Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

Analytical Method Development and Validation of an HPLC Method for Related Substances Analysis of Clopidogrel Impurity C in Rosuvastatin and Clopidogrel Tablets

Alexandar Sekar
Vinayaka mission's College of pharmacy, Vinayaka mission's research foundation (deemed to be university)
Nivetha G
SNS College of Pharmacy and Health Sciences
Karishma Jeevanandham
Vinayaka mission's College of pharmacy, Vinayaka mission's research foundation (deemed to be university)
Nithya S
Vinayaka mission's College of pharmacy, Vinayaka mission's research foundation (deemed to be university)
Pasupathi C
Vinayaka mission's College of pharmacy, Vinayaka mission's research foundation (deemed to be university)
Nallakumar P
SNS College of Pharmacy and Health Sciences
Ravisankar M
Srinivasan College of pharmaceutical sciences
Karthick Raja S
drkarthickrajaselvaraj@gmail.com

Saveetha University

Research Article

Keywords: Clopidogrel Impurity C, HPLC, Related Substances, Method Validation, Rosuvastatin, Pharmaceutical Analysis

Posted Date: November 24th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-11486570/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

Additional Declarations: No competing interests reported.

Analytical Method Development and Validation of an HPLC Method for Related Substances Analysis of Clopidogrel Impurity C in Rosuvastatin and Clopidogrel Tablets

S.Alexandar[a], G.Nivetha [b], J.Karishma [c], S.Nithya [d], C.Pasupathi [e], P.Nallakumar[f], M.Ravisankar[g] and S.Karthick Raja*[h]

[a] Associate Professor, Vinayaka mission's College of pharmacy, Vinayaka mission's research foundation (Deemed to be university), Salem, India. alexmpharm@gmail.com.

[b] Assistant Professor, SNS College of Pharmacy and Health Sciences, The Tamilnadu DR.MGR Medical University, Coimbatore, India. nivetha2003g@gmail.com.

[c] Assistant Professor Vinayaka mission's College of pharmacy, Vinayaka mission's research foundation (Deemed to be university), Salem, India. karishmakanishka99@gmail.com.

[d] Assistant Professor, Vinayaka mission's College of pharmacy, Vinayaka mission's research foundation (Deemed to be university), Salem, India. nithyasekar9711@gmail.com.

[e] Assistant Professor, Vinayaka mission's College of pharmacy, Vinayaka mission's research foundation (Deemed to be university), Salem, India. pasup1077@gmail.com.

[f] Professor, SNS College of Pharmacy and Health Sciences, The Tamilnadu DR.MGR Medical University, Coimbatore, India. nallakumarponnuswamy@gmail.com.

[g] Professor, Srinivasan College of pharmaceutical sciences, The Tamilnadu DR.MGR Medical University, Trichy, Tamilnadu, India. pharmaravi18@gmail.com.

[h] Research Scholar, Saveetha Medical college and Hospital, Department of Pharmacology, Saveetha Institute of Medical and Technical Sciences, Chennai, Tamilnadu. drkarthickrajaselvaraj@gmail.com.

Address Correspondence to:

S.Karthick Raja* Pharm.D Lecturer.

Department of Pharmacology,

SIMATS, Chennai, Tamilnadu

Mobile : 9994573352

Mail Id : drkarthickrajaselvaraj@gmail.com

Abstract

Objective

The goal of this study is to develop and validate an accurate High Performance Liquid Chromatography method to determine Clopidogrel Impurity C in the related substance analysis of Rosuvastatin and Clopidogrel tablet formulations.

Methods

We used an ULTRON ES-OVM Chiral (150 mm × 4.6 mm, 5 μm) column to separate the substances. The mobile phase was a mix of KH2P04 buffer and ACN in a ratio of 77:23. We set the flow rate at 1.0 mL/min. Used UV detection at 220 nm. We validated the method according to ICH guidelines for parameters like linearity, specificity, LOD, LOQ, precision, accuracy, robustness, solution stability and filter compatibility.

Results

The method showed excellent specificity without interference from blank, placebo, or related impurities. Theoretical plates numbered 3818, and the tailing factor was 0.856. The correlation coefficient was 1.0000 for Clopidogrel Impurity C and 0.99998 for Clopidogrel. Recovery values varied from 98.5% to 100.9%. The method proved precise, robust, and suitable for routine quality control testing.

Conclusion

The High Performance Liquid Chromatography method we established for determining Clopidogrel Impurity C in Rosuvastatin and Clopidogrel Tablets was demonstrated to be highly sensitive, accurate, precise and reliable.

Keywords

Clopidogrel Impurity C, HPLC, Related Substances, Method Validation, Rosuvastatin, Pharmaceutical Analysis

Introduction:

Cardiovascular disease is an underlying cause of illness and death all over the world, and it is a major problem in public health. Cardiovascular disorders such as coronary artery disease, atherosclerosis and acute coronary syndrome lead to a worldwide increase in the prescription of combination drugs that target disease progression and prevention of ischemic cardiovascular events [1, 2].

Among these, Rosuvastatin and Clopidogrel combination therapy has emerged as the standard of care in reducing risk for patients with high cardiovascular risk, since Rosuvastatin inhibit cholesterol biosynthesis by targeting HMG-CoA reductase and slows the progression of atherosclerosis, while Clopidogrel has long been established as the antiplatelet agent in preventing thrombotic events [3, 4].

Impurities in a drug substance are defined as any component of the drug substance other than that to which it purports to be. For drugs as specified in general chapters on drug substances or drug products, an impurity should be identified, qualified, and quantified. ICH Q3A (R2) guidance for impurities in drug substance and ICH Q3B (R2) guidance for impurities in new drug product [5, 6] require that all substantial impurities need to be controlled and even minimal quantities of the impurities could affect the drug stability, effectiveness, or safety, hence it necessary to develop proper method to quantify the amount of impurity present in any formulation and to detect impurities is essential for ensuring pharmaceutical quality and regulatory compliance.

Clopidogrel is a chiral thienopyridine derivative with a stereogenic center and exists as optical isomers. The marketed version contains the active (S)-enantiomer, while Clopidogrel

Impurity C is the (R)-enantiomer, considered an undesired stereoisomeric impurity [7, 8]. Since enantiomers have similar physicochemical properties, separating and measuring them pose significant analytical challenges. Accurately identifying and controlling Clopidogrel Impurity C is crucial for ensuring product quality and regulatory compliance.

Several analytical methods exist for determining Rosuvastatin and Clopidogrel in pharmaceutical formulations. Harshana Rothekar et al. (2017) and Pooja Pisal et al. (2018) created RP-HPLC methods to estimate these drugs in combined dosage forms [9, 10]. Harshal Kanubhai Trivedi and Mukesh C. Patel (2012) and Jinesh Bahubali Nagavi and Bannimath Gurupadayya (2014) reported stability-indicating chromatographic methods for Rosuvastatin and Clopidogrel, respectively [11, 12]. These methods showed satisfactory analytical performance for assays and impurity analysis. However, there is limited information on the selective determination of Clopidogrel Impurity C, the (R)-enantiomer, in combination tablets using a chiral chromatographic approach.

Thus, this study aims to develop and validate a selective, sensitive, precise, accurate and robust HPLC method for determining Clopidogrel Impurity C in Rosuvastatin and Clopidogrel tablet formulations using an ULTRON ES-OVM chiral column.

Drug profile:

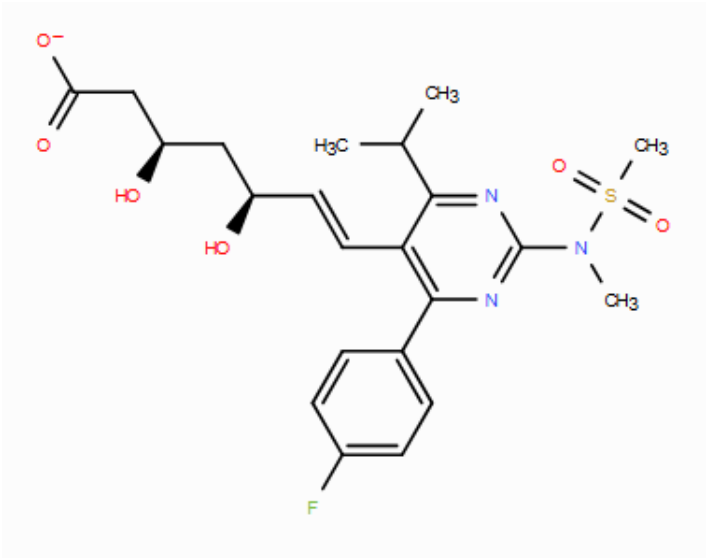

Fig. 1: Chemical structure of Rosuvastatin Calcium.

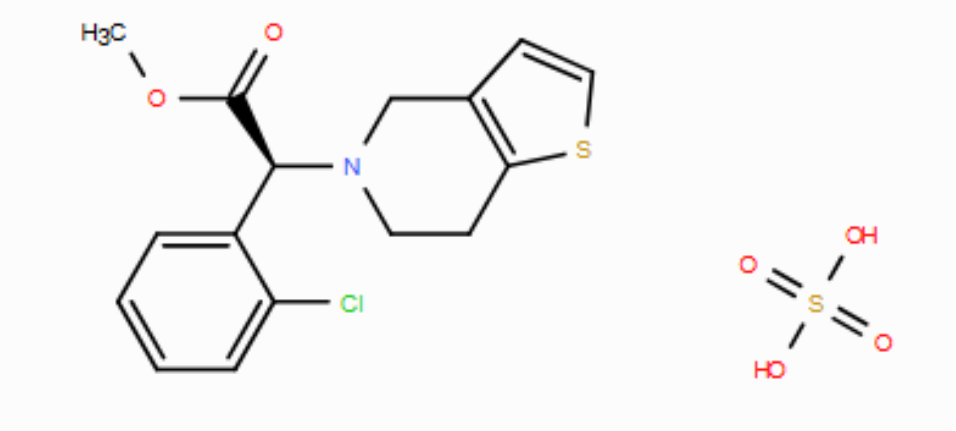

Fig 2: Chemical structure of Clopidogrel Bisulfate.

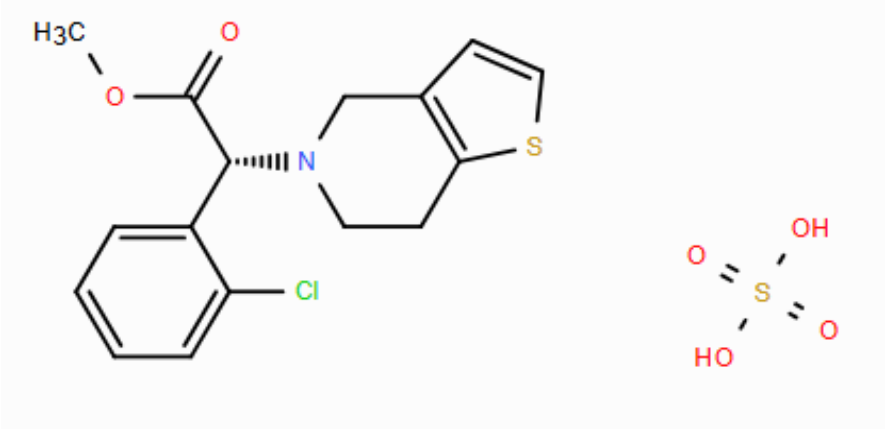

Fig 3: Chemical structure of Impurity C.

Materials & Methods:

Chemicals and Reagents

Clopidogrel Bisulphate working standard, Clopidogrel Impurity A, Clopidogrel Impurity B, and Clopidogrel Impurity C working standards were sourced from qualified suppliers.

Potassium dihydrogen phosphate, acetonitrile, methanol, and HPLC-grade purified water were used throughout the study. Rosuvastatin and Clopidogrel tablets were utilised for method development and validation.

Instrumentation

Analysis was carried out with a High Performance Liquid Chromatography system coupled with both a Photodiode Array (PDA) detector and Ultraviolet (UV) detector (Shimadzu LC-2050C 3D and LC-2050C, Shimadzu, Japan). Data acquisition and processing used validated chromatography software. Chromatographic analysis relied on both UV and photodiode array detectors. The UV detector focused on quantitative analysis during validation studies, including system suitability, linearity, accuracy, precision, and sensitivity evaluation. The PDA detector assessed specificity and forced degradation studies to check peak purity and confirm the stability-indicating ability of the developed method. Method development and sample preparation were assisted by a Sartorius Quintix-125D-1IN analytical balance and a PC 700 Eutech Instruments pH meter. In studies that looked at specificity and how things break down, an ULTRON ES-OVM Chiral column with dimensions 150 mm by 4.6 mm and a particle size of 5 micrometres, or a similar column was used.

Chromatographic Conditions:

The process of chromatographic separation was conducted using a specific ULTRON ES-OVM Chiral column measuring 150 mm in length and 4.6 mm in diameter, with a particle size of 5 µm. The mobile phase consisted of a mixture of KH2P04 buffer & ACN in a volumetric ratio of 77:23. For Buffer preparation, dissolve 1.36 g KH2P04 (Potassium dihydrogen phosphate) in water, and elution was performed isocratically at a flow rate of 1.0 mL/min. Detection of compounds was achieved through UV absorption at a wavelength of 220nm. The temperature of the chromatographic column was rigorously controlled at 30°C,

while the autosampler temperature was maintained at 15°C. A sample injection volume of 10 µL was used, and the entire chromatographic process lasted for a duration of 20 minutes.

Preparation of Buffer, Mobile Phase, and Diluent:

The buffer solution was formulated through the dissolution of 1.36 grams of KH2P04 in 1000 millilitres of deionized water, followed by thorough mixing. Subsequently, the mobile phase was concocted by combining the buffer solution with acetonitrile in a volumetric ratio of 77:23. Prior to utilization, the mobile phase underwent filtration and sonication procedures to eliminate any entrapped gases. This prepared mobile phase served as the diluting agent consistently throughout the experimental investigation.

Preparation of Standard, Impurity, and Sample Solutions:

Rosuvastatin Standard Solution: A solution of Rosuvastatin was prepared by accurately weighing approximately 40 mg of Rosuvastatin Calcium working standard and placing it into a 200 mL volumetric flask. The compound was dissolved in 5 mL of methanol and subjected to sonication for complete dissolution. The flask was then filled to the mark with a suitable diluent and mixed to reach a final conc. of 200 µg/ml.

Clopidogrel Standard Solution: For the Clopidogrel Standard Solution, around 37.5 mg of Clopidogrel bisulfate working standard was weighed and placed in a 100 mL volumetric flask. The substance was dissolved in 70 mL of methanol, followed by sonication to ensure complete dissolution. The flask was then topped up to the calibration mark with methanol, thoroughly mixed, and 2 mL of this solution was further diluted to 100 mL with a diluent, resulting in a conc. of 7.5µg/ml

System Suitability Solution employed in the analysis comprised the Clopidogrel Standard Solution.

Impurity Stock Solutions:

Preparation of Clopidogrel Impurity A stock solution:

Regarding the Impurity Stock Solutions, the preparation of Clopidogrel Impurity A stock solution involved weighing approximately 5 mg of Clopidogrel Impurity A and 100 mg of Clopidogrel standard in a 50 mL volumetric flask. The substances were dissolved in 10 mL of methanol and sonicated for 10 minutes to ensure complete dissolution. The solution was then adjusted to volume with methanol, thoroughly mixed, and further diluted by transferring 6 mL of this solution to a 100 mL volumetric flask with diluent, resulting in a concentration of 6µ/ml.

Preparation of Clopidogrel Impurity B and C stock solution:

The process of preparing stock solutions of Clopidogrel Impurity B and Clopidogrel Impurity C involves weighing approximately 5 mg of the respective impurity in a 100 mL volumetric flask, dissolving it in 10 mL of methanol, sonication for complete dissolution, dilution with additional methanol, and further dilution with a diluent to achieve concentrations of 2.5 µ/ml and 8µ/ml, respectively.

Placebo Solution and Sample Solution:

For the placebo solution, about 365 mg of powdered placebo is weighed in a 200 mL volumetric flask, dissolved in 5 mL of methanol, sonicated, diluted to volume with diluent, hand-shaken, and filtered through a 0.45µ Nylon membrane filter. Similarly, for the sample solution, an equivalent of 75.0 mg of Clopidogrel from crushed tablets is prepared similarly as the placebo solution.

Method Validation:

The validation of the method was conducted following the ICH Q2 (R2) guidelines for determining Clopidogrel Impurity C in Rosuvastatin and Clopidogrel tablets. Validation parameters included such as system suitability, precision, linearity, accuracy, robustness,

specificity, Limit of Detection (LOD), Limit of Quantification (LOQ), LOQ Precision, solution stability, and filter compatibility have been demonstrated.

System suitability was determined by injecting blank and standard solution replicates to evaluate key parameters.

Specificity was verified by assessing potential interferences at the retention time of Clopidogrel and Clopidogrel Impurity C.

LOD and LOQ were calculated using residual standard deviation based on calibration data at low concentrations.

LOQ Precision was evaluated by determining % Relative Standard Deviation of responses from multiple injections.

Precision was assessed by examining method precision through the analysis of six separate sample preparations conducted under identical analytical conditions, and intermediate precision through the analysis of six distinct sample preparations performed by a different analyst on a separate day.

Linearity was verified within the range spanning from the limit of quantification (LOQ) to 150% of the specified concentration level. Calibration curves plotted peak area against concentration.

Accuracy was obtained through recovery studies at LOQ, 50%, 100%, and 150% concentration levels by spiking known amounts of Clopidogrel Impurity C into placebo preparations. **Solution Stability** was analyzed standard and sample solutions at appropriate time intervals, monitoring the changes in percentage purity or relative percentage purity compared to fresh solutions, to assess stability.

Filter compatibility was assessed through the comparison of impurity outcomes derived from unfiltered (centrifuged) samples with those filtered through 0.45 µm nylon and 0.45 µm PVDF filters.

Robustness was evaluated by minor modifications in chromatographic conditions, including variations in wavelength, flow rate, and column temperature.

RESULTS AND DISCUSSION:

Method Development and Optimisation:

We started method development based on the related substances method described in the United States Pharmacopoeia for Clopidogrel tablets.

Trial 1

First, we carried out chromatographic separation using a mobile phase buffer-acetonitrile ratio (v/v) 75:25, wherein Clopidogrel Impurity C was eluting very early and without resolving a sharp peak, which was not appropriate for quantifying.

Trial 2

In order to enhance retention, we reduced the organic composition in the mobile phase (80:20 buffer-acetonitrile (v/v)) wherein retention was enhanced and peak separation was improved but the peak shape was not sharp, therefore, mobile phase was further modified.

Trial 3

We attempted a further optimisation with mobile phase buffer-acetonitrile ratio (77:23 v/v) which resulting in a favourable balance between the resolution, retention, and peak shape of all analytes.

Trial 4

We performed experiments using optimum mobile phase by increasing column temperature from 25 degree to 30 degree centigrade; the improved peak symmetry and efficiency could be

seen and resolution was fairly resolved. The increase in temperature enhances peak symmetry, retention reproducibility and chromatographic efficiency.

Trial 5

The flow rate was augmented from 1.0 mL/min to 1.2 mL/min to determine any impact on the chromatographic performance. While the retention time was decreased, the efficiency of the chromatography declined marginally. The higher flow rate was thus not utilized.

We made various attempts by trying different mobile phases, column temperature and flow rates to get proper resolution between Clopidogrel impurity C and Clopidogrel and some other related impurities. According to these trials the most suitable conditions for the estimation were found with an ULTRON ES-OVM Chiral column by using mobile phase having KH_2P0_4 Buffer & Acetonitrile in the ratio 77:23 (v/v) respectively, a flow rate 1.0 mL/min and column temperature 30° C gave well-formed peaks, resolution, and retentions. We accepted the final operating parameters (1.0mL/min, 30 °C, and 77:23 v/v mobile phase ratio) and validation parameters were found to comply with acceptance criteria. Under these optimized settings, Clopidogrel Impurity C eluted at around 10 minutes with good peak symmetry, theoretical plates greater than 2000, and a tailing factor under 2.0. All system suitability criteria were met, confirming the method was suitable for validation.

Optimized Chromatographic Conditions:

From the optimization results, we selected the final chromatographic conditions:

• Column: ULTRON ES-OVM Chiral (150 mm × 4.6 mm, 5 µm)

• Mobile Phase: Buffer: Acetonitrile (77:23, v/v)

• Flow Rate: 1.0 mL/min

• Detection Wavelength: 220 nm

• Column Temperature: 30°C

• Injection Volume: 10 µL

• Run Time: 20 min

Under these optimized conditions, Clopidogrel Impurity C eluted at around 10 minutes with good peak symmetry, theoretical plates exceeding 2000, and a tailing factor below 2.0. All system suitability criteria were met, confirming the method was suitable for validation.

Validation of the method:

Specificity:

This developed method specificity was determined by analysing samples of Blank, placebo, Clopidogrel standard solution, individual impurity standards such as Impurity A, B, and C, spike solutions and test solutions. The absence of any interfering peaks in blank and placebo solutions at Clopidogrel Impurity C retention time shows that this developed chiral RP-HPLC method was selective. The retention times for Impurity A, Impurity B, Clopidogrel, and Impurity C were approximately 2.64, 4.67, 5.17, and 10.33 min, respectively. Adequate chromatographic resolution was ensured between Clopidogrel Impurity C and all related substances, which confirms the selectivity of the developed chiral method

Peak purity analysis using the PDA detector showed purity index values greater than 0.950 for Clopidogrel and Clopidogrel Impurity C in all tested solutions. The purity values ranged from 0.971 to 1.000, confirming no co-eluting peaks and demonstrating the spectral purity of

the analyte peaks. Hence, , it is affirmed that established method is specific and it can accurately determine Clopidogrel Impurity C in the presence of formulation excipients and related impurities. Acceptance Criteria: No interference with peak from blank or placebo with reference at the Rt of impurity. Peak purity >0.950.

Table 1: Specificity results:

S.No	Sample ID	Peak Name	Retention time	Peak Purity index
1	Blank	Blank peak	Refer chromatogram	Not applicable
2	Impurity C standard	Impurity C	10.41	0.999
3	Standard Preparation	Clopidogrel	5.97	1.000
4	Placebo	Placebo peak	Refer chromatogram	Not applicable
5	Impurity A WS	Impurity A	2.63	1.000
6	Impurity B WS	Enantiomer-1	4.76	0.999
		Enantiomer-2	6.66	0.999
7	Impurity C WS	Impurity C	10.42	0.999
8	Clopidogrel WS + Impurity A WS	Impurity A	2.65	1.000
		Clopidogrel	5.61	1.000
9	Rosuvastatin WS	Rosuvastatin	5.41	Not applicable
10	Clopidogrel WS	Clopidogrel	5.28	1.000
11	Placebo + Clopidogrel WS + Impurity A WS + Impurity B WS +	Impurity A	2.64	1.000
		Impurity B	4.67	0.999
		Clopidogrel	5.17	1.000

	Impurity C WS	Impurity C	10.33	0.999
12	Test Solution	Impurity A	2.65	0.999
		Impurity B	4.28	1.000
		Clopidogrel	5.37	0.971
		Impurity C	10.45	0.976

Acceptance criteria:

i) There should not be any interference due to Blank and placebo peak with analyte.

ii) Peak purity should not be less than 0.950 according to Lab Solution Software.

Results and Conclusion:

The Blank and Placebo peaks are not interfere with retention time of Impurity C peak in test solution and Peak purity passes within specified limits. Hence method is selective and specific.

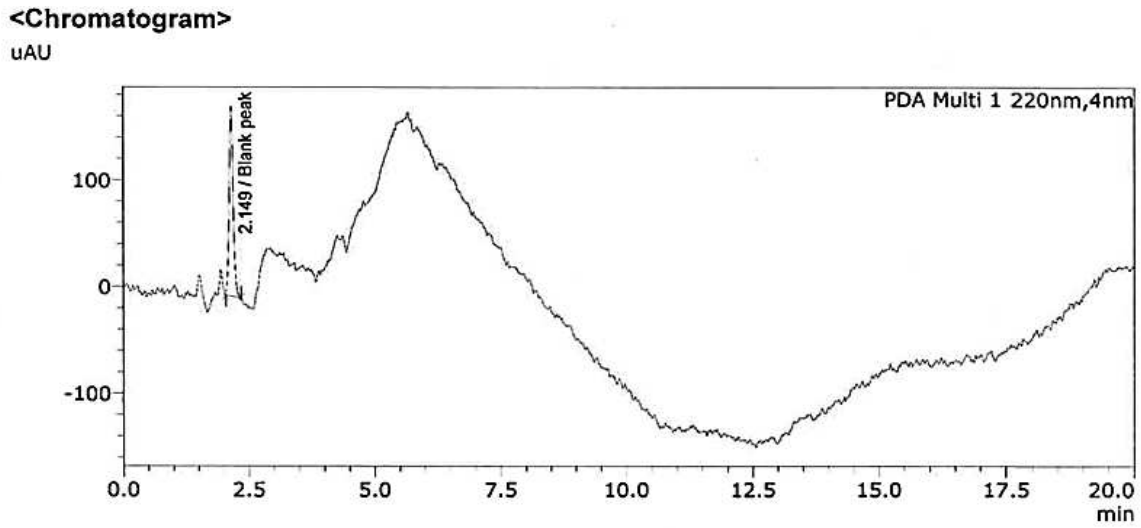

Fig. 4: Chromatogram of Blank (Specificity)

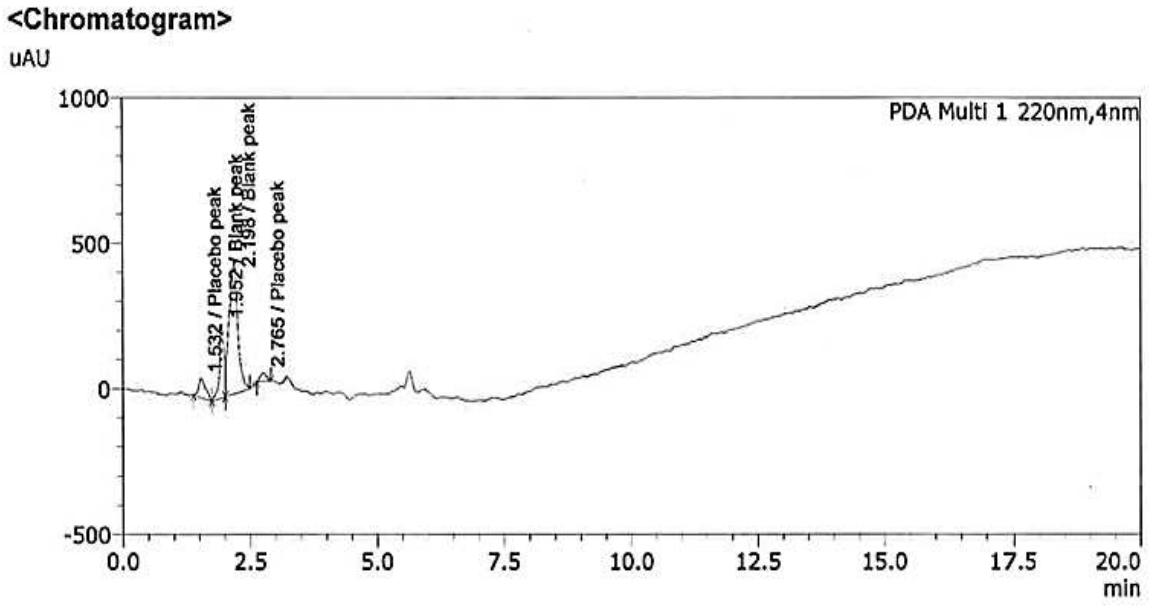

Fig. 5: Chromatogram of Plain Placebo (Specificity)

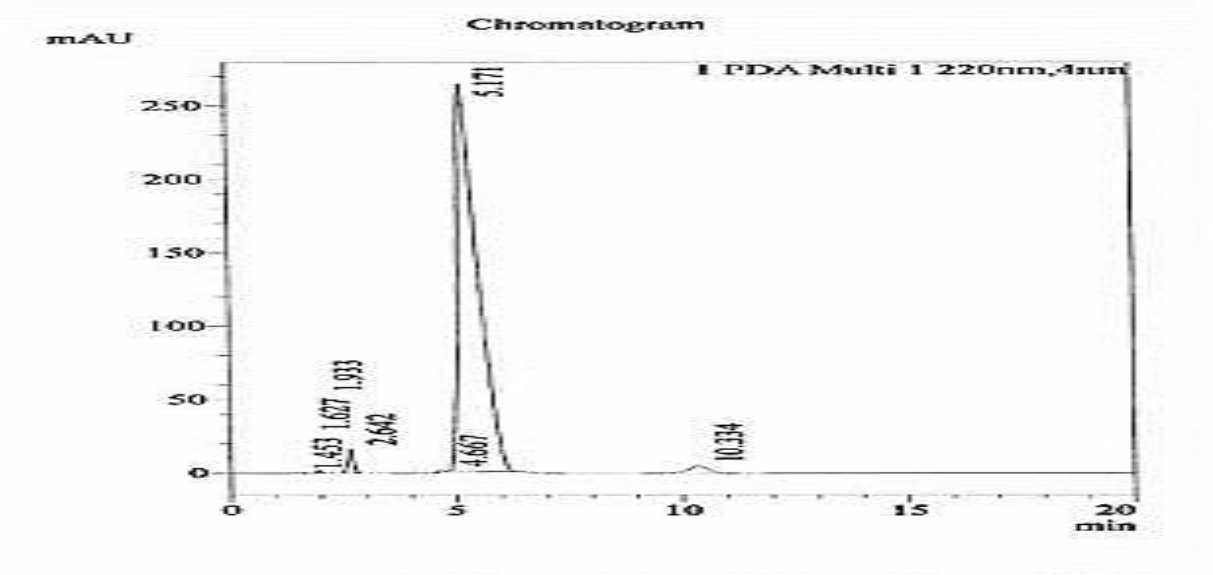

Fig. 6: Specificity Chromatogram of Clopidogrel in presence of Placebo and Impurity standards (A, B, and C)

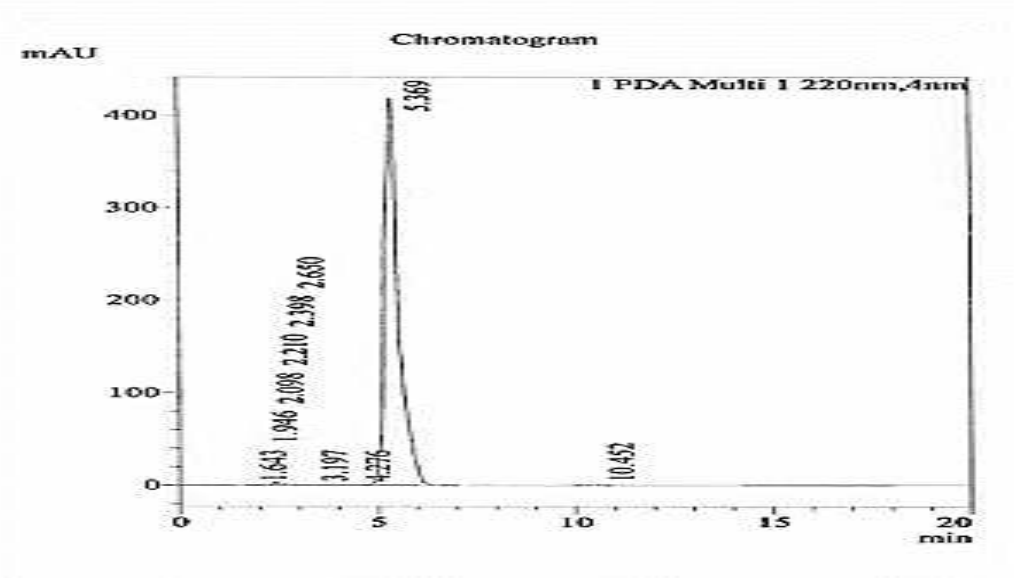

Fig. 7: Chromatogram of Test sample (Specificity)

Limit of Detection (LOD) and Limit of Quantitation (LOQ):

A sensitivity study was conducted utilising the residual standard deviation method to establish the LOD and LOQ for Clopidogrel Impurity C and Clopidogrel. The calibration curves were plotted by using a number of low level concentrations by slope & standard error obtained by regression of calibration plot. LOD and LOQ for both Clopidogrel Impurity C and Clopidogrel have been calculated using the values obtained by regression of calibration plot, Slope and Standard error obtained from the plots.

LOD & LOQ of Clopidogrel Impurity C: SLOPE= 16162; STANDARD ERROR= 261; LOD = 0.053 ppm; LOQ = 0.161 ppm

LOD & LOQ of Clopidogrel: SLOPE= 18290; STANDARD ERROR= 407; LOD = 0.073 ppm; LOQ=0.223ppm.

It shows that observed & calculated values were equal for both the analyte & therefore calculation were done correct. The low values obtained for LOD and LOQ suggest a high

sensitivity of the developed method for analysis of trace amounts of Clopidogrel Impurity C and Clopidogrel.

Table 2: LOD and LOQ Results

Analyte	Slope	Standard Error	LOD (ppm)	LOQ (ppm)
Clopidogrel Impurity C	16162	261	0.053	0.161
Clopidogrel	18290	407	0.073	0.223

Acceptance Criteria: The determination of limits of detection (LOD) and quantification (LOQ) by employing the residual standard deviation technique on the calibration dataset, and report the observed concentrations.

Result: LOD and LOQ of Clopidogrel Impurity C was 0.053ppm and 0.161ppm, respectively. LOD and LOQ of Clopidogrel was 0.073ppm and 0.223ppm, respectively. So, the proposed method possesses sufficient sensitivity to perform regular analysis for the related substances.

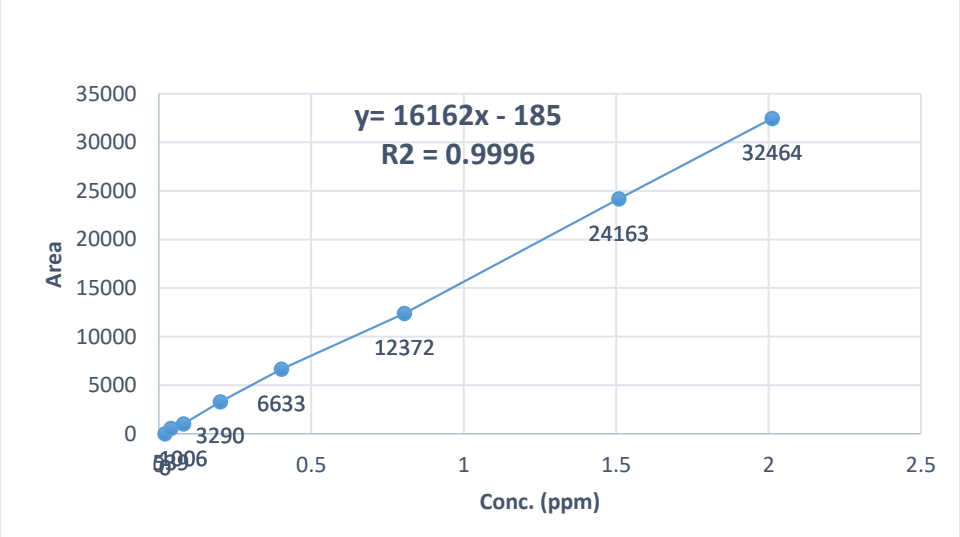

Fig. 8: Calibration curve used for determination of LOD and LOQ of Clopidogrel Impurity C.

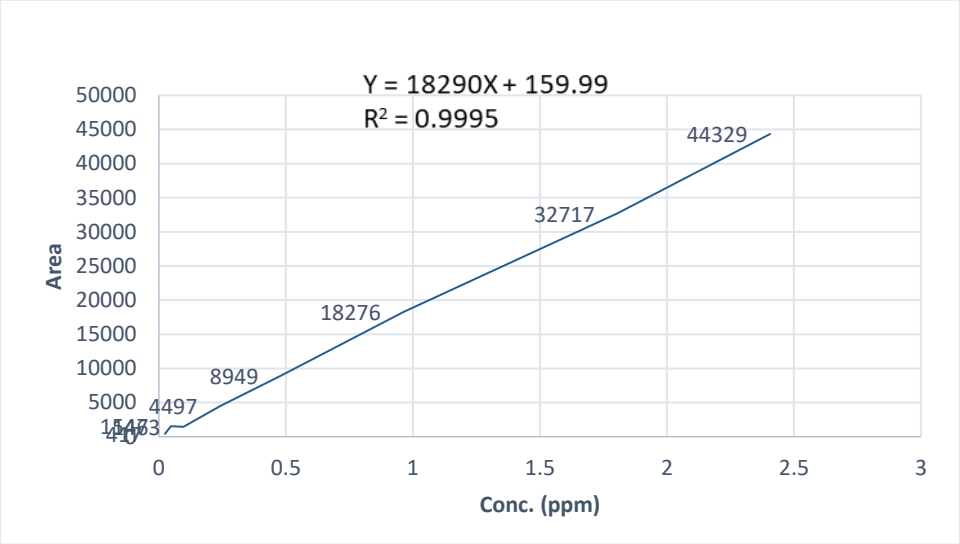

Fig. 9: Calibration curve used for determination of LOD and LOQ of Clopidogrel.

Precision at the Limit of Quantitation (LOQ):

We determined the precision at LOQ concentration by analyzing 6 replicate injection of Clopidogrel Impurity C and Clopidogrel solutions in LOQ concentration. We recorded the retention times and peak areas and calculated the % relative standard deviation for repeatability at quantification limit.

Table 3: LOQ Precision Results

Analyte	**Mean Retention Time (min)**	**Mean Peak Area**	**%RSD of Retention Time**	**%RSD of Peak Area**
Clopidogrel Impurity C	9.99	2419	0.390	7.508
Clopidogrel	5.68	4138	0.414	3.491

The %RSD values for peak areas were 7.508% for Clopidogrel Impurity C and 3.491% for Clopidogrel, both within the acceptance criterion of not exceeding 10.0%. These results show acceptable precision and reproducibility of the developed method at the quantitation limit, confirming its suitability for trace-level quantification of Clopidogrel Impurity C and Clopidogrel.

Acceptance Criterion: It was stipulated that the %Relative Standard Deviation for peak areas from six replicate injections at the LOQ level should not exceed 10.0%.

Precision:

Precision assessment involved the evaluation of repeatability (method precision) and intermediate precision of the developed method. We checked the method precision with the six independent sample preparations. We determined the intermediate precision with the help of different analysts on different day on the same analytical procedure.

Table 4: Precision Results for Clopidogrel Impurity C

Parameter	**Mean (%)**	**%RSD**
Method Precision	0.37	1.49
Intermediate Precision	0.33	7.98
Overall Precision (Cumulative)	0.35	7.59

The method precision study resulted in a mean impurity level of 0.37% with a %RSD of 1.49%, showing excellent repeatability of the analytical procedure. Intermediate precision, assessed under different conditions, gave a mean impurity level of 0.33% with a %RSD of 7.98%. The cumulative precision data showed a %RSD of 7.59%, indicating good agreement between analysts and analysis days.

We found that all the %RSD values were within the accepted limits as per specified criteria, and the proposed method possesses excellent precision and reproducible accuracy in the quantification of Clopidogrel Impurity C in pharmaceutical dosage forms.

Acceptance Criteria: The %RSD for impurities above 0.1% should not exceed 15.0%. The cumulative %RSD from method precision and intermediate precision studies should also meet this limit.

Linearity:

The linearity of the developed method for Clopidogrel Impurity C and Clopidogrel was determined for the range of concentrations from LOQ level to 150 % target concentration. A calibration curve was constructed by plotting the mean peak area against concentration. This plot, accompanied by its calculated average linear regression and slope, served the purpose of establishing the linearity of the relationship between mean peak area and concentration.

Table 5: Linearity Results

Analyte	**Concentration Range (ppm)**	**Slope**	**Intercept**	**Correlation Coefficient (r)**	**Coefficient of Determination (R^2)**
Clopidogrel Impurity C	0.161-12.072	16260	-471.49	1.00000	1.0000
Clopidogrel	0.230-11.520	18189	-204.80	0.99998	1.0000

A good linear response was observed for Clopidogrel Impurity C over the concentration range of 0.161-12.072 ppm with a correlation coefficient of 1.00000 and R value 1.0000. Similarly, Clopidogrel demonstrated linearity in the concentration range of 0.230-11.520 ppm with a correlation coefficient of 0.99998 and R2 value of 1.0000.

The calculated correlation coefficients were within the accepted limit of 0.990, demonstrating a good linear correlation between the concentration levels and the response of the detector. Consequently, the devised methodology is deemed suitable for estimation of Clopidogrel Impurity C and Clopidogrel in the concentration range as mentioned above.

Acceptance Criterion: The coefficient of determination (R^2) should not be less than 0.990.

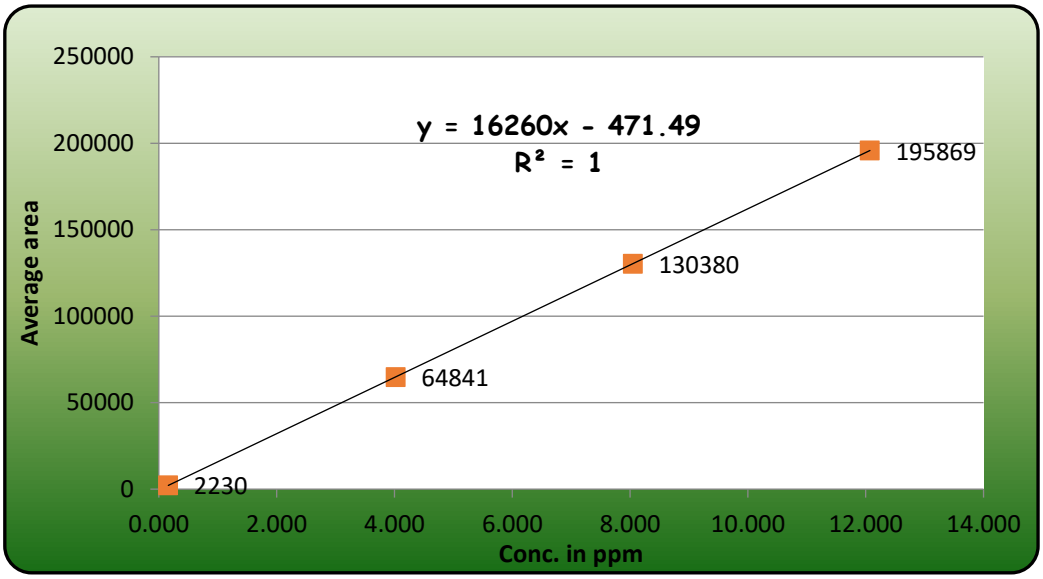

Fig. 10 Linearity Graph for Clopidogrel Impurity C

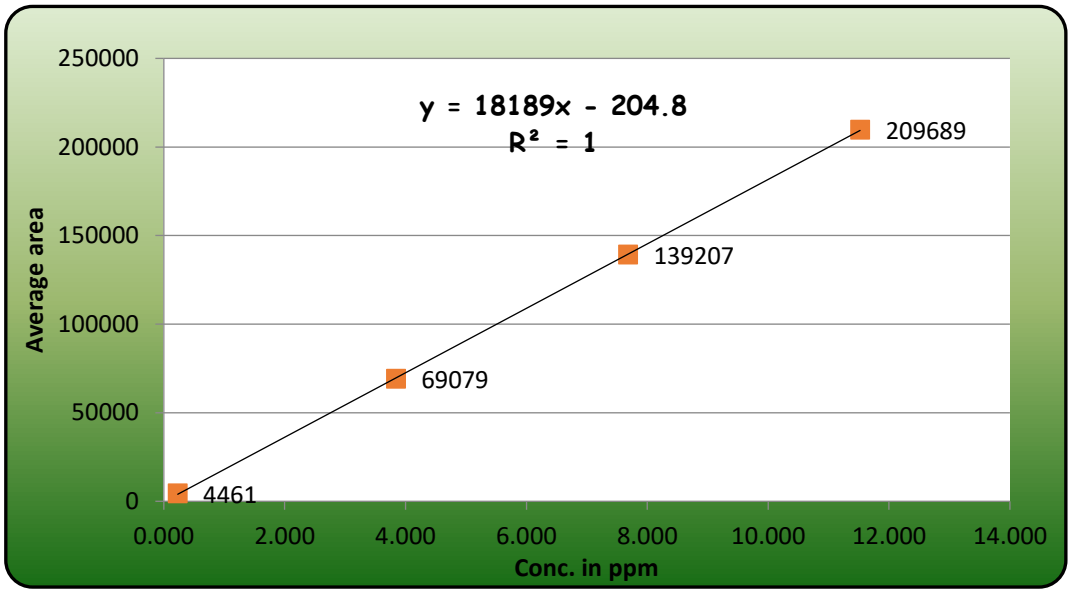

Fig no.11: Linearity Graph for Clopidogrel

Accuracy:

The method's accuracy was assessed by examining the retrieval of Clopidogrel Impurity C at various concentration levels, including LOQ, 50%, 100%, and 150% of the target concentration. This evaluation involved the generation of three sample replicates at each concentration level and the subsequent determination of the mean percentage recovery.

Table no.6: Accuracy Results

Level	**Mean Recovery (%)**	**%RSD**
LOQ	99.0	1.846
50%	98.5	0.777
100%	99.5	0.338
150%	100.9	0.206

The mean recovery values at LOQ, 50%, 100% and 150% were 99.0%, 98.5%, 99.5%, 100.9%, respectively. The %RSD values at all the concentration levels were less than 2.0%, which indicates that the recoveries are consistent and precise. All recovery values are within the acceptance range 90.0-110.0%. The method showed good accuracy.

Acceptance Criterion: The average percentage recovery at each level should be within the range of 90.0%-110.0%.

Stability of Analytical Solutions:

Stability study was performed on standard and sample solution under refrigerated conditions (15 °C). Percentage changes of peak area of the solution were observed compared with its initial value at an interval of 24 hours The results are presented in the Table.

Table no.7 Stability of Analytical Solutions:

Time (h)	Standard Solution (% change)	Sample Solution (% change)
0	0.00	0.00
2	-0.06	1.40
4	-0.48	1.15
6	-0.46	2.36
8	0.12	4.23
10	-0.38	6.23
12	0.05	6.63
16	-1.24	8.18
20	-0.68	12.39
24	-0.15	13.90

Minimal changes in peak area were observed for the standard solution, while the sample solution shows some drift over a study period of 24 h. The sample solution demonstrated stability for 16 hours when stored under refrigerated conditions.

The values of percent change at each interval are within the acceptable limit of ±10%, which shows the stability of the analytical solution.

Acceptance Criterion: The % change in peak area should be within ±10% of the initial value.

Filter Study:

The effect of filtration was evaluated on 0.45 m nylon and PVDF membrane filters and compared to the centrifuged sample which was kept unfiltered. The percentage difference in the impurity content was evaluated.

Table no.8 Filter Study Results

Filter Type	Sample Area	Impurity C (%)	% Difference from Unfiltered
Unfiltered (Centrifuged)	30786	0.39	Not applicable
Nylon Filter	30657	0.39	0.22
PVDF Filter	31427	0.40	2.08

The percent difference between the filtered and unfiltered samples remained within the acceptance limits. No significant loss of Clopidogrel Impurity C was noted. Consequently, either nylon or PVDF filters may be utilized.

Acceptance Criteria:

- NMT 15% for impurities >0.1%
- NMT 20% for trace impurities (0.05-0.1%)

Robustness:

The robustness of the established method was evaluated by making slight adjustments to the chromatographic conditions, including wavelength (± 3 nm), flow rate (± 0.1 mL/min), and column oven temperature (± 5°C). The results obtained are presented below.

Table no. 9 Robustness Study:

Condition	Impurity C%	Tailing Factor	Theoretical Plates	%RSD
217 nm	0.42	0.89	3264	3.87
223 nm	0.43	0.89	3276	0.37
0.9 mL/ min	0.42	0.88	3545	0.21
1.1 ml/min	0.44	0.89	3045	0.40
25°C	0.45	0.90	3035	0.15
35°C	0.46	0.89	3498	0.32

System suitability parameters (theoretical plates, tailing factor) did not significantly change when the above conditions were varied within the specified limits. %Relative Standard Deviation was found within the limits i.e. Well within 5.0% with unaffected peak shape and efficiency.

Acceptance Criteria:

• %RSD: No more than 5.0%

• Tailing factor: No more than 2.0

• Theoretical plates: Not less than 2000

• No significant change in system suitability parameters

CONCLUSION:

A simple, fast, accurate, precise, and reliable chiral RP-HPLC method has been developed and validated for the quantification of Clopidogrel Impurity C in pharmaceutical formulations, adhering to the International Council for Harmonisation Q2 (R2) guidelines.

Method has demonstrated specificity with no interference of placebo and related substances. A good linear relationship was observed between the concentration of Clopidogrel Impurity C and response. The method has good accuracy with percentage recoveries at the levels of LOQ, 50%, 100% and 150% ranging from 98.5% to 100.9%.

The method is sensitive as it exhibited low values for LOD and LOQ. The analytical solutions were stable over the entire period of study. Filter study showed no significant loss of analyte during filtration. Robustness studies revealed that minor changes in analytical parameters did not have significant impact on the method performance. Thus, the proposed method is very convenient for routine analysis of Clopidogrel Impurity C.

References:

1. World Health Organization. Cardiovascular diseases (CVDs). Geneva: WHO; Available from: https://www.who.int

2. Tsao CW et al., Heart Disease and Stroke Statistics-2023 Update: A Report From the American Heart Association, *Circulation,* Feb 21; 147 (8):e93-e621, (2023). doi: 10.1161/CIR.0000000000001123. Epub Jan 25.

3. Fergus McTaggart, Comparative pharmacology of Rosuvastatin, *Atheroscler Suppl.*, Mar; 4 (1):9-14, (2003) doi: 10.1016/s1567-5688(03)00004-7.

4. Pierre Savi and Jean-Marc Herbert, Clopidogrel and ticlopidine: P2Y12 adenosine diphosphate-receptor antagonists for the prevention of atherothrombosis*, Semin Thromb Hemost*, Apr; 31(2):174-83, (2005). doi: 10.1055/s-2005-869523.

5. International Council for Harmonisation (ICH). Q3A (R2): Impurities in new drug substances. Geneva: ICH, (2006).

6. International Council for Harmonisation (ICH). Q3B (R2): Impurities in new drug products. Geneva: ICH; (2006).

7. United States Pharmacopeia (2019). USP Monographs, Clopidogrel Bisulfate. USP-NF. Rockville, MD: United States Pharmacopeia. DOI: https://doi.org/10.31003/USPNF_M18680_02_01

8. Jean-Marie Pereillo, Structure and Stereochemistry of the Active Metabolite of Clopidogrel, *Drug Metabolism and Disposition: the Biological Fate of Chemicals,* November, 30(11):1288-1295, (2002). DOI:10.1124/dmd.30.11.1288

9. Rothekar H et al., Development and validation of RP-HPLC method for simultaneous estimation of Rosuvastatin Calcium and Clopidogrel Bisulphate in pharmaceutical dosage forms, *Asian Journal of Pharmaceutical Analysis,* May, 5(12):475-482, (2017) DOI:10.13140/RG.2.2.10087.19361.

10. Pisal P et al., Stability-indicating RP-HPLC method for simultaneous determination of Aspirin, Rosuvastatin and Clopidogrel in pharmaceutical dosage forms*, International Journal of Pharmacy and Pharmaceutical Sciences*, Vol 10, Issue 10, ISSN: 2656-0097, (2018).

11. Trivedi HK, Patel MC, Stability-indicating RP-UPLC method for determination of Rosuvastatin and related substances in pharmaceutical dosage forms, *Scientia Pharmaceutica,* Mar 26;80(2):393–406, (2012). doi: 10.3797/scipharm.1201-09.

12. Nagavi JB, Gurupadayya BM, Stability-indicating RP-HPLC method for estimation of Clopidogrel Bisulphate, *International Journal of PharmTech Research*, 4(6), ISSN: 2249-3387, (2014).

Competing Interests (Conflict of Interest):"Declaration of competing interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper."

Consent to publish declaration: “Not applicable"

Consent to Participate declaration: “Not applicable"

Clinical Trial: Not Applicable

Funding/Acknowledgments: "This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors."

CRediT Author Statement: Briefly define the individual contributions of each co-author using standard roles

Conceptualization: J.Karishma, S.Nithya , C.Pasupathi and P.Nallakumar

Methodology: M.Ravisankar, & S.Karthick Raja

Writing – Original draft: S.Alexandar, G.Nivetha

Data Availability: All data generated or analysed during this study are included in this published article

Ethics Approval (if applicable): Not Applicable