Development and Validation of Stability indicating HPLC Method for Determination of Nirogacestat in Bulk and Pharmaceutical Dosage Form

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ABSTRACT:
A simple, rapid, precise, sensitive and reproducible reverse phase high performance liquid chromatography (HPLC) method has been developed for the quantitative analysis of Nirogacestat in pharmaceutical dosage form. Chromatographic separation of Nirogacestat was achieved on Waters alliance e-2695 HPLC, by using Symmetry shield RP-18 (150 x 4.6 mm, 3.5 µ) column and the mobile phase containing 1.15 gms ammonium acetate is dissolved in 1lt of HPLC water pH-3.0/OPA & ACN in the ratio of 70:30 % v/v. The flow rate was 1.0 mL/min; detection was carried out by absorption at 269 nm using a photodiode array detector at ambient temperature. The number of theoretical plates and tailing factor for Nirogacestat were NLT 2000 and should not more than 2 respectively. % Relative standard deviation of peak areas of all measurements always less than 2.0. The percentage degradation observed for acid, alkali, peroxide, reduction, thermal, photolytic, and hydrolysis conditions were 10.2 %, 12.4 %, 14.1 %, 8.6 %, 1.2 %, 0.8 %, and 1.7 %, respectively. The proposed method was validated according to ICH Q2(R1) guidelines. The method was found to be simple, economical, suitable, precise, accurate & robust method for quantitative analysis of Nirogacestat.

Key words: HPLC, Nirogacestat, Degradation studies, ICH Guidelines.

Introduction

Nirogacestat, a selective γ-secretase inhibitor, has garnered significant attention in recent years due to its potential as a novel treatment for desmoid tumors. The molecular mechanism of Nirogacestat is centered around its ability to inhibit the γ-secretase enzyme, which plays a crucial role in the Notch signaling pathway [1]. Dysregulation of Notch signaling is known to contribute to the development and progression of desmoid tumors, making it an attractive target for therapeutic intervention.

Several studies have explored the efficacy of Nirogacestat in desmoid tumors. The Phase 3 DeFi study demonstrated the drug's ability to significantly reduce tumor size and improve progression-free survival compared to placebo [2]. This large-scale trial supported Nirogacestat's effectiveness in treating patients with progressing desmoid tumors, particularly those with advanced or unresectable tumors. Furthermore, a study by Kummar et al. [3] highlighted the pathway to approval for Nirogacestat as the first FDA-approved treatment for desmoid tumors, addressing a significant unmet medical need.

The safety profile of Nirogacestat has been closely examined in multiple studies, with a focus on potential side effects such as ovarian toxicity. Loggers et al. [4] reported that ovarian toxicity was a notable adverse event in female patients, particularly those of reproductive age, but these effects were generally reversible upon discontinuation of the drug. In pediatric populations, Nirogacestat has also demonstrated a favorable safety profile, with manageable side effects observed [5]. These findings suggest that the drug can be safely administered to a diverse patient population, including children and young adults.

In addition to its use in desmoid tumors, Nirogacestat's application in other cancers is being actively investigated. Shearer et al. [6] examined its potential in combination therapies for multiple myeloma, where it was found to enhance the expression of B-cell maturation antigen (BCMA) on plasma cells. Chemical structure of nirogacestat is shown in Figure1.

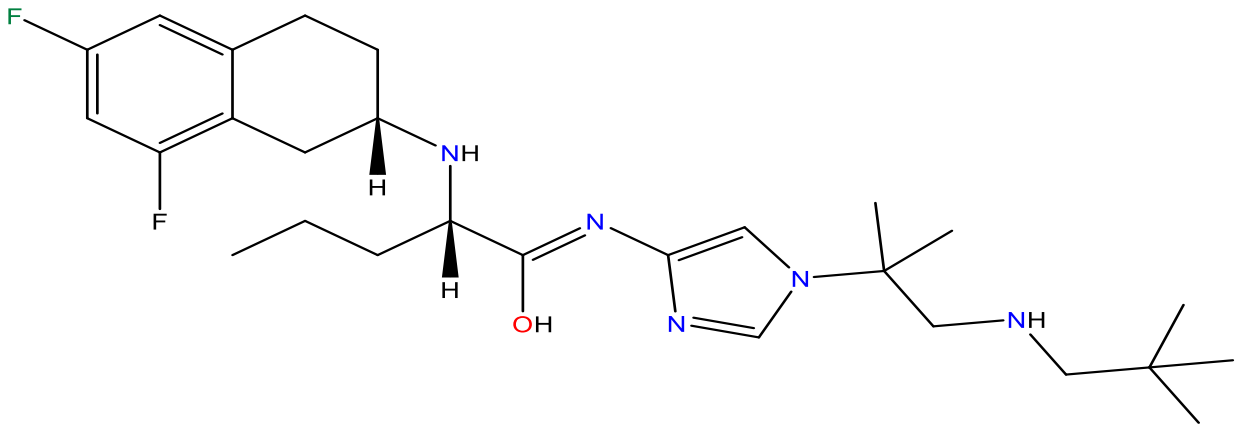


Figure 1: Chemical structure of Nirogacestat

The role of Nirogacestat in treating desmoid tumors with APC mutations, which are common in this disease, was highlighted in a study by Kasper et al. [7]. Research has also extended to the drug's broader applicability in combination with other therapies. For example, Campos and Kasper [8] examined the potential of combining Nirogacestat with other systemic treatments for desmoid tumors. Their findings suggest that the drug may enhance the effectiveness of existing therapies, contributing to better overall patient outcomes.

Moreover, the chemical synthesis of Nirogacestat has been extensively studied to improve its pharmacokinetic properties. Shen et al. [9] reviewed the chemical synthesis and drug development process of Nirogacestat, providing insights into how its formulation and delivery can be optimized for better patient outcomes.The comprehensive literature review indicates that, to date, no HPLC analytical method has been established for the quantification of Nirogacestat. As a result, there is a considerable need for the development of a simple, robust, accurate, and efficient method for its estimation. High-Performance Liquid Chromatography (HPLC) is recognized as a highly effective and sensitive technique for the separation, identification, and quantification of compounds.

MATERIAL AND METHODS

The HPLC System of model acquity manufacturer Water e2695 ALLIANCE. The analytes were separated on Symmetry Shield RP-18 column (150 × 4.6 mm, 3.5 µm column, and the mobile phase containing 1.15 g ammonium acetate dissolved in 1 L of HPLC grade water, pH -3.0/OPA and ACN in the ratio of 70:30 %v/v. The flow rate was maintained as 1 mL/min and injection volume was 10 µL. The run time was set as 5 min. HPLC grade water and acetonitrile (ACN, HPLC grade) were used throughout the analysis.

Determination of Working Wavelength (λ max):

The wavelength of maximum absorption of the solution of the drug in mixture of Acetonitrile and Ammonium acetate pH-3.0/OPA (30:70) were scanned using PDA Detector within the wavelength region of 200–400 nm against Acetonitrile and Ammonium acetate pH-3.0/OPA (30:70) as blank. The absorption curve shows maximum absorbance at 269 nm. Thus 269 nm was selected as detector wavelength for the HPLC chromatographic method. PDA Spectrum of nirogacestat is shown in figure 2.

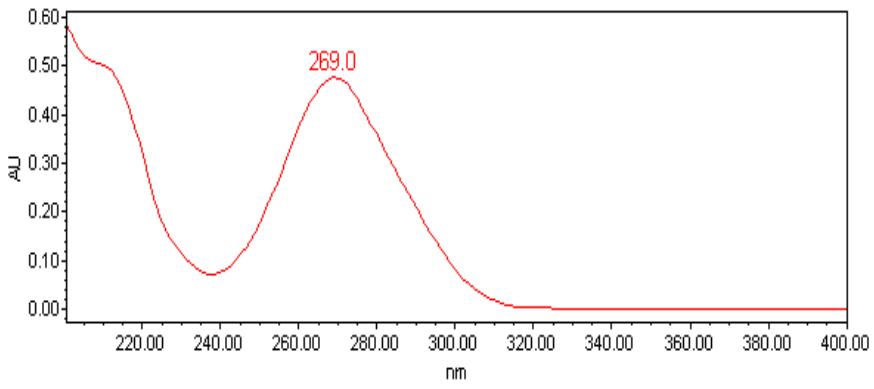


Figure 2: PDA Spectrum of nirogacestat

Chromatographic conditions:
During the selection of chromatographic conditions, numbers of trails were carried out and the best trail was selected for optimized method. Optimization of chromatographic conditions are shown in Table 1.

Preparation of standard stock &Sample solution:
Weigh 8 mg of Nirogacestat working standard and dissolve in diluent to make a 10 mL stock solution. For the sample, weigh 28 mg of Nirogacestat, dissolve in diluent, sonicate, centrifuge, and filter to make a 10 mL stock solution. Pipette 1mL of each stock solution into a 10mL flask and dilute to 80 ppm. Filter the sample solution before dilution.

Table 1. Trails in optimization of chromatographic conditions

Trails	Column	Mobile phase	Wavelength (nm)	Flow Rate (mL/min)	Injector Volume (µl)	Run time (min)	Observation
Trail 1	Inertsil ODS (250 mm×4.6 mm,5 µ).	ACN ＋ 0.1% Formic acid (80:20)	200 - 400 nm	1mL/min	10 µL	10 min	System is sutability conditions are not with in limit
Trail 2	Inertsil ODS (250 mm × 4.6 mm, 5 µ).	ACN ＋ 0.1% Formic acid (70:30)	269 nm	1mL/min	10 µL	10 min	Unknown peak is observed.
Trail 3	Inertsil ODS (250 mm × 4.6 mm, 5 µ).	ACN ＋ 0.1% Formic acid (65:35)	269 nm	1mL/min	10 µL	10 min	Retention time is not with in the limit.
Trail 4	Symmetry shield RP-18 (150 × 4.6 mm, 3.5 µ).	Acetonitrile and ammonium acetate PH-3.0/OPA (50:50).	269 nm	1mL/min	10 µL	6 min	Base line is not good
Trail 5	Symmetry shield RP-18 (150 × 4.6 mm, 3.5 µ).	Acetonitrile and ammonium acetate PH-3.0/OPA(40:60).	269 nm	1mL/min	10 µL	10 min	Respone of the peak is very high.
Trail 6	Symmetry shield RP-18 (150 × 4.6 mm, 3.5 µ).	Acetonitrile and ammonium acetate PH-3.0/OPA(30:70).	269 nm	1mL/min	10 µL	5 min	This method is suitable for validation.

The Nirogacestat peak was observed at 2.782 min with peak area 2981428, tailing factor 0.69 and the USP plate count was 18878. Eventually this trial was optimized.

General preparations
Preparation of Ammonium acetate buffer:
Take 1.15 g of Ammonium acetate dissolved in 1litre of HPLC grade water and pH-3.0 adjusted with OPA. Filter through 0.45 µ nylon filter.

Preparation of Mobile Phase:
Mobile phase was prepared by mixing Ammonium acetate pH-3.0/OPA and ACN taken in the ratio 70:30. It was filtered through 0.45 µ membrane filter to remove the impurities which may interfere in the final chromatogram.

Preparation of diluent: Acetonitrile is used as diluent.

Preparation of standard stock and Sample Solution
Weigh 8 mg of Nirogacestat working standard and dissolve in diluent to make a 10 mL stock solution. For the sample, weigh 28 mg of Nirogacestat, dissolve in diluent, sonicate, centrifuge, and filter to make a 10 mL stock solution. Pipette 1mL of each stock solution into a 10 mL flask and dilute to 80 ppm. Filter the sample solution before dilution.

Procedure:
Inject 10 µL of the standard, sample into the chromatographic system and measure the areas for Nirogacestat peak and calculate the % Assay by using the formula.

SYSTEM SUITABILITY:
Tailing factor for the peak due to Nirogacestat in Standard solution should not be more than 2.0. Theoretical plates for the Nirogacestat peak in Standard solution should not be less than 2000.
Formula for Assay:

$$\%\ Assay = \frac{AT}{AS} * \frac{WS}{DS} * \frac{DT}{WT} * \frac{Average\ weight}{Label\ Claim} * \frac{P}{100} * 100$$

Where,

AT = Average area counts of test (sample) preparation
AS = Average area counts of standard preparation.
WS = Weight of working standard taken in mg.
DS = Dilution of working standard in mL.
DT = Dilution of test (sample) in mL.
WT = Weight of test (sample) taken in mg.

METHOD VALIDATION SUMMARY:
Specificity:
Specificity of an analytical method is ability to measure specifically the analyte of interest without interference from blank and known impurities. For this purpose blank chromatogram, standard chromatogram and sample chromatogram were recorded. The chromatogram of blank shows no response at the retention times of drugs which confirms the response of drug was specific.

LINEARITY:
Preparation of stock solution:
Accurately weigh and transfer 8mg of Nirogacestat working standard into a 10 mL clean dry volumetric flask add diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)
Level – I :0.25 mL (20.0 ppm of Nirogacestat)
Level – II :0.50 mL (40.0 ppm of Nirogacestat)
Level – III:0.75 mL (60.0 ppm of Nirogacestat)
Level – IV:1.00 mL (80.0 ppm of Nirogacestat)
Level –V:1.25 mL (100.0 ppm of Nirogacestat)
Level – VI:1.50 mL (120.0 ppm of Nirogacestat)
Inject each level into the HPLC 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.

Range:
The Range of an analytical method is the interval between the upper and lower levels of analyte (including these levels) that have been demonstrated with precision, accuracy and linearity

Acceptance Criteria:
Correlation coefficient should be not less than 0.999.

Preparation Accuracy Sample solutions:
For preparation of 50 % solution (With respect to target Assay concentration):
Accurately weigh and transfer 14 mg of Nirogacestat sample into a 10 mL clean dry volumetric flask add diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 1mL of the above stock solutions into a 10 mL volumetric flask and dilute up to the mark with diluent. (40 ppm of Nirogacestat).

For preparation of 100 % solution (With respect to target Assay concentration):
Accurately weigh and transfer 28 mg of Nirogacestat Sample into a 10 mL clean dry volumetric flask add diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 1 mL of the above stock solutions into a 10 mL volumetric flask and dilute up to the mark with diluent. (80 ppm of Nirogacestat)

For preparation of 150 % solution (With respect to target Assay concentration):
Accurately weigh and transfer 42 mg of Nirogacestat sample into a 10 mL clean dry volumetric flask add diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 1 mL of the above stock solutions into a 10 mL volumetric flask and dilute up to the mark with diluent. (120 ppm of Nirogacestat).

Procedure:
Inject the standard solution, Accuracy - 50 %, Accuracy -100 % and Accuracy -150 % solutions.

Acceptance Criteria:
The % Recovery for each level should be between 98.0 to 102.0 %

Precision
Precision is the degree of repeatability of an analytical method under normal operation conditions. Precision is of 3 types
1. System precision 2. Method precision
3. Intermediate precision (a. Intra-day precision, b. Inter day precision)
System precision is checked by using standard chemical substance to ensure that the analytical system is working properly. In this peak area and % of drug of six determinations is measured and % RSD should be calculated.
In method precision, a homogenous sample of single batch should be analyzed 6 times. This indicates whether a method is giving constant results for a single batch. In this analyze the sample six times and calculate the % RSD. The precision of the instrument was checked by repeatedly injecting (n = 6) solutions.

Acceptance Criteria:
The % RSD for the absorbance of six replicate injections results should not be more than 2 %.

ROBUSTNESS:
As part of the Robustness, deliberate change in the Flow rate, Mobile Phase composition, Temperature Variation was made to evaluate the impact on the method.

A. The flow rate was varied at 0.9 mL/min to 1.1 mL/min.
Standard solution 80 ppm of Nirogacestat was prepared and analysed using the varied flow rates along with method flow rate. On evaluation of the above results, it can be concluded that the variation in flow rate affected the method significantly. Hence it indicates that the method is robust even by change in the flow rate ± 10 %.

B. The variation of Organic Phase ratio.
Standard solution of 80 ppm of Nirogacestat was prepared and analyzed using the varied in mobile phase ratio.

Limit of detection (LOD) and limit of quantification (LOQ):
The limit of detection (LOD) limit of quantification (LOQ) of the drug carry was calculated using the following equation as per international conference harmonization (ICH)guidelines.
$LOD = 3.3 \times \sigma / S$, $LOQ = 10 \times \sigma / S$
LOD for Nirogacestat was found to be 0.48 µg/mL and LOQ for Nirogacestat was found to be 1.60 µg/mL.
Acceptance Criteria: s/n value for LOD is 3 and LOQ is 10.

DEGRADATION STUDIES:
Preparation of stock:
Accurately weigh and transfer 28 mg of Nirogacestat sample into a 10 mL clean dry volumetric flask add Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Acid degradation
Pipette 1 mL of above solution into a 10 mL volumetric flask and 1 mL of 1N HCl was added. Then, the volumetric flask was kept at 60 °C for 1 hour and then neutralized with 1 N NaOH and make up to 10 mL with diluent. Filter the solution with 0.45 microns syringe filters and place in vials.

Alkali degradation
Pipette 1 mL of above solution into a 10 mL volumetric flask and add 1mL of 1N NaOH was added. Then, the volumetric flask was kept at 60 ºC for 1 hour and then neutralized with 1N HCl and make up to 10 mL with diluent. Filter the solution with 0.45 microns syringe filters and place in vials.

Peroxide degradation
Pipette 1 mL above stock solution was added to a 10 mL vacuum flask, 1 mL of 10 percent w/v hydrogen peroxide was added to the flask and the volume was built up to the mark using diluent. The vacuum flask was then maintained at 60 ºC for 1 hour. After that, the vacuum flask was left at room temperature for 15 minutes. Filter the solution using 0.45 micron syringe filters and transfer to bottles.

Reduction degradation
Pipette 1 mL above stock solution was added to a 10 mL vacuum flask, 1 mL of 10 percent w/v Sodium bisulphite was added to the flask and the volume was built up to the mark using diluent. The vacuum flask was then maintained at 60 ºC for 1 hour. After that, the vacuum flask was left at room temperature for 15 minutes. Filter the solution using 0.45 micron syringe filters and transfer to bottles.

Hydrolysis degradation
Pipette 1mL of above-stock solution was added to a 10 mL vacuum flask, 1mL of HPLC grade water was added to a flask and the volume was built up to the required volume with diluent. The vacuum flask was then maintained at 60 ºC for 1 hour. After that, the vacuum flask was left at room temperature for 15 minutes. Filter the solution using 0.45 micron syringe filters and transfer to bottles.

Photolytic degradation
Nirogacestat sample was placed in photo stability chamber for 3 hours. Then the sample was taken and diluted with diluents and injected into HPLC and analyzed.

Thermal degradation
Nirogacestat sample was taken in petridish and kept in Hot air oven at 105 ºC for 3 hours. Then the sample was taken and diluted with diluents and injected into HPLC and analysed.

RESULTS AND DISCUSSION
In the optimization of chromatographic conditions for the HPLC method, six trials were conducted to achieve optimal parameters. Initial trials presented variations in retention time, area, USP tailing, and plate count. Trial-1 showed a retention time of 1.162 minutes with an area of 912869, USP tailing of 2.16, and plate count of 392, indicating the need for further optimization. Trials - 2 to 5 adjusted parameters such as mobile phase composition, leading to significant improvements in peak symmetry and efficiency. Trial - 5, with a retention time of 2.638 minutes and plate count of 2768, approached acceptable limits but required further refinement for validation. In Trial-6, all parameters met the required specifications, resulting in an optimized method suitable for validation. This final method displayed a retention time of 2.782 minutes, area of 2981428, USP tailing of 0.69, and a plate count of 18878, confirming enhanced separation and resolution. The optimized conditions used Waters Alliance e-2695 HPLC with a 10 µL injection volume. The mobile phase consisted of acetonitrile and ammonium acetate at pH 3.0/OPA (70:30), with an RP-18 column (150 x 4.6 mm, 3.5 µ), detection at 269 nm, a flow rate of 1 mL/min, runtime of 5 minutes, ambient temperature (25 °C), and isocratic mode of separation. This setup demonstrated consistent and reproducible results across validation trials. Figure 3 shows the optimization of chromatographic conditions through trails 1- 6.

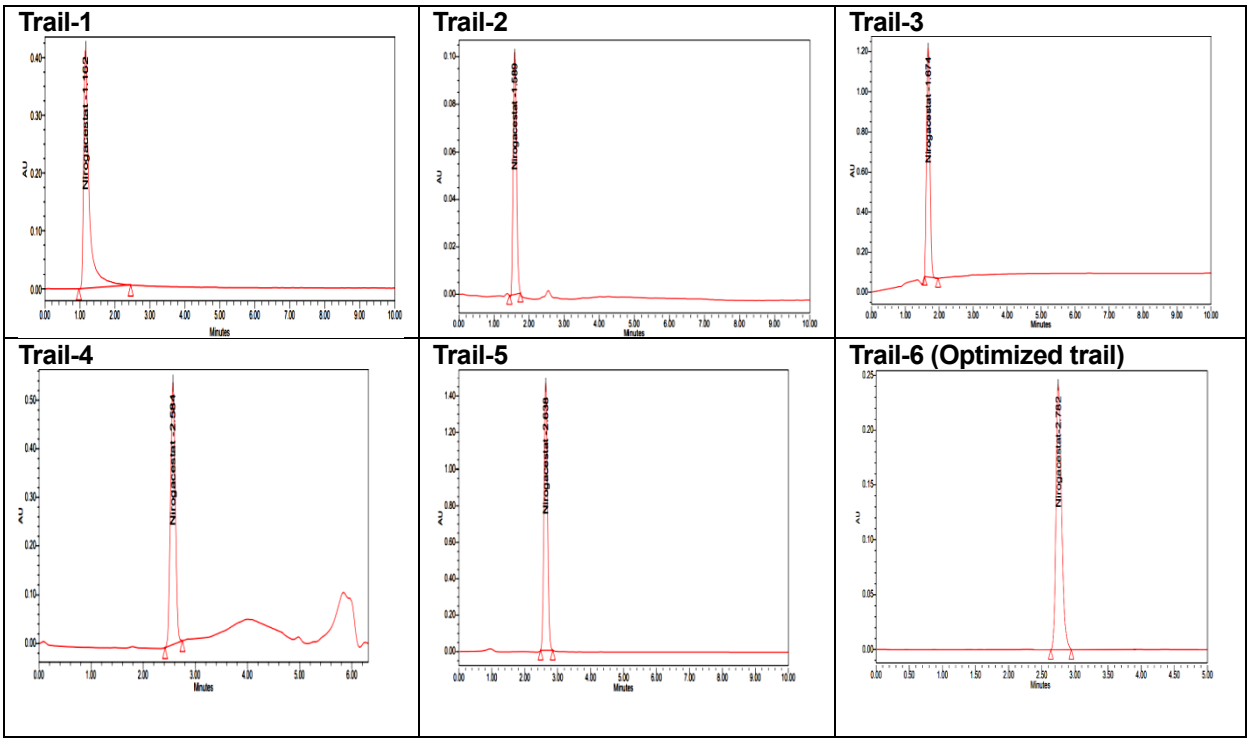


Figure 3: Optimization of Chromatographic Conditions through Trials 1–6.

ANALYTICAL METHOD VALIDATION (HPLC)

The method was validated for its linearity range, accuracy, precision and specificity. Method validation was carried out as per ICH guidelines.[10-15].

Table 2. Optimized chromatographic conditions and system suitability parameters for nirogacestat

PARAMETERS	OBSERVATION
Instrument used	Waters Alliance e-2695 HPLC
Injection volume	10 µL
Mobile Phase	Acetonitrile and Ammonium acetate pH-3.0/OPA (30:70)
Column	Symmetry shield RP-18 (150 x 4.6 mm, 3.5 µ).
Detection Wave Length	269 nm
Flow Rate	1 mL/min
Runtime	5 minutes
Temperature	Ambient (25° C)
Mode of separation	Isocratic mode
System suitability parameters for nirogacestat	
Retention time	2.782
Plate count	18878
Tailing factor	0.69
% RSD	0.31

System suitability: All the system suitability parameters were within the range and satisfactory as per ICH guidelines system suitability parameters and optimized chromatographic conditions are shown in Table 2.

Specificity:

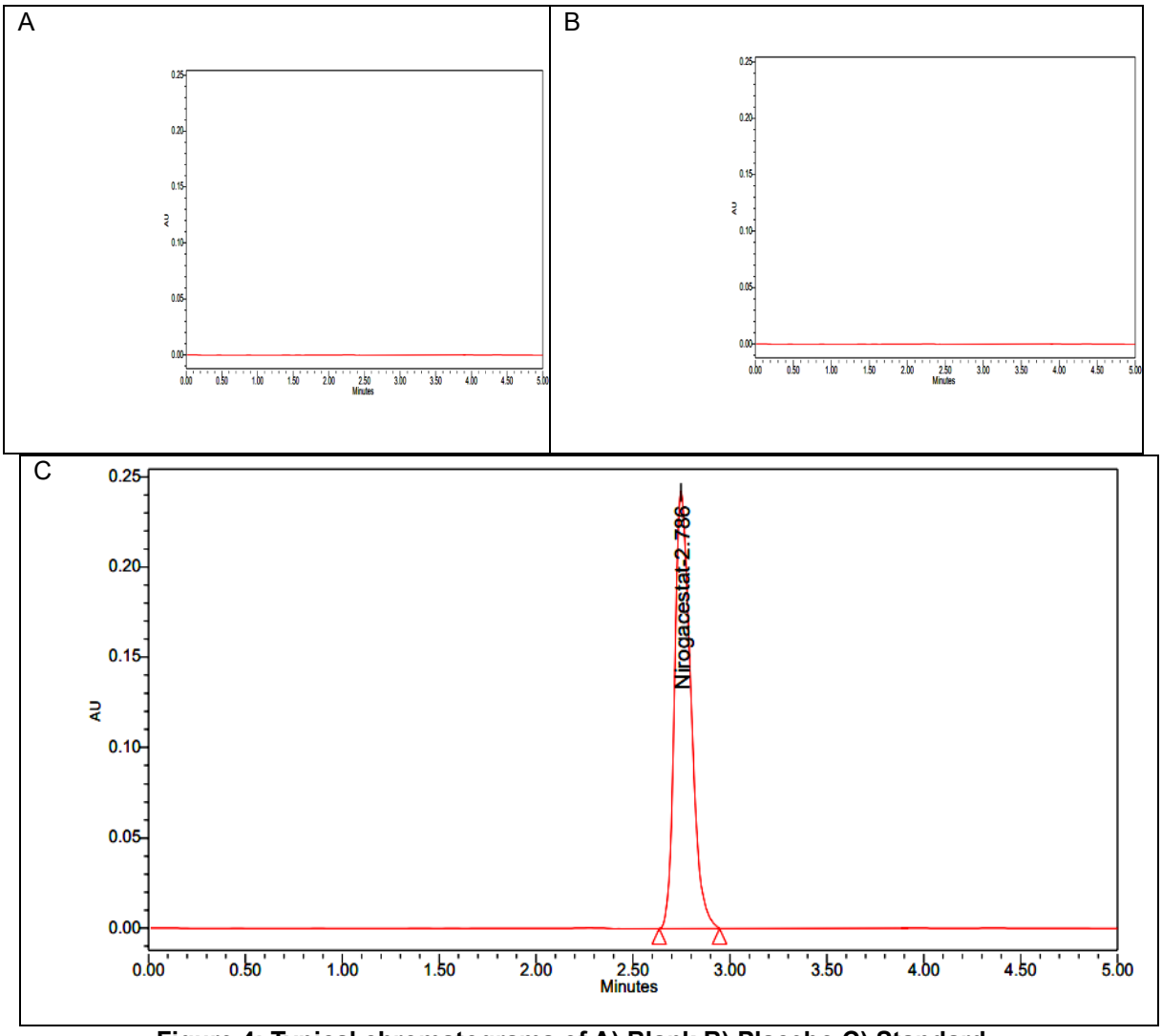


Figure 4: Typical chromatograms of A) Blank B) Placebo C) Standard

Retention times of Nirogacestat were 2.786 min. We did not found and interfering peaks in blank and placebo at retention times of these drugs in this method. So this method was said to be specific. Typical chromatograms of blank, placebo and standard are shown in Fig.4.

Linearity:

A	B	C

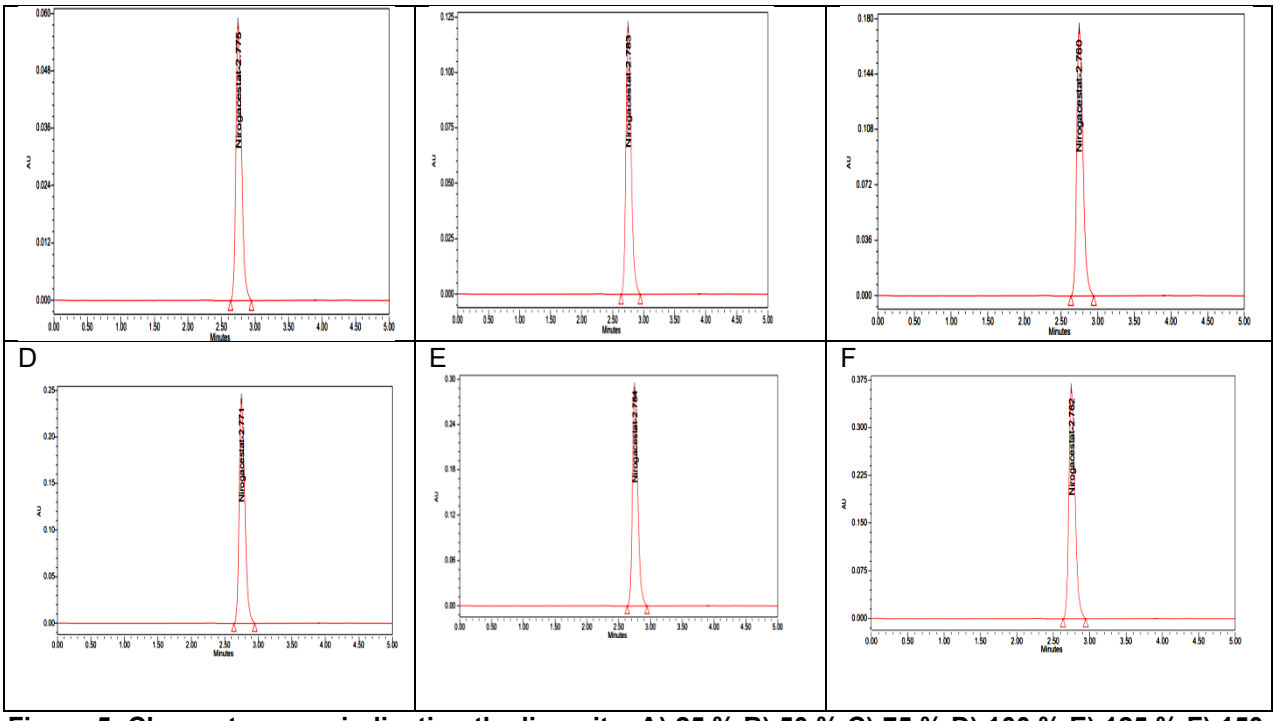


Figure 5: Chromatograms indicating the linearity: A) 25 % B) 50 % C) 75 % D) 100 % E) 125 % F) 150 % concentrated solutions of selected drug

Table 3. Linearity for Nirogacestat

S.NO	Nirogacestat	
	Conc.(µg/mL)	Peak area
1	20.00	748412
2	40.00	1497520
3	60.00	2241087
4	80.00	2984513
5	100.00	3748511
6	120.00	4387478
Regression equation	y = 36874.33x+17186.04	
Slope	36874.33	
Intercept	17186.04	
R^2	0.99981	

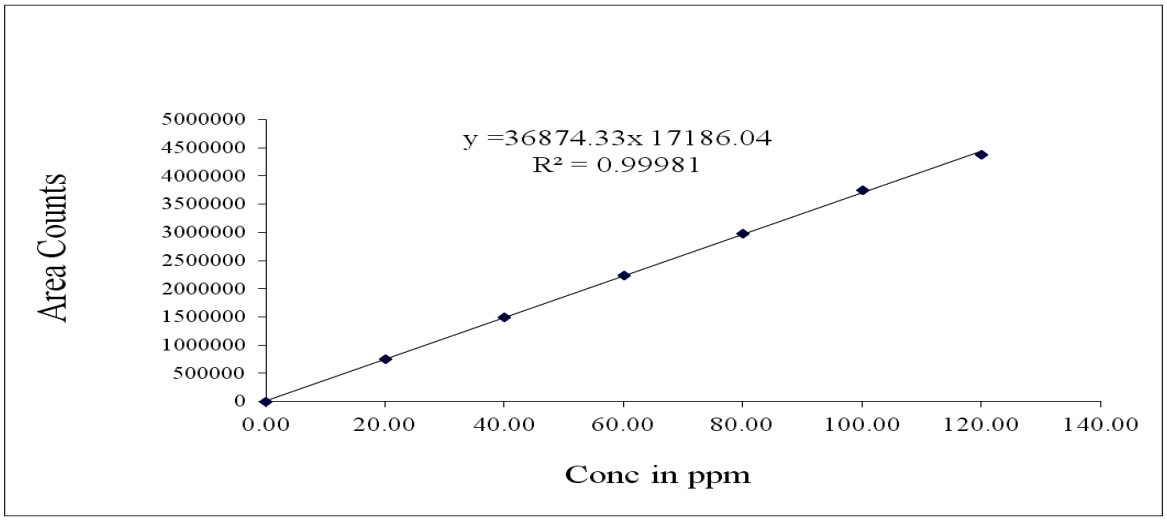


Figure 6: Calibration curve for Nirogacestat

Linearity in HPLC is the ability of an analytical procedure to produce test results that are directly proportional to the concentration of an analyte in a sample. The linearity data is presented in Table 3, the chromatograms indicating linearity are shown in Figure 5, and the calibration curve for Nirogacestat is depicted in Figure 6.

PRECISION:

Table 4. System Precision

S. No	Concentration Nirogacestat (µg/mL)	Area of Nirogacestat
1.	80	2981428
2.	80	2996572
3.	80	2970654
4.	80	2982898
5.	80	2990947
6.	80	2978854
Mean		2983559
S.D		9138.65
% RSD		0.31

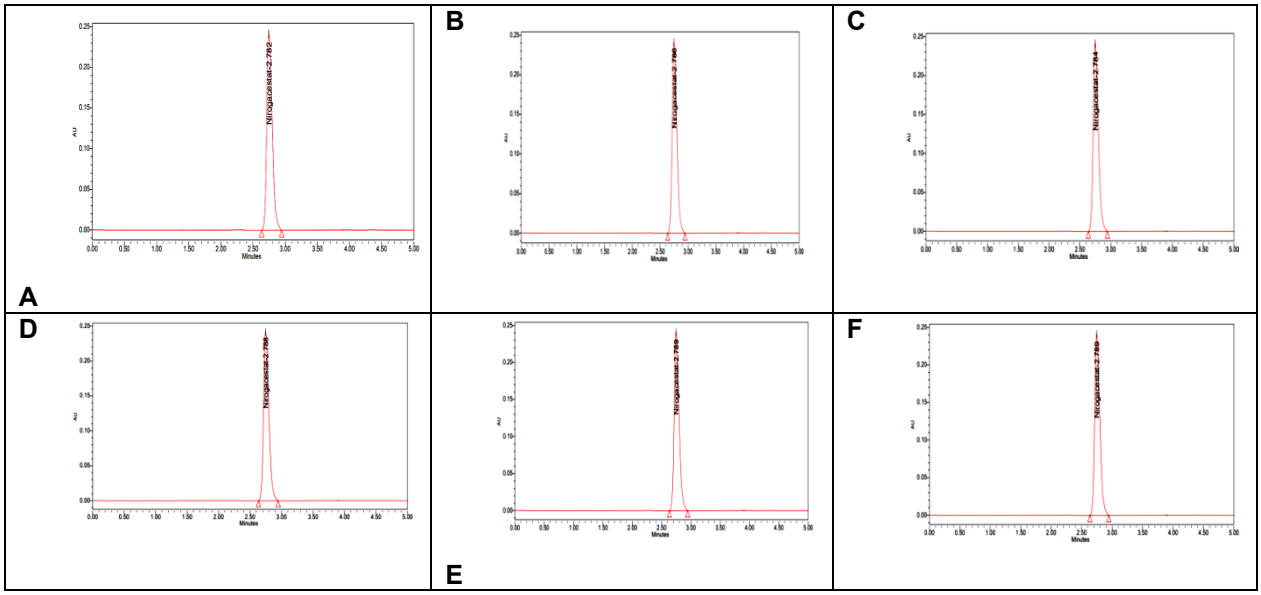


Figure 7: System Precision Chromatograms (A-F)

Discussion: From a single volumetric flask of working standard solution six injections were given and the obtained areas were mentioned above. Average area, standard deviation and % RSD was calculated for Nirogacestat. % RSD obtained as 0.31 for Nirogacestat. As the limit of Precision was less than "2" the system precision was passed in this method. Table 4 and Table 5 shows the system precision and method precision respetively, and the Fig. 7 represents system precision chromatograms. Method precision for Nirogastat is represented in Table 5 and the Figure 8 shows the repeatability chromatograms. Intermedicate precision is represented in Table 6. Interday (Day-1) and Interday (Day-2) chromatograms are shown in Fig.9 and Fig.10 respectively.

Repeatability:

Table 5. Method Precision for Nirogacestat by HPLC method

S. No.	Area for Nirogacestat
1	2971984
2	2955491
3	2960482
4	2989431
5	2966820
6	2977890
Average	2970350
SD	12281.973
% RSD	0.41

Acceptance Criteria: The % RSD for the area of six standard injections results should not be more than 2 %.

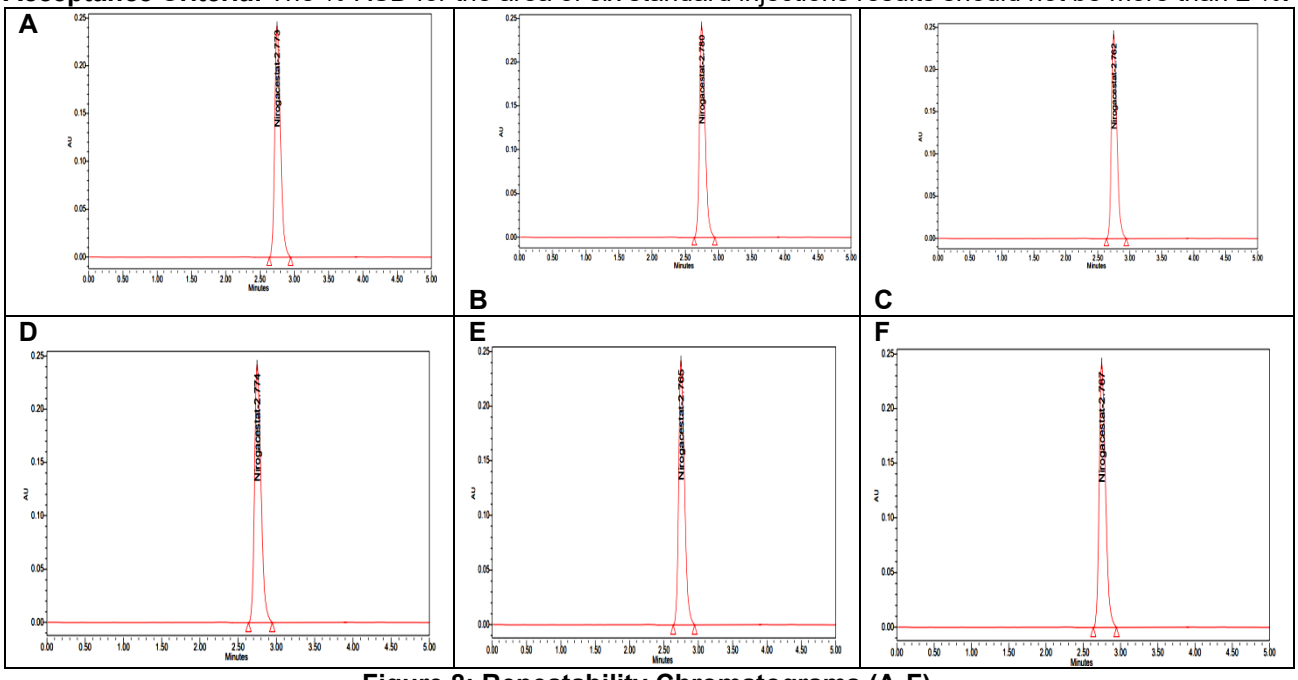


Figure 8: Repeatability Chromatograms (A-F)

Intermediate precision (Day_ Day Precision):

Table 6. Intermediate Precision (Day variation) for Nirogacestat by HPLC method

S. No.	Day 1 Area	Day 2 Area
1	2972415	2965875

2	2984960	2952154
3	2945612	2955520
4	2985206	2944875
5	2997823	2961420
6	2968679	2976632
Average	2975783	2959413
SD	18060.295	11148.824
% RSD	0.61	0.38

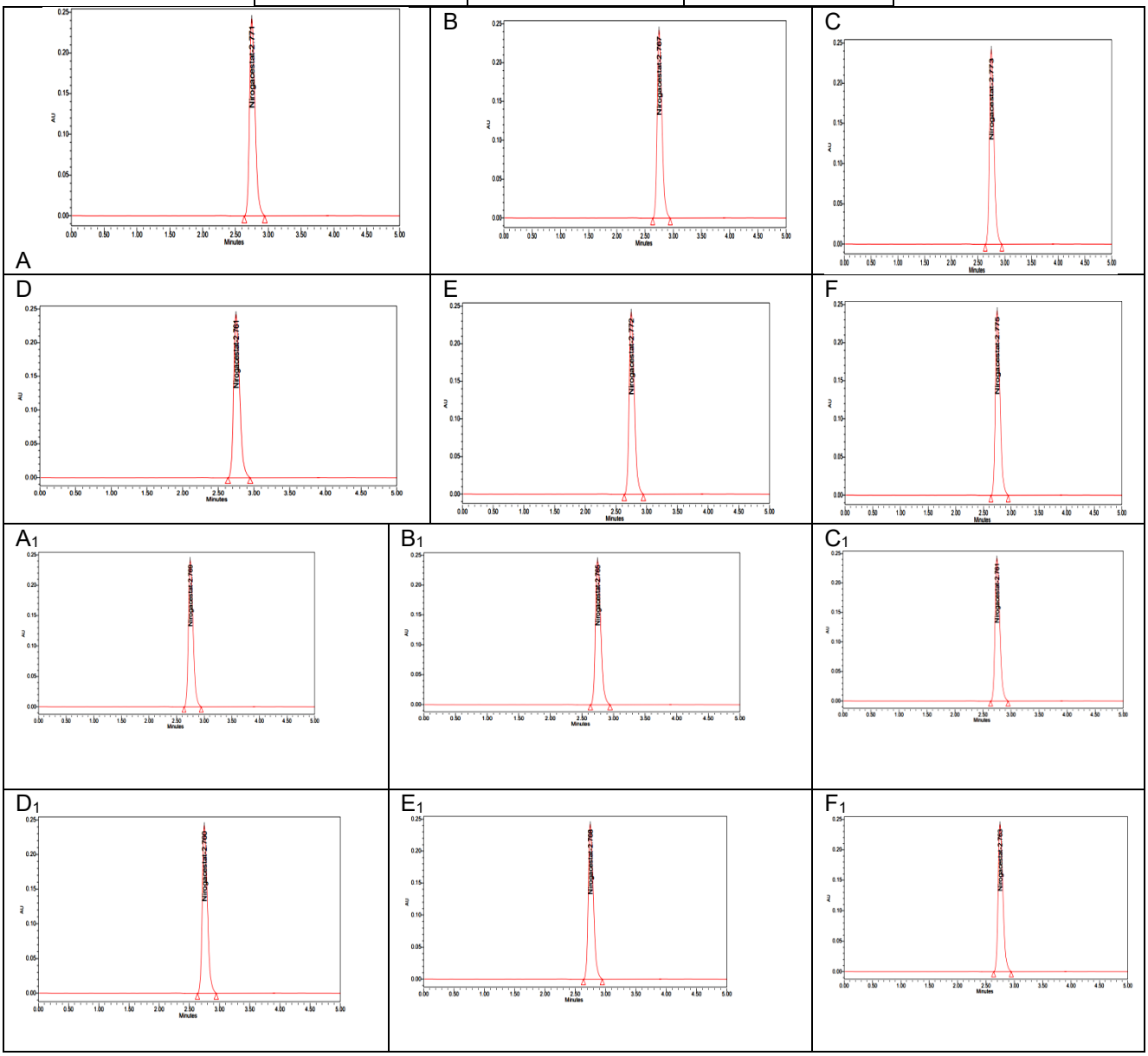


Figure 9 and Figure 10: Inter-day precision chromatograms for Day 1 (A-F) and Day 2 (A1-F1), respectively, illustrating the consistency and reproducibility of chromatographic measurements across two different days.

Acceptance Criteria: The % RSD for the area of six standard injections results should not be more than 2 %.

Accuracy:

Table 7. Accuracy results of Nirogacestat by HPLC method

Concentration(at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean % Recovery
50 %	1484840	4.00	3.98	99.5	99.7
	1496487	4.00	4.01	100.3	
	1479951	4.00	3.97	99.3	
100 %	2961578	8.00	7.94	99.3	99.6
	2959854	8.00	7.94	99.3	
	2992602	8.00	8.02	100.3	
150 %	4475895	12.00	12.00	100.0	99.9
	4483174	12.00	12.02	100.2	
	4456842	12.00	11.95	99.6	

Discussion: Triplicate injections were given for each level of accuracy and mean % Recovery was obtained as 99.7 % for Nirogacestat respectively.Accuracy table is shown in Table 7 and the accuracy chromatograms are depicted in Fig.11.

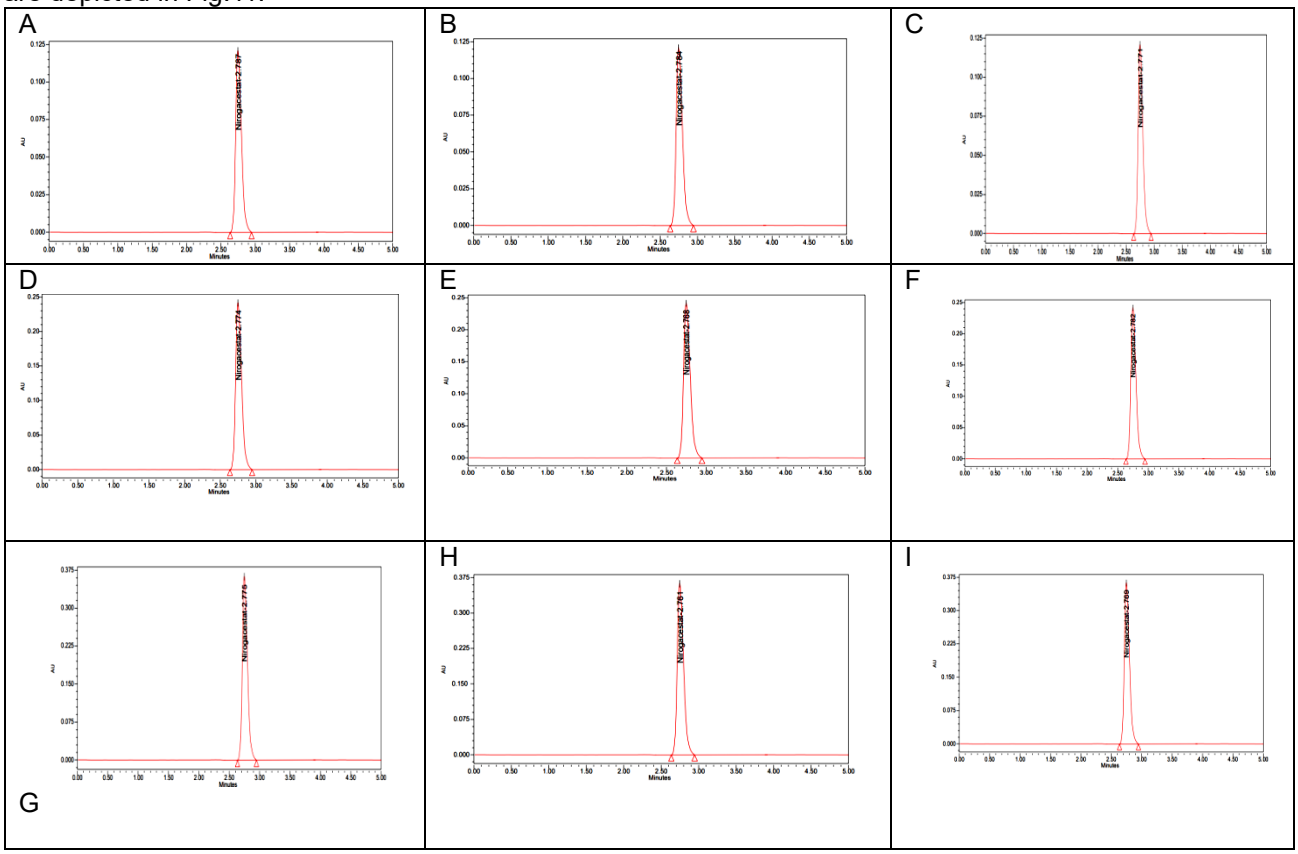


Figure 11: Chromatograms representing accuracy levels at 50 % (A, B, C), 100 % (D, E, F), and 150 % (G, H, I)

Robustness:

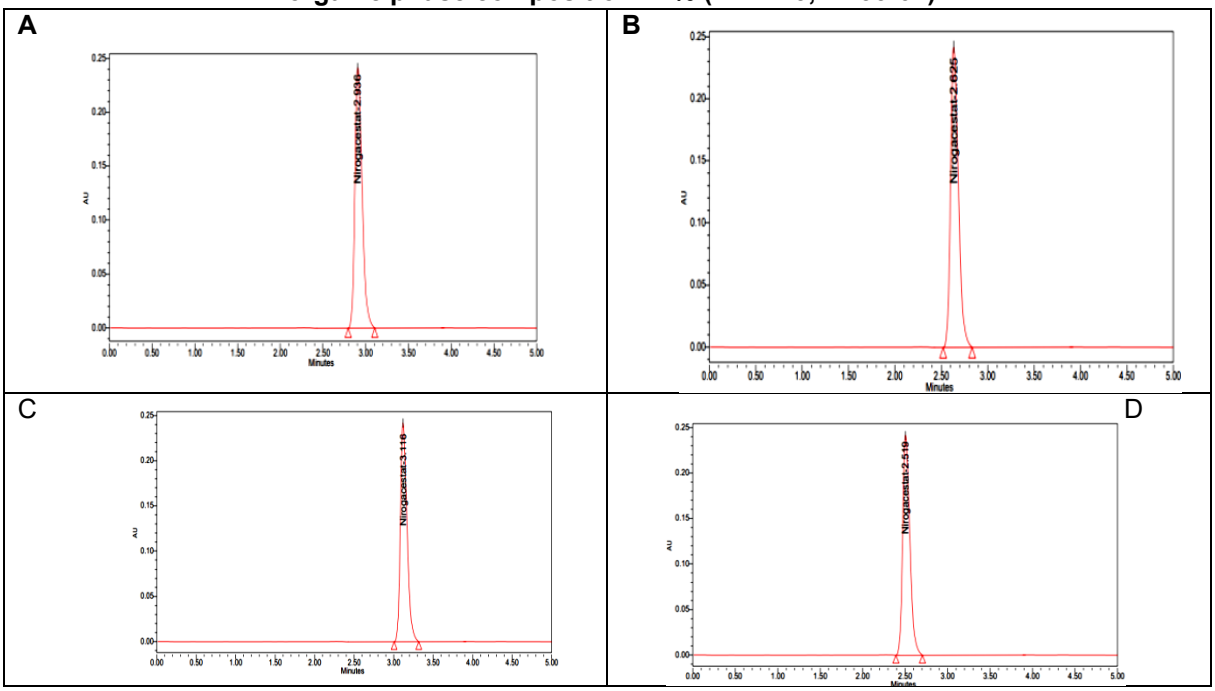
Robustness in HPLC assesses the method's reliability against small, deliberate variations in parameters like flow rate, mobile phase composition. It ensures consistent and accurate results under varied analytical conditions. Robustness results are shown in Table 8. Chromatograms showing the effects of flow rate (A: 0.9 mL/min, B: 1.1 mL/min) and organic phase composition in % (C: 27:73, D:33:67) are shown in Fig.12. Robustness results of Nirogacestat is represented in Table 8.

Table 8. Robustness results of Nirogacestat

Parameter	Nirogacestat					
	Condition	Retention time(min)	Peak area	Tailing	Plate count	% RSD
Flow rate	Less flow (0.9 mL)	2.936	2844441	0.72	18775	0.51

Change (mL/min)	Actual flow (1.0 mL)	2.782	2981428	0.69	18878	0.31
	More flow (1.1mL)	2.625	3171852	0.62	18940	0.56
Organic Phase change	Less Org (27:73)	3.116	2771204	0.76	18620	0.73
	Actual (30:70)	2.786	2996572	0.68	18851	0.31
	More Org (33:67)	2.519	3254122	0.59	18989	0.36

Figure 12: Chromatograms showing the effects of flow rate (A: 0.9 mL/min, B: 1.1 mL/min) and organic phase composition in % (C:27:73, D: 33:67)



LOD and LOQ (µg/mL):

LOD (Limit of Detection) is the lowest concentration of an analyte that can be detected but not quantified, typically with a signal-to-noise ratio of 3:1. LOQ (Limit of Quantification) is the lowest concentration that can be quantified accurately and precisely, with a signal-to-noise ratio of 10:1. Chromatograms for LOD and LOQ are shown in Fig.13. and the Sensitivity parameters (LOD & LOQ) are shown in Table 9.

Table 9. Sensitivity parameters (LOD & LOQ) by HPLC

Name of drug	LOD (µg/mL)	s/n	LOQ (µg/mL)	s/n
Nirogacestat	0.48	3	1.60	10

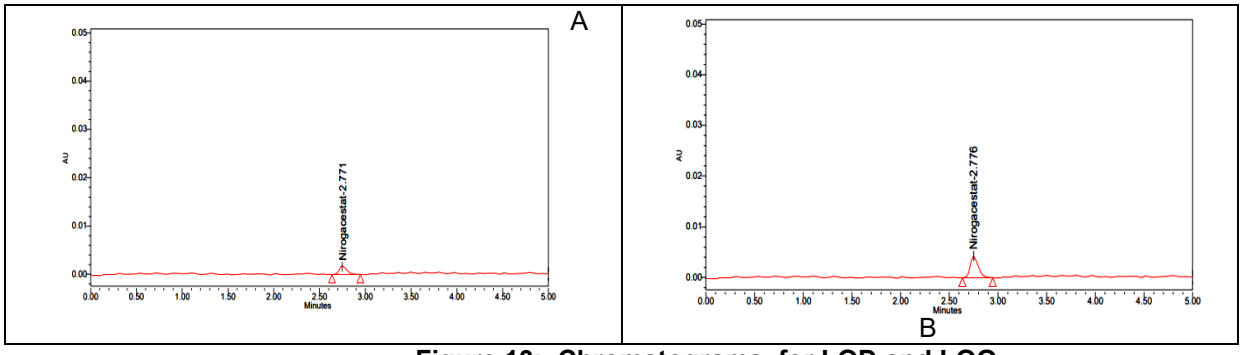


Figure 13: Chromatograms for LOD and LOQ

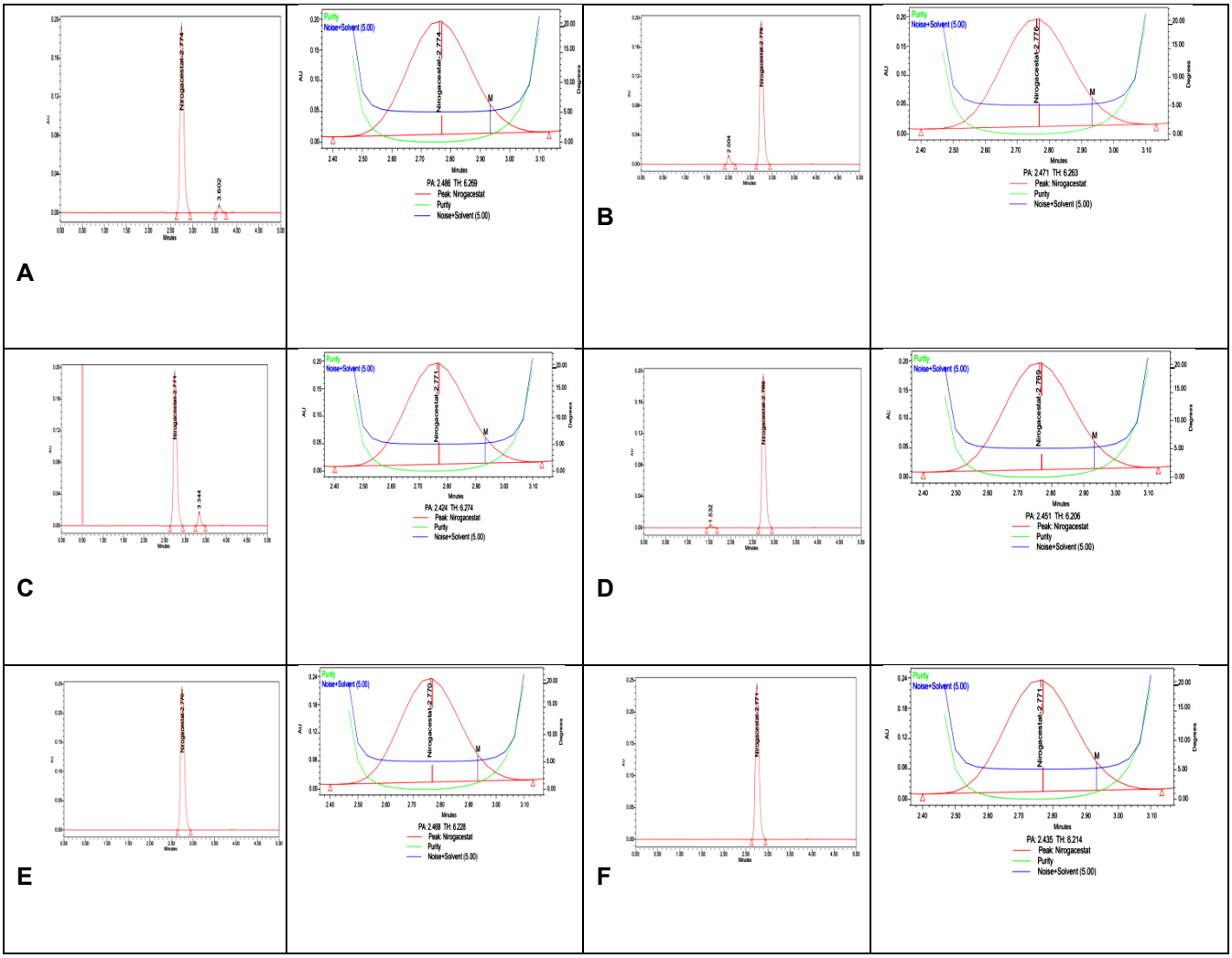
Forced degradation studies:

Forced degradation studies play a vital role in assessing the stability of pharmaceutical compounds under various stress conditions. These evaluations help identify degradation pathways and detect potential impurities that may arise due to environmental factors. In the case of Nirogacestat, tests were carried out under a range

of stress conditions, including acidic, alkaline, oxidative (peroxide), reductive, hydrolytic, thermal, and photolytic stress. The resulting chromatograms provide a detailed view of the compound's behavior under these conditions. This data is instrumental in determining the stability profile of Nirogacestat, which is crucial for optimizing its formulation and ensuring proper storage. Table 10 outlines the results of the forced degradation studies, while Fig. 14 visually represents the corresponding chromatograms.

Table 10. Forced Degradation results for Nirogacestat

Stress conditions	Sample Wt in mg	Area	Mean area count	% Label Claim	Purity Angle	Purity Threshold	% Degradation	Pass/ Fail
Control	28	2983672	2983672	100	2.435	6.214	0	Pass
Acid	28	2681536	2681536	89.8	2.486	6.269	10.2	Pass
Alkali	28	2614395	2614395	87.6	2.471	6.263	12.4	Pass
Peroxide	28	2564130	2564130	85.9	2.424	6.274	14.1	Pass
Reduction	28	2726422	2726422	91.4	2.451	6.206	8.6	Pass
Thermal	28	2947367	2947367	98.8	2.436	6.251	1.2	Pass
Photolytic	28	2960405	2960405	99.2	2.468	6.228	0.8	Pass
Hydrolysis	28	2933348	2933348	98.3	2.458	6.243	1.7	Pass



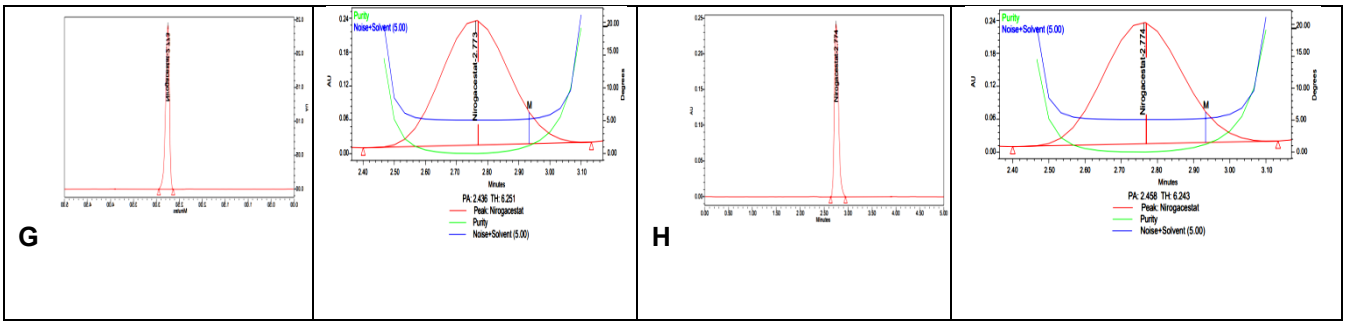


Figure 14: Chromatograms of forced degradation studies: A.Chromatograms of control and purity plot of cntrol degradation, B) Acid degradation C) Alkali degradation D) Peroxide degradation E) Reduction degradation F) Hydrolysis Degradation G) Thermal Degradation H) Photolytic Degradation

Assay:

The assay of Nirogacestat is performed by comparing the chromatographic area counts of the sample with those of a standard preparation. Key factors considered include the sample weight, dilution factors, and standard purity. Using these parameters, a formula calculates the percentage assay, providing the final result.are presented in the following (Table 11) and chromatograms are represented in Fig.15.

Assay of Nirogacestat and formula for Assay:

$$\%\ Assay = \frac{AT}{AS} * \frac{WS}{DS} * \frac{DT}{WT} * \frac{Average\ weight}{Label\ Claim} * \frac{P}{100} * 100$$

Where: AT = average area counts of test (sample) preparation.
AS = average area counts of standard preparation.
WS = Weight of working standard taken in mg.
DS = Dilution of working standard in mL.
DT = Dilution of test (sample) in mL.
WT = Weight of test (sample) taken in mg.
P = Percentage purity of working standard , LC = Label Claim mg/mL.

Table 11. Assay of Nirogacestat

Brand	Drug	Sample Area	Avg sample area (n=5)	Std. wt (mg)	Sample wt. (mg)	Label amount (mg)	Std purity	Amount found (µg/mL)	% assay
Ogsiveo	Nirogacestat	2984563 2974154	2979359	8.0	28	50	99.9	7.99	99.9

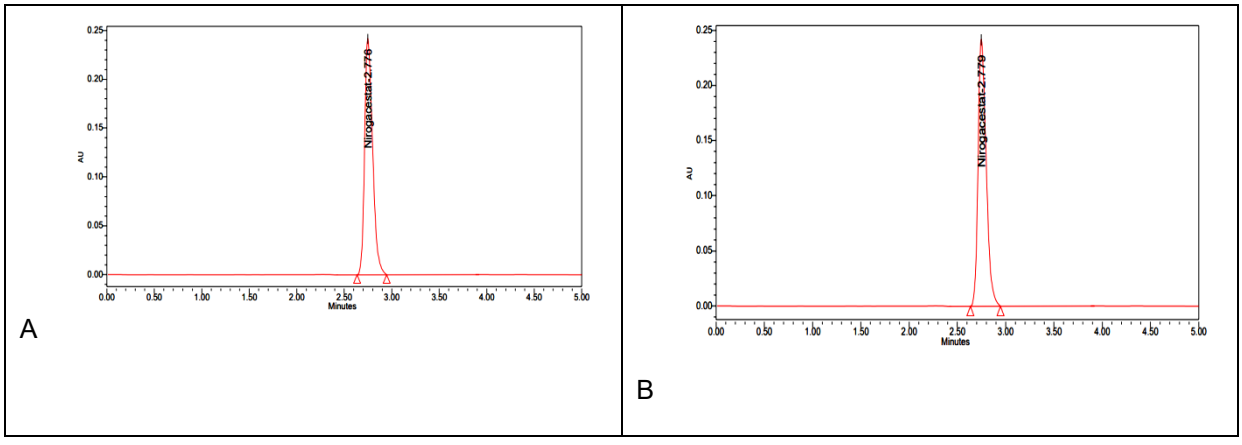


Figure 15: Chromatogram of Assay-A and B.

Conclusion:

The developed stability-indicating HPLC method for the estimation of Nirogacestat is precise simple, rapid, accurate, robust, and economical. The mobile phase and solvents are easy to prepare, cost-effective, and reliable, making the method highly efficient for laboratory use. The results of the method validation, including linearity, precision, accuracy, ruggedness, and robustness, comply with ICH guidelines, confirming the reliability of the developed HPLC method. The retention time of Nirogacestat was identified as 2.782 minutes, ensuring a short analysis time without interference from excipients or degradation products. The recoveries of Nirogacestat were found to be within acceptable limits (98–102 %), supporting the accuracy of the method for pharmaceutical dosage forms. Additionally, the method demonstrated no interference from formulation excipients making it suitable for routine quality control and stability analysis.In the case of Nirogacestat, tests were carried out under a range of stress conditions, including acidic, alkaline, oxidative (peroxide), reductive, hydrolytic, thermal, and photolytic stress. The resulting chromatograms provide a detailed view of the compound's behavior under these conditions. This data is instrumental in determining the stability profile of Nirogacestat, which is crucial for optimizing its formulation and ensuring proper storage. In conclusion, the stability-indicating assay method using HPLC is specific, reproducible, and well-suited for the routine analysis of Nirogacestat in bulk and pharmaceutical formulations.

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