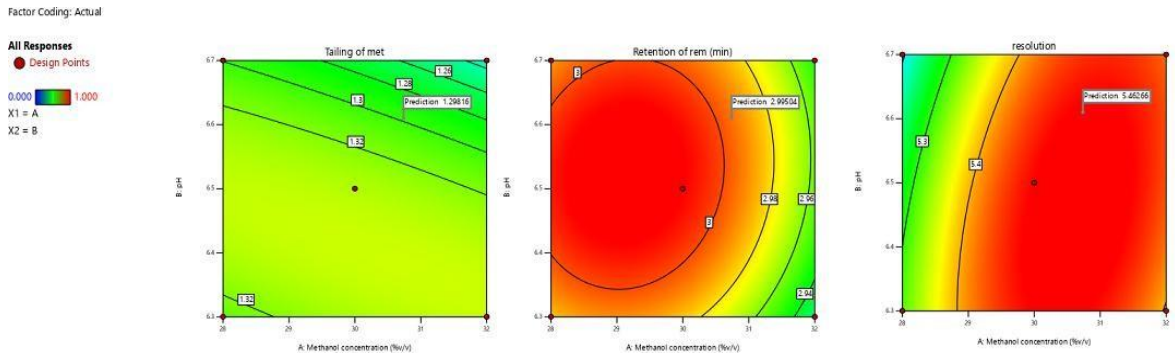

Fig. 9: Desirability Plots for Selected Responses.

Checkpoint Analysis

Check point analysis was performed by taking three random batches from 100 solutions given by DOE Software. Run no. 22, 35 and 98 were selected, from input variables Responses were measured by putting responses coded value in polynomial equation.[33] Predicted and measured values found for selected runs as per Table 14.

Table 14: Check Point Analysis.

Run No.	X1 Methanol Conc.	X3 pH	Y1 (Resolution)		Y2 (Retention Time of Rosuvastatin)		Y3 (Tailing)	
			Predicted	Measured	Predicted	Measured	Predicted	Measured
39	29.83	6.51	5.50	5.670	3.12	3.13	1.19	1.20
55	**30.572**	**6.648**	**5.452**	**4.43**	**2.995**	**3.01**	**1.286**	**1.20**
79	28.011	6.398	5.302	5.35	3.00	3.05	1.332	1.37

When measured Resolution, Retention Time, and Tailing values were compared with Predicted Resolution, Retention Time of Rosuvastatin and Tailing, the values were found significant. Thus, it can be concluded that the obtained mathematical equation is valid for predicted value.

Optimization

An optimization technique using a desirable approach to develop a new method for the desired responses. This was the most important part of the response surface Methodology. The optimum method development was selected based on the criteria of attaining appropriate Resolution, Tailing and the retention time. Overlay plot of responses generates an optimized area as per desired criteria of Resolution 5.50, Tailing 1.20 and the retention time 3.12 min. So, it can be concluded that by adopting a systemic Analytical approach one can reach to an optimum Resolution, Tailing and the retention time.[34-35]

The % Predicted error was calculated using formula
% Predicted error = (Experimental – Predicted) /Predicted *100

Table-15: % Predicted Error Results.

Optimum Condition	pH	Mobile Phase	Resolution	Retention time of Clopidogrel (min)	Tailing of Rosuvastatin (min)
1	6.51	29.83			
	Predicted		**5.50**	**3.12**	**1.19**
	Experimental		**5.670**	**3.13**	**1.20**
	Predicted Error (%)		**3.09**	**0.3**	**1.83**

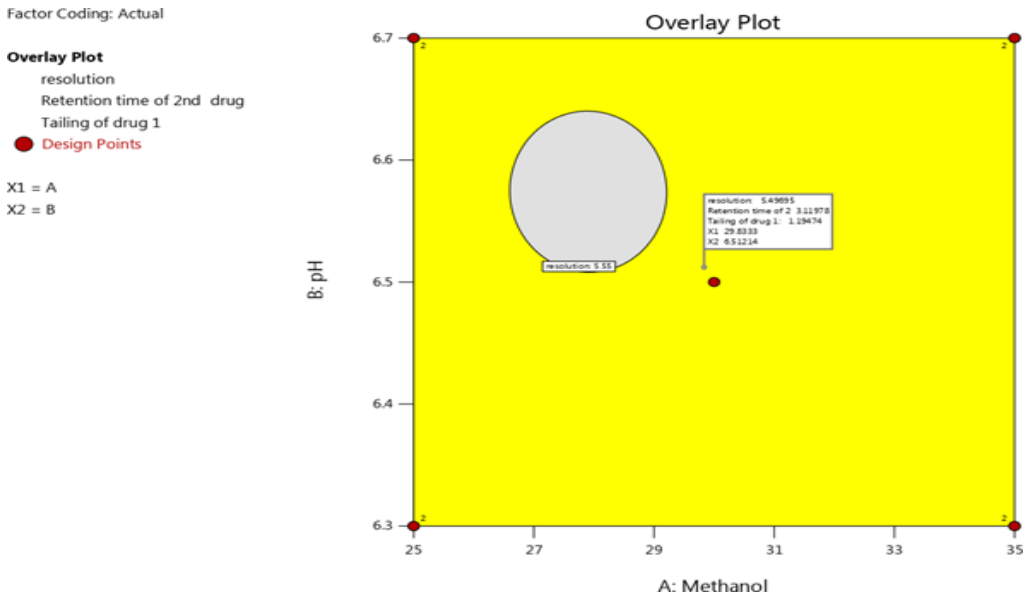

Fig. 10: Overlay Plot for Clopidogrel and Rosuvastatin using DOE.

Optimized Conditions for Clopidogrel and Rosuvastatin

After applying Design of Experiment conditions, optimized RP-HPLC conditions are given in Table 16.

Table-16: Optimized Conditions for Clopidogrel and Rosuvastatin.

Column	Phenomenex Gemini (250mmx4.6mm) 5µm Particle size
Flow rate	1 ml/minute
Detection	246 nm
Column Temperature	Ambient
Injection Volume	20 µl
Runtime	7 minutes
Diluents	Mobile phase
Mobile phase	Methanol : KH2PO4 buffer pH 4.2 (30:70%v/v)

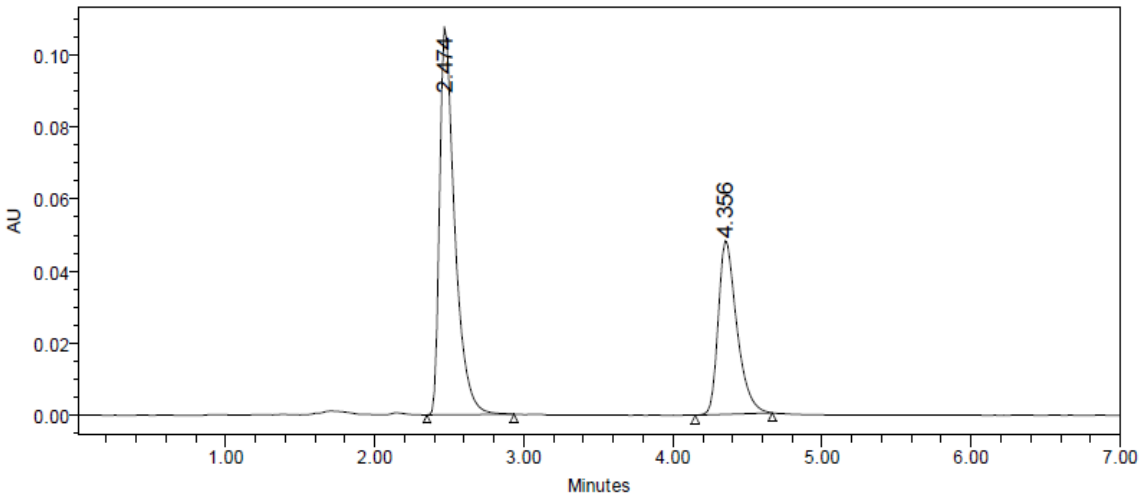

Fig-11: Chromatogram of Clopidogrel and Rosuvastatin in Methanol: buffer (pH 4.2) (30:70) %v/v (Final Trial)

Table 17: Results of Optimized Chromatographic Condition.

S. No.	Peak Name	R_t	Area	Height	USP Resolution	USP Tailing	USP Plate Count
1	Clopidogrel	2.474	8658972	125642	5.28	1.34	6358
2	Rosuvastatin	4.356	536584	46858		1.28	8476

Method Validation Parameters

As per ICH guidelines various Validation parameters are performed.[36]

System Suitability Test

Table 18: Results of System Suitability for Clopidogrel.

S. No.	Name	Rt	Area	Height	USP plate count	USP Tailing
1	Clopidogrel	2.459	8659854	126585	6368	1.34
2	Clopidogrel	2.466	8659745	126985	6392	1.34
3	Clopidogrel	2.472	8652695	125989	6385	1.35
4	Clopidogrel	2.452	8653698	126584	6374	1.34
5	Clopidogrel	2.450	8659875	126532	6328	1.35
Mean			**8657173**			
Std. Dev			**3648.008**			
% RSD			**0.042139**			

Table 19: Results of System Suitability for Rosuvastatin.

S.No.	Name	Rt	Area	Height	USP plate count	USP Tailing	USP Resolution
1	Rosuvastatin	4.322	422674	50988	5949	1.5	3.2
2	Rosuvastatin	4.323	424692	49813	5890.0	1.5	3.3
3	Rosuvastatin	4.342	421255	49826	5952.5	1.4	3.2
4	Rosuvastatin	4.300	415235	51804	5926.4	1.50	3.2
5	Rosuvastatin	4.295	416260	51274	5898.5	1.49	3.2
Mean			**420023.2**				
Std. Dev			**724.7845**				
% RSD			**0.17**				

Specificity

The ICH documents define specificity as the ability to assess unequivocally the analyte in the presence of components that may be expected to be present, such as impurities, degradation products, and matrix components.

Analytical method was tested for specificity to measure accurately quantitates Clopidogrel and Rosuvastatin in drug product.[37]

Assay (Standard)

Table-20: Peak Results For Assay Standard.

S.No.	Name	Rt	Area	Height	USP Resolution	USP Tailing	USP plate count	Injection
1	Clopidogrel	2.456	8675895	126582	5.29	1.34	6359	1
2	Rosuvastatin	4.312	536985	46859		1.28	8462	1
3	Clopidogrel	2.457	8635986	126985	5.28	1.35	6384	2
4	Rosuvastatin	4.308	532648	46358		1.29	8494	2
5	Clopidogrel	2.456	8625984	126354	5.29	1.34	6329	3
6	Rosuvastatin	4.312	534587	46325		1.28	8536	3

Assay (Sample)

Table-21: Peak results for Assay sample.

S.No.	Name	Rt	Area	Height	USP Resolution	USP Tailing	USP plate count	Injection
1	Clopidogrel	2.465	8756895	126585	5.30	1.35	6358	1
2	Rosuvastatin	4.337		46859		1.28	8566	1
3	Clopidogrel	2.474	8754258	126985	5.31	1.34	6397	2
4	Rosuvastatin	4.356		46258		1.29	8534	2
5	Clopidogrel	2.465	8725642	126859	5.30	1.35	6324	3
6	Rosuvastatin	4.337		46256		1.28	8695	3

$$\%ASSAY = \frac{\text{Sample area}}{\text{Standard area}} \times \frac{\text{Weight of standard}}{\text{Dilution of standard}} \times \frac{\text{Dilution of sample}}{\text{Weight of sample}} \times \frac{\text{Purity}}{100} \times \frac{\text{Weight of tablet}}{\text{Label claim}} \times 100$$

The % purity of Clopidogrel and Rosuvastatin in pharmaceutical dosage form was found to be 99.63% and 99.79%.

Linearity
Chromatographic Data for Linearity Study
Table 22: Linearity Data for Clopidogrel.

Concentration µg/ml	Average Peak Area
20	2869587
40	5685225
60	8459858
80	11265886
100	13858985

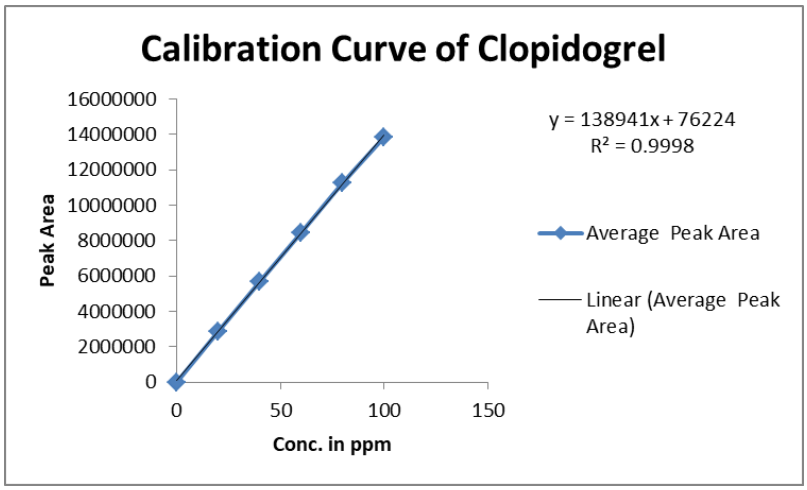

Fig. 12: Calibration Graph for Clopidogrel.

Linearity Plot: The plot of Concentration (x) versus the Average Peak Area (y) data of Clopidogrel is a straight line.
Y = mx + c
Slope (m) = 13894
Intercept (c) = 76224
Correlation Coefficient (r) = 0.999

Validation Criteria: The response linearity is verified if the Correlation Coefficient is 0.99 or greater.

Conclusion: Correlation Coefficient (r) is 0.99, and the intercept is 76224. These values meet the validation criteria.

Table-23: Linearity Data for Rosuvastatin.

Concentration µg/ml	Average Peak Area
40	265867
60	405698
80	536985
100	685685
120	822568

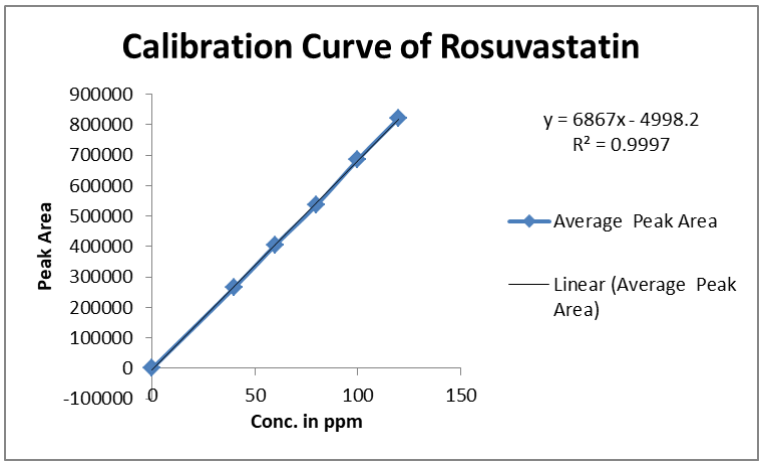

Fig. 13: Calibration Graph for Rosuvastatin.

Linearity Plot: The plot of Concentration (x) versus the Average Peak Area (y) data of Rosuvastatin is a straight line.
Y = mx + c
Slope (m) = 6867
Intercept (c) = 4998
Correlation Coefficient (r) = 0.999

Validation Criteria: The response linearity is verified if the Correlation Coefficient is 0.99 or greater.

Conclusion: Correlation Coefficient (r) is 0.99, and the intercept is 4998. These values meet the validation criteria.

Precision
The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions.[38]

Repeatability
Obtained Five (5) replicates of 100% accuracy solution as per experimental conditions. Recorded the peak areas and calculated % RSD.

Table-24: Results of Repeatability for Clopidogrel.

S.No.	Name	Rt	Area	Height	USP plate count	USP Tailing
1	Clopidogrel	2.453	8658785	125698	6359	1.36
2	Clopidogrel	2.455	8652474	126985	6485	1.35
3	Clopidogrel	2.453	8659865	126587	6459	1.36
4	Clopidogrel	2.452	8659328	125498	6359	1.35
5	Clopidogrel	2.450	8657487	126525	6375	1.36
Mean			**8657588**			
Std. Dev			**2992.003**			
% RSD			**0.034559**			

Table 25: Results of Method Precision for Rosuvastatin.

S.No.	Name	Rt	Area	Height	USP plate count	USP Tailing	USP Resolution
1	Rosuvastatin	4.289	536985	46985	1.29	8548	5.38
2	Rosuvastatin	4.309	534887	46536	1.28	8498	5.39
3	Rosuvastatin	4.306	536588	46365	1.29	8426	5.38
4	Rosuvastatin	4.300	532642	46359	1.28	8425	5.36
5	Rosuvastatin	4.295	536985	46825	1.29	8457	5.38
Mean			**535617.4**				
Std. Dev			**1875.447**				
% RSD			**0.350147**				

Intermediate Precision

Table 26: Results of Intermediate Precision for Clopidogrel.

S. No.	Name	Rt	Area	Height	USP plate count	USP Tailing
1	Clopidogrel	2.465	8758685	136528	6452	1.36
2	Clopidogrel	2.472	8756846	136598	6435	1.38
3	Clopidogrel	2.467	8769852	135264	6435	1.38
4	Clopidogrel	2.466	8745985	136582	6582	1.37
5	Clopidogrel	2.472	8758472	136598	6529	1.36
6	Clopidogrel	3.424	8759864	136582	6547	1.38
Mean			**8758284**			
Std. Dev			**7613.73**			
% RSD			**0.086932**			

Table-27: Results of Intermediate Precision for Rosuvastatin.

S.No.	Name	Rt	Area	Height	USP plate Count	USP Tailing	USP Resolution
1	Rosuvastatin	4.323	548568	47586	8587	1.29	5.30
2	Rosuvastatin	4.343	547854	47568	8569	1.30	5.31
3	Rosuvastatin	4.324	542578	47526	8547	1.29	5.31
4	Rosuvastatin	4.323	542365	47258	8692	1.29	5.30
5	Rosuvastatin	4.342	548752	47895	8567	1.30	5.31
6	Rosuvastatin	4.323	542689	47568	8693	1.31	5.30

Mean			**545467.7**				
Std. Dev			**3218.422**				
% RSD			**0.59003**				

Table-28: Results of Intermediate Precision Day 2 for Clopidogrel

S.No.	Name	Rt	Area	Height	USP plate count	USP Tailing
1	Clopidogrel	2.456	8569853	136598	6298	1.38
2	Clopidogrel	2.457	8579869	135894	6235	1.39
3	Clopidogrel	2.456	8585865	135876	6198	1.38
4	Clopidogrel	2.459	8545852	136589	6258	1.39
5	Clopidogrel	2.467	8549585	135687	6285	1.38
6	Clopidogrel	2.459	8594872	135698	6295	1.39
Mean			**8570983**			
Std. Dev			**19808.27**			
% RSD			**0.231109**			

Table 29: Results of Intermediate Precision for Rosuvastatin.

S. No.	Name	Rt	Area	Height	USP plate Count	USP Tailing	USP Resolution
1	Rosuvastatin	4.312	526985	458655	8365	1.27	5.27
2	Rosuvastatin	4.308	524653	457892	8426	1.28	5.26
3	Rosuvastatin	4.312	526538	456825	8396	1.27	5.27
4	Rosuvastatin	4.322	526985	458624	8345	1.26	5.26
5	Rosuvastatin	4.324	528473	452658	8412	1.26	5.26
6	Rosuvastatin	4.322	524865	452315	8452	1.28	5.27
Mean			**526416.5**				
Std. Dev			**1442.735**				
% RSD			**0.274067**				

Accuracy: Accuracy at different concentrations (50%, 100%, and 150%) was prepared and the % recovery was calculated.[39]

Table 30: The Accuracy Results for Clopidogrel.

% Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	493113.3	30	30.004	100.013%	100.20%
100%	912300.3	60	60.175	100.291%	
150%	1330473	90	90.272	100.302%	

Table 31: The Accuracy Results for Rosuvastatin.

%Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	281726	40	40.298	100.745%	100.25%
100%	554209.7	80	79.978	99.972%	
150%	829292	120	120.036	100.030%	

Limit of Detection
Result: Clopidogrel: = 0.98µg/ml **& Rosuvastatin:** =1.27µg/ml

Limit of Quantitation
Result: Clopidogrel: = 2.94µg/ml **& Rosuvastatin:** = 3.81µg/ml

Robustness
Table 32: Results for Robustness of Clopidogrel.

Parameter used for sample analysis	Peak Area	Retention Time	Theoretical plates	Tailing factor
Actual Flow rate of 1.0 mL/min	8658972	2.474	6358	1.34
Less Flow rate of 0.9 mL/min	9122485	2.741	6587	1.39
More Flow rate of 1.1 mL/min	8587852	2.270	6152	1.35
Less organic phase	8326585	3.266	6258	1.36
More organic phase	8256854	2.147	6354	1.37

Table 33: Results for Robustness of Rosuvastatin.

Parameter used for sample analysis	Peak Area	Retention Time	Theoretical plates	Tailing factor
Actual Flow rate of 1.0 mL/min	536584	4.356	8476	1.28
Less Flow rate of 0.9 mL/min	612548	4.830	8859	1.30
More Flow rate of 1.1 mL/min	546584	3.979	8622	1.29
Less organic phase	526587	6.778	8854	1.31
More organic phase	512586	3.309	8726	1.28

FORCED DEGRADATION STUDIES

The results of the forced degradation studies indicated the specificity of the developed method that has been developed. Clopidogrel Bisulphate and Rosuvastatin Calcium were stable only in alkaline and thermal stress conditions.[40-45] The results of stability studies are given in the following Table-34.

Table 34: Results of Forced Degradation Studies of Clopidogrel Bisulphate and Rosuvastatin Calcium.

Stress Condition	Time(hours)	Assay of Active Substance	Degraded Products	Mass Balance (%)
Basic Hydrolysis (0.1N NaOH)	24Hrs.	96.305	3.695	100.00
Thermal Degradation (60 ^{0}C)	24Hrs.	94.216	5.784	100.00
Acid Hydrolysis (0.1N HCl)	24Hrs.	73.138	26.862	100.00
UV (254nm)	24Hrs.	72.255	27.745	100.00
3% Hydrogen Peroxide	24Hrs.	70.131	29.869	100.00

SUMMARY AND CONCLUSION

QbD assisted RP-HPLC and Stability indicating methods were developed for the estimation of some newer drugs which are used to prevent heart attack, angina, and stroke. For this study, two combinational drugs namely Clopidogrel Bisulfate and Rosuvastatin Calcium were selected for analytical Method Development. RP-HPLC methods were developed for the estimation of Clopidogrel Bisulfate and Rosuvastatin Calcium in bulk powder as well as in marketed formulations. In RP-HPLC method, C18 column was found to be most suitable for the drugs under study. The newly developed HPLC method was validated for specificity, linearity, accuracy, method precision, intra-day and inter-day precision, limit of detection and limit of quantification. Newly developed QbD assisted RP-HPLC methods are simple, accurate, sensitive, specific, precise, reproducible, and can be used for the routine analysis of respective drugs in bulk as well as in bulk and marketed pharmaceutical dosage forms in an economic Manner as they do not require any sample pre-treatment and costly solvents. QbD assisted method development is more economical than conventional RP-HPLC method Development.

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