A Quality by Design-Driven Stability indicating UPLC Method for the Development and Validation of Simultaneous Nirogacestat and Impurity Quantification

Umamaheswara Rao K.V. and Shorgar Neetu*
Department of Chemistry, Faculty of Science, Pacific Academy of Higher Education and Research University, Udaipur-313001, Rajasthan, INDIA
*nshorgar@gmail.com

Abstract

The present study intended to propose a robust and reliable UPLC method for the simultaneous quantification of Nirogacestat and its impurities 1 to 5. A systematic quality by design (QbD) approach was employed to optimize critical method parameters that ensure enhanced resolution, peak symmetry and sensitivity. The chromatographic conditions were carefully selected based on an extensive literature review and theoretical physicochemical assessments. Various column chemistries and mobile phase compositions were evaluated to achieve optimal separation of Nirogacestat and its impurities. A total of 13 experimental trials were conducted and the results were analyzed through perturbation charts, contour plots and 3D surface plots to determine optimal chromatographic conditions. Finally, the optimized conditions comprise of an X-Bridge C18 (50 mm × 4.6 mm, 2.1 µm) column, acetonitrile and 0.1% trifluoroacetic acid in a 40:60 (v/v) ratio at 0.3 mL/min flow as mobile phase and 299 nm wavelength. This method exhibits excellent linearity over the studied concentration ranges, with correlation coefficients (R^2) greater than 0.999 for Nirogacestat (50–300 µg/mL) and its impurities (2.5–15.0 µg/mL).

The method demonstrated high accuracy with mean recovery rates of 98-102 % for Nirogacestat and its impurities across three concentration levels. Sensitivity assessment reveals sensitive detection and quantification limits for Nirogacestat and its impurities respectively ensures reliable detection of trace impurities. The robustness study indicates that minor variations in flow rate and organic modifier composition had minimal impact on chromatographic performance. Forced degradation study confirms the method's stability-indicating nature. The purity angle and purity threshold assessment in stress study confirm the absence of co-elution proving the method's capability for impurity profiling. In conclusion, this study establishes a novel, precise and reliable chromatographic method for the simultaneous determination of Nirogacestat and its impurities for routine pharmaceutical analysis and quality control applications.

Keywords: Nirogacestat, impurity profiling, quality by design approach, method optimization, forced degradation studies.

Introduction

The analytical quality by design (AQbD) framework plays a crucial role in the development of reliable, reproducible and regulatory-compliant analytical methods for analysis of impurities in a pharmaceutical compound. Impurity profiling is a critical aspect in pharmaceutical quality control because impurities at trace level can impact drug efficacy and safety[12]. The conventional analytical method development approach relies on trial-and-error based optimization that leads to inconsistencies, lack of robustness and the need for frequent revalidation. In contrast, AQbD provides a systematic, risk-based strategy that exhibit enhanced method understanding, reduces variability and ensures consistency throughout the lifecycle of method[8].

AQbD approach defines analytical target profile (ATP) and ensures that the method meets predefined performance characteristics such as specificity, sensitivity, accuracy and precision. This approach minimizes uncertainty in results with enhanced regulatory compliance in alignment with guidelines set by the ICH[6] and FDA[3]. AQbD tool identify and optimize the critical method parameters (CMPs) that influence impurity detection and quantification. This approach utilizes risk assessment tools including Ishikawa diagrams and FMEA (Failure Mode and Effects Analysis) to identify potential method variability sources during the early stage of development[7]. Further, the Design of Experiments (DoE) approach facilitates the optimization of CMPs by correlating their interactions with critical quality attributes (CQAs) such as resolution, retention time and limit of detection (LOD).

This procedure ensures that the method operates within a well-defined design space and provides flexibility to modify parameters without requiring regulatory re-approval. Additionally, this approach maintains method performance over time by integrating robust control strategies such as system suitability, continuous monitoring and lifecycle validation[9].

Nirogacestat (Figure 1A) belongs to selective γ-secretase inhibitor drug that was developed for the treatment of desmoid tumors which are also known as aggressive fibromatosis that arise from fibroblasts and exhibit unpredictable growth patterns, often causing pain, functional impairment and significant morbidity despite being non-

metastatic[2]. Nirogacestat exhibits therapeutic effect by inhibiting γ-secretase which is a crucial enzyme in the Notch signalling pathway that plays a key role in cell proliferation, differentiation and survival[4].

Additionally, nirogacestat exhibits potential application in combination therapies for the treatment of hematologic malignancies such as multiple myeloma. The common adverse effects associated with nirogacestat include gastrointestinal disturbances, fatigue and ovarian dysfunction in premenopausal women[5].

Nirogacestat has been previously reported in the literature with an analytical method based on RP-HPLC for its quantification in formulations[1]. However, this method focused solely on the determination of nirogacestat and did not extend the method applicability for quantification of its related impurities.

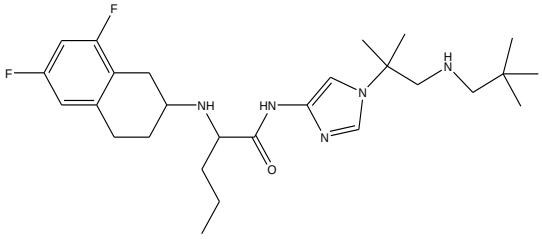


Figure 1A
Nirogacestat
Molecular Formula: $C_{27}H_{41}F_2N_5O$
Molecular Weight: 489.3279 g/mol
IUPAC Name: *(S)-2-((S)-5,7-difluoro-1,2,3,4-tetrahydronaphthalen-3-ylamino)-N-(1-(2-methyl-1-(neopentylamino)propan-2-yl)-1H-imidazol-4-yl)pentanamide*

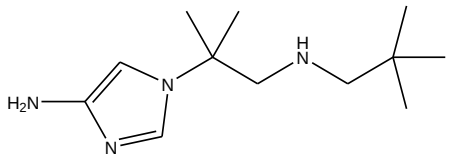


Figure 1B
Impurity 1
Molecular Formula: $C_{12}H_{24}N_4$
Molecular Weight: 224.2001 g/mol
IUPAC Name: *1-(2-methyl-1-(neopentylamino)propan-2-yl)-1H-imidazol-4-amine*

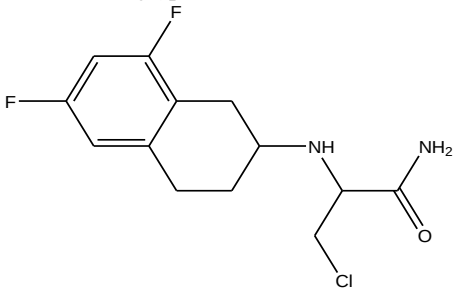


Figure 1C
Impurity 2
Molecular Formula: $C_{13}H_{15}ClF_2N_2O$
Molecular Weight: 288.0841 g/mol
IUPAC name: *2-(5,7-difluoro-1,2,3,4-tetrahydronaphthalen-3-ylamino)-3-chloropropanamide*

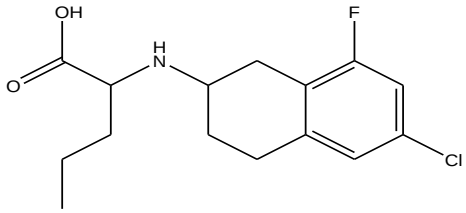


Figure 1D
Impurity 3
Molecular Formula: $C_{15}H_{19}ClFNO_2$
Molecular Weight: 299.1088 g/mol
IUPAC Name: *(S)-2-((R)-7-chloro-5-fluoro-1,2,3,4-tetrahydronaphthalen-3-ylamino)pentanoic acid*

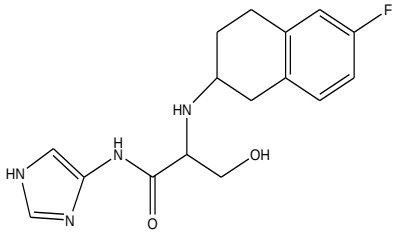


Figure 1E
Impurity 4
Molecular Formula: $C_{16}H_{19}FN_4O_2$
Molecular Weight: 318.1492 g/mol
IUPAC Name: *2-(6-fluoro-1,2,3,4-tetrahydronaphthalen-2-ylamino)-3-hydroxy-N-(1H-imidazol-4-yl)propanamide*

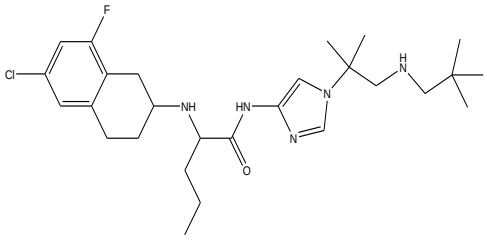


Figure 1F
Impurity 5
Molecular Formula: $C_{27}H_{41}ClFN_5O$
Molecular Weight: 505.2984 g/mol
IUPAC Name: *2-((S)-7-chloro-5-fluoro-1,2,3,4-tetrahydronaphthalen-3-ylamino)-N-(1-(2-methyl-1-(neopentylamino)propan-2-yl)-1H-imidazol-4-yl)pentanamide*

Figure 1: Details of Nirogacestat and its impurities in the study

Keep in consideration of growing importance of impurity profiling in pharmaceutical quality control. There is a need to develop a comprehensive and robust analytical approach for quantification of nirogacestat impurities in formulations. Hence, this study aimed to develop and validate a QbD-based RP-UPLC method for the simultaneous quantification of nirogacestat and its impurities. The structure of nirogacestat and its impurities in the study was presented in figure 1.

Material and Methods

Materials: The active pharmaceutical ingredient of nirogacestat along with its impurity 1 to 5 was kindly provided as a gift sample by Jubilant Biosys Ltd., Bengaluru, Karnataka, India. The chromatographic analyses were performed through HPLC-grade solvents which were procured from Merck, Mumbai, India. In addition to these solvents, other analytical reagent grade chemicals were used throughout the study, which were also sourced from Merck, Mumbai.

Instrumentation: In this study, the ACQUITY UPLC system (Waters, Massachusetts, USA) equipped with a Photodiode Array (PDA) detector was used for the analysis. The instrument contains a quaternary solvent manager (QSM) pump for precise solvent delivery and a sample manager with flow-through needle (SM-FTN) injector for efficient and accurate sample introduction. The desired column temperature for efficient resolution was maintained using a column heater (CH-A) and empower 2 (Waters) software was utilized for acquisition and processing of data.

Chromatographic conditions: The separation of nirogacestat and its five studied impurities was achieved using X-Bridge C18 column (50 × 4.6) mm, 2.1 µm that provides efficient resolution and peak separation. The injection volume of 5 µL was finalized to ensure optimal sample loading for accurate quantification. The column temperature and sample temperature were maintained at ambient temperature for stable and reproducible analysis of samples.

The mobile phase consists of a mixture of acetonitrile and 0.1% aqueous TFA in 40:60 (v/v), at 0.3 mL/min flow rate. This mobile phase conditions facilitate optimal separation of nirogacestat and its impurities with a total run time of 6 min.

Preparation of Standard and calibration Solutions: A stock solution of nirogacestat was prepared by accurately weighing 10 mg of the drug and transferred into a 10 mL volumetric flask. The compound was dissolved in equal volume of 0.1 % aqueous TFA and acetonitrile which serves as diluent and the final volume in flask was made up to 10 mL to obtain a final concentration of 1 mg/mL (1000 µg/mL).

This stock solution was appropriately diluted to achieve working standard solutions in the range of 50–300 µg/mL. Similarly, the impurity solutions in 2.50–15.0 µg/mL were prepared individually using the same diluent and these solutions were stored at 4°C for further use.

Method development: The UPLC method for the simultaneous estimation of nirogacestat and its impurities was optimized by evaluating various chromatographic conditions that produce adequate separation, resolution and peak symmetry[13]. Different stationary phases that include Phenomenex (50 mm) C18, X-Bride Phenyl (150 mm) and X-Bridge (50 mm) C18 column were screened to achieve optimal retention and selectivity. The mobile phase composition was systematically varied using different ratios of aqueous buffers such as formic acid and TFA along with acetonitrile as organic modifier under isocratic elution mode. The pH of the aqueous phase was adjusted within an appropriate range to enhance peak resolution. The flow rate, column temperature and detection wavelength were also optimized to ensure the best sensitivity and separation efficiency. Further, the robust and rugged method conditions were finalized based on QbD approach[11].

QbD approach: The final optimization of UPLC method for the simultaneous quantification of nirogacestat and its impurities was performed through QbD approach. The key quality target product profile (QTPP) attributes such as retention time, theoretical plates, peak asymmetry and resolution between nirogacestat and its impurities were identified to ensure method performance. The critical quality attributes (CQAs) such as mobile phase composition and flow rate were controlled to maintain analytical precision. A central composite experimental design was employed to systematically evaluate the effects of these parameters using Design Expert® software (Version 11.0, Stat-Ease Inc.).

The final method was selected based on its ability to achieve optimal separation with robust and reproducible results. Risk assessment, as per ICH Q8 and Q9 guidelines, was conducted to evaluate method robustness and ruggedness by ensuring appropriate stability under small variations in operating conditions and across multiple analysts. The optimized method demonstrates consistent performance that makes it ideal for routine analysis of nirogacestat and its impurities.

Method Validation: The developed UPLC method for the quantification of nirogacestat and its impurities was validated as per ICH Q2 (R1) guidelines to ensure its reliability and suitability for routine analysis[10]. The method linearity was established by analyzing six concentrations within the range of 50–300 µg/mL for nirogacestat and 2.50–15.0 µg/mL for its impurities. This linearity range produces strong correlation coefficient for nirogacestat and its impurities.

The method precision was evaluated through repeatability studying selected mid-range concentration level of nirogacestat and its impurities. Intra-day precision was

determined by analyzing six replicates at two-hour intervals on the same day whereas inter-day precision was assessed over two consecutive days. The %RSD (relative standard deviation) of the peak area response was calculated and a % RSD of less than 2% indicates acceptable method precision.

The method accuracy was confirmed through recovery studies at three concentration levels (low, mid and high) within the linearity range and recoveries results between 98–102% were determined to be precise. The sensitivity was determined by calculating the limit of detection (LOD) and limit of quantification (LOQ) using the standard deviation of the y-intercept (σ) and slope (SD) of the calibration curve by applying the formulas LOD = 3.3 × σ/SD and LOQ = 10 × σ/SD. Method robustness was evaluated by introducing minor variations in flow rate and mobile phase composition to confirm that these changes did not significantly affect method performance.

System suitability was verified through parameters such as retention time, theoretical plates and peak asymmetry to ensure the method's consistency and reproducibility for the analysis of nirogacestat and its impurities.

Forced degradation studies: Forced degradation studies were conducted to evaluate the stability of nirogacestat under various stress conditions[15]. In acid degradation, 50.8 mg of nirogacestat was transferred into a 10 mL volumetric flask and was treated with 1 mL of 1N HCl. This mixture was left for 15 min to induce degradation and then the solution was neutralized with 1N NaOH followed by dilution with diluent. Alkali degradation was performed similar to acid stress study using 1 mL of 1N NaOH, followed by neutralization with 1N HCl.

The oxidative degradation involves treating 50.8 mg of nirogacestat with 1 mL of 10% H_2O_2 for 15 min whereas reductive degradation utilizes 1 mL of 10% sodium bisulfite under identical conditions. Then, 1 mL aliquot of these stress induced solutions was diluted to 10 mL using same diluent, filtered and transferred to vials for analysis in the proposed method.

In thermal degradation, 50 mg of nirogacestat was exposed to 105 °C for 6 h and 20 mg of the exposed sample was dissolved in 7 mL of diluent. This solution was sonicated for 5 min and diluted prior to analysis. Photolytic degradation was assessed by exposing 70 mg of nirogacestat to a photostability chamber for 6 h whereas the hydrolysis degradation was performed by incubating nirogacestat with 1 mL of HPLC water for 15 min, followed by dilution, sonication and filtration. All degraded samples were analyzed to assess the formation of degradation products and evaluate method specificity.

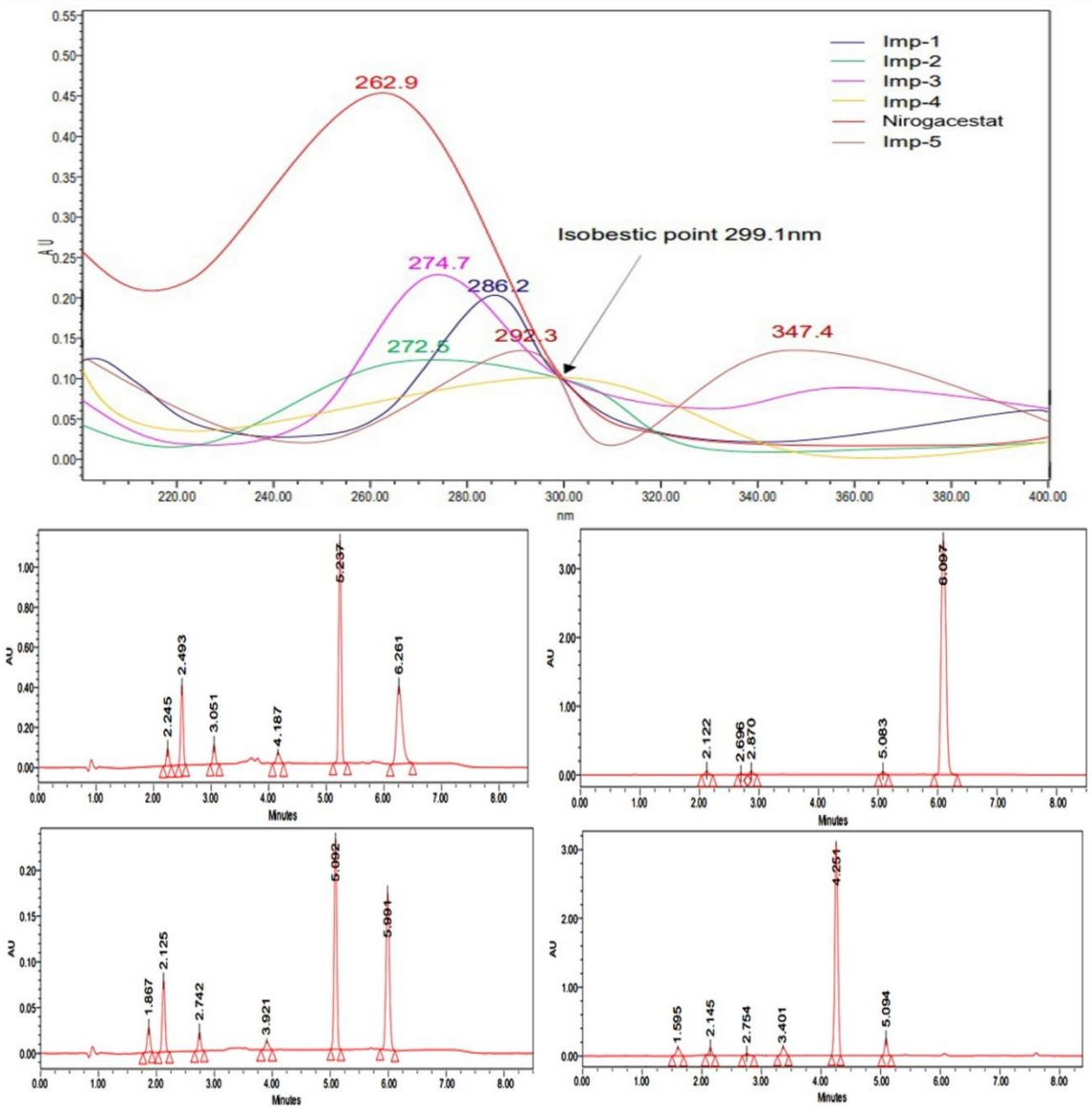
Results and Discussion

In this study, the method optimization process was initiated to develop a robust and reliable chromatographic method for the simultaneous determination of nirogacestat and its impurities. The comprehensive literature review along with theoretical assessment of the physicochemical properties of nirogacestat and its impurities was considered during the optimization of suitable analytical conditions. The chromatographic parameters that include column chemistry, mobile phase composition, flow and detection wavelength were carefully optimized to achieve best resolution, peak symmetry and sensitivity. An iso-absorption wavelength of 299.1 nm (Figure 2A) was selected using a PDA detector as detector wavelength to ensure accurate quantification of nirogacestat and its impurities.

Initially, a Phenomenex C18 (50 mm × 1.7 mm, 2.1 µm) column with a mobile phase composition of 0.3 mL/min flow of acetonitrile and aqueous 0.1% formic acid in 20:80 (v/v) was tested. This condition produces inadequate resolution of nirogacestat and its impurities suggesting that the studied column does not resolve analytes (Figure 2B). Subsequent trials using an X-Bridge Phenyl (150 mm × 4.6 mm, 3.5 µm) column with a mobile phase ratio of 30:70 (acetonitrile: 0.1% formic acid) produce improved separation of analytes but detected only five peaks suggesting that the column does not resolve analytes completely (Figure 2C).

Further modifications with X-Bridge C18 (50 mm × 4.6 mm, 2.1 µm) column with a mobile phase composition of 35:65 (acetonitrile: 0.1% formic acid) led to an unstable baseline indicating that the chromatographic conditions were suboptimal for the resolution of analytes (Figure 2D). Further, formic acid was replaced with 0.1% trifluoroacetic acid (TFA) in the mobile phase (30:70 acetonitrile: TFA) resulting in the appearance of unknown peaks with potential interference or degradation (Figure 2E). The changes in the composition of mobile phase (40:66 of acetonitrile: 0.1% formic acid) produced well resolved peaks and were further finalized through QbD approach.

The fine tune of analytical method was conducted through QbD approach and this approach ensures robust and reliable method for the analysis of nirogacestat and its impurities. Based on preliminary method optimization runs, resolution, peak asymmetry and theoretical plates were confirmed as QTPPs to ensure high-resolution separation, symmetrical peak shapes and reproducible retention times. Meanwhile, the composition of acetonitrile in the mobile phase and the flow rate were identified as CQAs because these parameters significantly influence the peak resolution, retention as well as the overall method robustness. A quadratic central composite design and response surface methodology were employed to evaluate the effect of CQAs on the method performance. A total of 13 experimental runs were conducted based on these design principles that allow an in-depth assessment of interactions between mobile phase composition and flow rate. The results obtained from these optimization trials, along with the corresponding CQAs, were compiled in table 1.



(A) Overlay UV Scanning spectrum Nirogacestat and its impurities (B-E) method optimization chromatograms noticed in the study

Figure 2: UV Scanning and UPLC method optimization chromatograms observed in the study for the analysis of Nirogacestat and its impurities

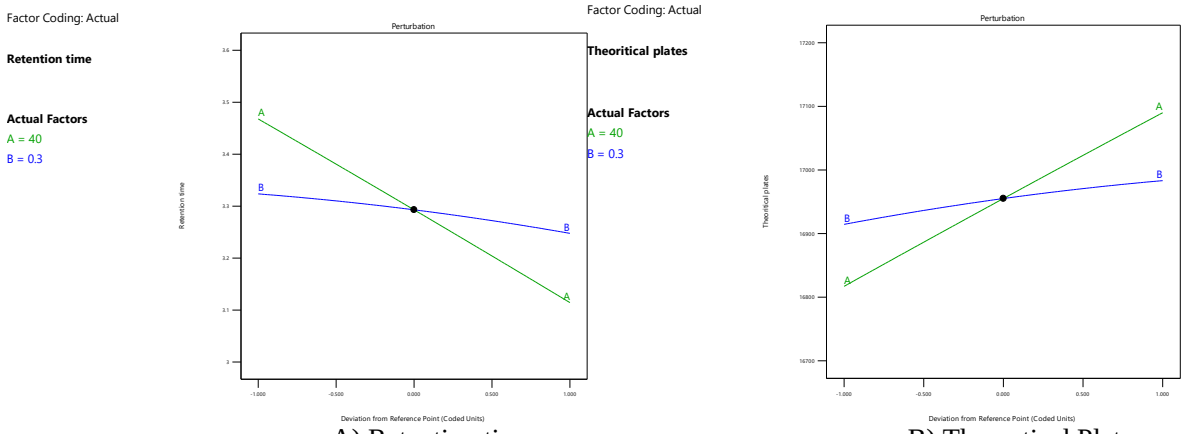
Table 1

Parameters optimized for the analysis of Nirogacestat and its impurities using central composite design

Run	Factor 1	Factor 2	Response 1	Response 2
	A:Acetonitrile	B:Flow rate	Retention time	Theoretical plates
1	47.07	0.37	3.063	17101
2	47.07	0.23	3.15	17056
3	40	0.3	3.29	16945
4	40	0.3	3.292	16960
5	40	0.4	3.222	17005
6	50	0.3	3.04	17149
7	32.93	0.23	3.487	16760
8	40	0.3	3.294	16951
9	30	0.3	3.54	16773
10	32.93	0.37	3.433	16837
11	40	0.2	3.336	16898
12	40	0.3	3.295	16966
13	40	0.3	3.294	16954

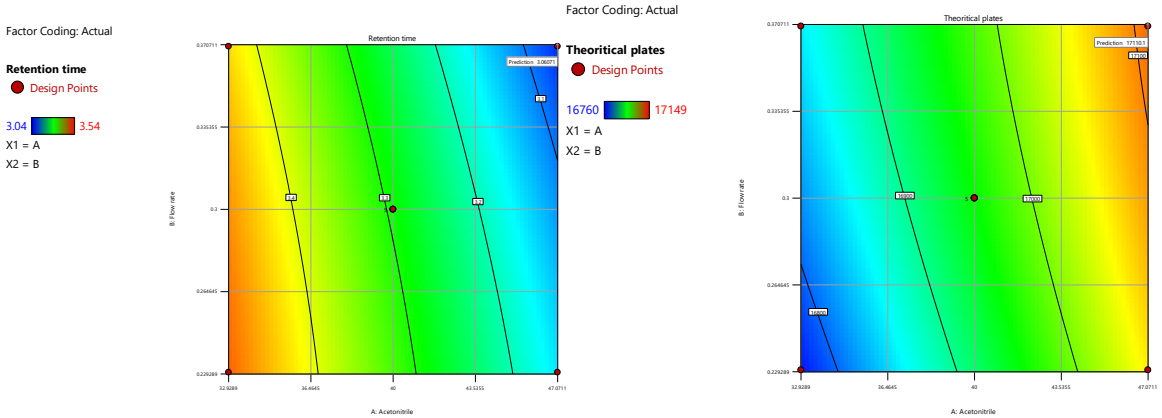
The perturbation charts were used to understand the influence of individual factors on method developed for analysis of nirogacestat and its impurities. These charts help to visualize the method response towards the change in specific factors while keeping all other parameters constant at a reference point. This approach allows clearer assessment of specific factors that shows most significant impact on method performance. The perturbation charts related to retention time (Figure 3A) and theoretical plates (Figure 3B) provide insight into how variations in chromatographic conditions affect peak behavior.

Additionally, 2D contour plots and 3D surface plots were generated to further analyze the relationships between method variables. The contour plots, surface plots and overlay plots corresponding to the theoretical plates and tailing factor of nirogacestat in design of experiments (DoE) studies are illustrated in figures 4, 5 and 6 respectively.



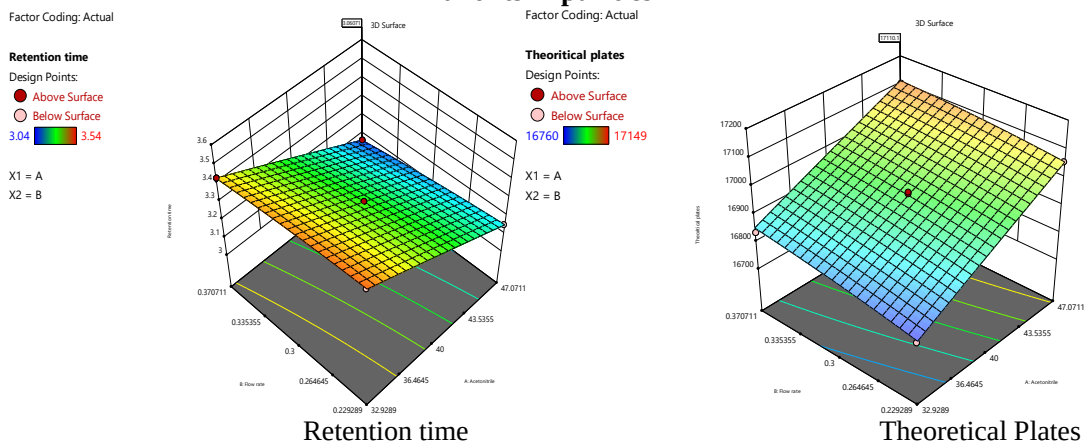
A) Retention time B) Theoretical Plates

Figure 3: Perturbation charts observed during optimization of analytical method for the analysis of Nirogacestat and its impurities



Retention time Theoretical Plates

Figure 4: 2D Contour plot observed during optimization of analytical method for the analysis of Nirogacestat and its impurities



Retention time Theoretical Plates

Figure 5: 3D Surface plots observed during optimization of analytical method for the analysis of Nirogacestat and its impurities

These graphical representations help to identify the optimal conditions for achieving high separation efficiency, well-shaped peaks and reproducible retention times for the analysis of nirogacestat and its impurities.

The results achieved in QbD DoE suggest the finalized chromatographic conditions for the analysis of nirogacestat and its impurities were established through a systematic method optimization process. The optimized method employs X-Bridge C18 column (50 × 4.6 mm, 2.1 µm) using mobile phase composition of acetonitrile and 0.1% TFA in a 40:60 (v/v). This column and solvent at 0.3 mL/min produce efficient separation of all analytes with minimal peak tailing whereas the minimal flow rate ensures better elution time and enhanced resolution without compromising the overall run time.

The injection volume was optimized to 5 µL to maintain reproducible peak areas and to prevent column overloading. Detection was carried out at the iso-absorption wavelength of 299 nm using PDA detector that ensures consistent quantification of nirogacestat and its impurities. The chromatogram noticed in the optimized conditions was presented in figure 7.

Method validation: The analysis of standard solution in the proposed method demonstrates the method effectiveness in achieving well-resolved, symmetrical and reproducible peaks for nirogacestat and its impurities[14]. The specificity study confirms that there was no interference from blank or placebo samples, as no peaks were observed in their respective chromatograms. This indicates that the method is highly selective for nirogacestat and its impurities, with no co-eluting peaks from excipients or other formulation components. The USP tailing factor for nirogacestat and its impurities remains within acceptable limits (ranging from 1.04 to 1.12) confirming good peak symmetry and minimal peak distortion.

The USP plate count which is a measure of column efficiency, was found to be highest for nirogacestat (17,195) whereas impurities exhibited in the range of 4,435 to 7,905 indicate satisfactory column performance and efficient separation. The USP resolution values were above the baseline acceptance criteria ensuring proper separation between peaks. The specificity results along with the chromatographic performance parameters (Table 2) proved that the method was suitable for routine analysis and quality control of nirogacestat and its impurities.

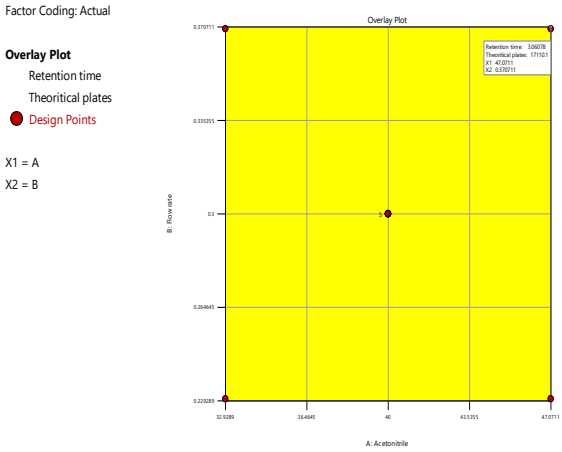


Figure 6: Retention time and theoretical plates overlay plots noticed during optimization of analytical method for quantification of Nirogacestat and its impurities

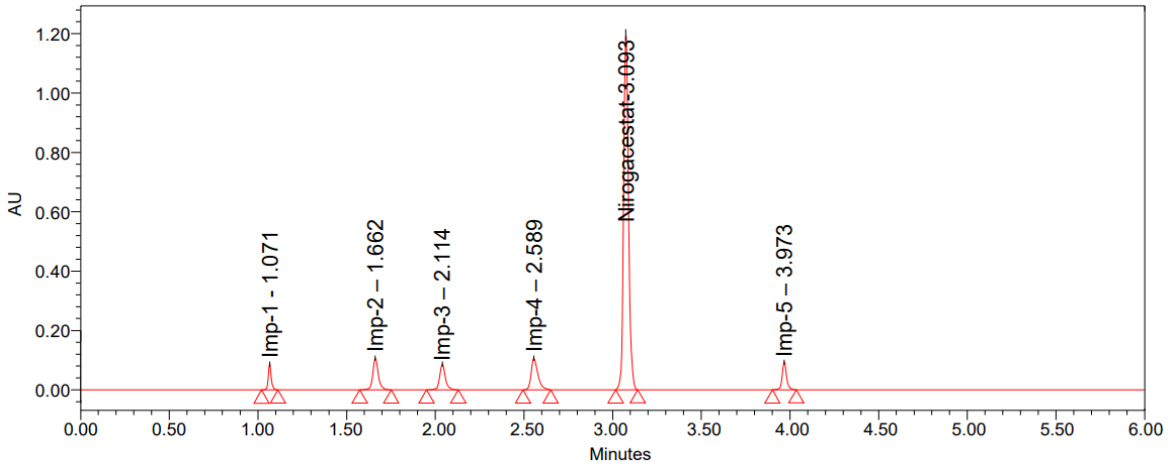


Figure 7: Chromatogram observed in the optimized conditions for the analysis of Nirogacestat and its impurities

The linearity study of the proposed method was assessed by preparing and analysing the calibration curve dilutions over a wide concentration range. The results demonstrated an excellent linear relationship between the analyte concentration and peak area response over a concentration range of 50–300 µg/mL for nirogacestat and 2.5–15.0 µg/mL for impurities. The correlation coefficient (R^2) values for nirogacestat and its impurities in the calibration range were found to be greater than 0.999 indicating strong linearity across the studied concentration ranges. These results (Table 3) confirm that the proposed method is highly linear over the specified concentration ranges that make it suitable for the precise and reproducible quantification of nirogacestat and its impurities in pharmaceutical formulations.

The precision of method proposed for the analysis of nirogacestat and its impurities was evaluated through method precision and intermediate precision studies. The method precision study involves six consecutive injections in 100 % precision level in linearity range analyzed under the same analytical conditions and the %RSD of peak area response was assessed for all the analytes. The %RSD values were exhibited to be within regulatory acceptance limits of 1.634% for nirogacestat and impurities in the range between 0.293% and 0.426%.

Table 2
System suitable results noticed in the proposed method for the analysis of Nirogacestat and its impurities

S.N.	Name	Area	USP Tailing	USP Plate Count	USP Resolution
1	Nirogacestat	16458425	1.11	17195	4.19
2	Impurity 1	65362	1.12	4435	-
3	Impurity 2	83487	1.07	5446	4.67
4	Impurity 3	75593	1.04	6878	3.83
5	Impurity 4	81324	1.12	7905	3.72
6	Impurity 5	70543	1.10	7137	7.64

Table 3
Linearly results noticed in the method proposed for the analysis of Nirogacestat and its impurities

S.N.	Concentration in µg/mL	Area response	Concentration in µg/mL	Area response of impurity				
				1	2	3	4	5
1	50	4184752	2.5	16589	19847	18974	20316	18547
2	100	8246824	5.0	32547	42856	37985	40845	36854
3	150	12504236	7.5	48215	63524	55653	61074	55142
4	200	16524201	10.0	65523	83365	75421	81320	70726
5	250	20365947	12.5	81052	104577	93652	100653	89563
6	300	24152368	15.0	92564	124563	112945	121426	108431
Correlation coefficient		0.99980		0.99903	0.99985	0.99995	0.99997	0.99979
Slope		80783.48		6279.91	8337.97	7508.96	8077.53	7159.96
Intercept		165096.39		970.64	141.21	58.54	223.39	480.75

Table 4
Precision results achieved in the method proposed for the analysis of Nirogacestat and its impurities

Parameter	Area values					
	Nirogacestat	Impurity 1	Impurity 2	Impurity 3	Impurity 4	Impurity 5
Method precision	16389629	65635	83732	75947	81654	70320
	16292627	65230	83956	75842	81325	70458
	16706485	65574	83514	75365	81405	70154
	16162126	65821	83059	75208	81984	70845
	16169053	65493	83369	75948	81632	70263
	15914548	65182	83085	75471	81759	70138
% RSD	**1.634**	**0.374**	**0.426**	**0.426**	**0.293**	**0.375**
Intermediate precision	16458425	68023	83458	75648	81498	70534
	16239847	68563	83263	75421	81457	70321
	16854127	68457	83320	75845	81156	70658
	16024635	68514	83421	75295	81248	70465
	16189452	68362	83263	75584	81496	70123
	16142316	68956	83201	75618	81742	70489
% RSD	**1.832**	**0.442**	**0.120**	**0.252**	**0.255**	**0.265**

The consistent area values obtained for nirogacestat and its impurities indicates high precision and reproducibility of the method. Similarly, in the intermediate precision study, the %RSD was noticed to be 1.832 for nirogacestat and impurities in the range of 0.120 to 0.442. The low %RSD values in both precision studies (Table 4) confirm the reliability and reproducibility of method optimized for the routine quantification of nirogacestat and its impurities.

The robustness of proposed method for the analysis of nirogacestat and its impurities was evaluated by deliberately varying critical parameters such as flow rate and organic modifier composition. The results achived in this study are used to assess the impact of method variations on peak area response. The results indicate that the method is resilient to minor changes in chromatographic conditions.

When the flow rate was increased, the % change in peak area response was noticed to be 0.399% for nirogacestat and impurities in the range of 0.429% and 1.891%. Conversely, when the flow rate was decreased, a slightly higher variation was observed, with nirogacestat exhibiting a % change of 1.576% and impurities in the range of 0.400% to 1.545%.

Similarly, the change in organic modifier concentration also shows very nominal change in peak area response suggesting minor but controlled effects on response (Table 5). These findings confirm that the proposed method is robust and capable of delivering consistent and reliable results even under slightly altered chromatographic conditions.

The accuracy of method proposed for quantification of nirogacestat and its impurities was evaluated through recovery studies and was conducted at three different concentration levels: 50%, 100% and 150%. At the 50% recovery level, a prepared concentration of 100 µg/mL results in a mean estimated concentration of 100.60±0.252 µg/mL, yields a mean recovery of 100.6% with a %RSD of 0.25.

Similarly, at the 100% level, the prepared concentration of 200 µg/mL shows a mean estimated concentration of 200.20±1.822 µg/mL, corresponds to a mean recovery of 100.1% with a %RSD of 0.91. At 150% level, the prepared concentration of 300 µg/mL results in a mean estimated concentration of 299.10±1.645 µg/mL, with a mean recovery of 99.7% and a %RSD of 0.55.

Table 5
Robustness results achieved in the method proposed for the analysis of Nirogacestat and its impurities

Parameter	% change in peak area response					
	Nirogacestat	Impurity 1	Impurity 2	Impurity 3	Impurity 4	Impurity 5
+ change in flow rate	0.399	0.429	1.393	1.891	0.899	1.010
- change in flow rate	1.576	0.400	1.009	1.545	0.696	1.243
+ change in organic modifier	1.643	0.911	1.358	0.225	0.298	0.328
- change in organic modifier	0.013	1.625	0.745	1.660	0.032	0.279

Table 6
Recovery results achieved in the method proposed for the analysis of Nirogacestat and its impurities

S.N.	Analyte	Recover level	Concentration prepared in µg/mL	Amount estimated mean±SD	% recovery mean±SD	% RSD
1	Nirogacestat	50%	100	100.60±0.252	100.6±0.252	0.25
2		100%	200	200.20±1.822	100.1±0.907	0.91
3		150%	300	299.10±1.645	99.7±0.551	0.55
4	Impurity 1	50%	5	4.96±0.038	99.1±0.757	0.76
5		100%	10	9.95±0.015	99.5±0.153	0.15
6		150%	15	14.87±0.037	99.1±0.252	0.25
7	Impurity 2	50%	5	5.03±0.027	100.6±0.529	0.53
8		100%	10	10.03±0.036	100.3±0.361	0.36
9		150%	15	14.82±0.113	98.8±0.751	0.76
10	Impurity 3	50%	5	4.99±0.021	99.7±0.416	0.42
11		100%	10	9.98±0.031	99.8±0.306	0.31
12		150%	15	14.90±0.052	99.3±0.351	0.35
13	Impurity 4	50%	5	4.96±0.026	99.2±0.529	0.53
14		100%	10	9.97±0.015	99.7±0.153	0.15
15		150%	15	14.94±0.076	99.6±0.503	0.51
16	Impurity 5	50%	5	4.96±0.020	99.2±0.400	0.40
17		100%	10	9.98±0.042	99.8±0.416	0.42
18		150%	15	14.96±0.139	99.7±0.929	0.93

SD = standard deviation (n = 3)

In terms of impurities, the mean recovery values ranges from 98.8% to 100.6% with %RSD values below 1% for all the impurities in the studied concentrations indicating minimal variability. Each impurity shows consistent recovery across all concentration levels and confirms the method's reliability. The low %RSD values further support the reproducibility of the method that makes it suitable for the accurate quantification of nirogacestat impurities in pharmaceutical formulations.

The LOD and LOQ were determined for nirogacestat and its impurities to evaluate the sensitivity of the developed method. The LOD value represents the lowest detectable concentration and was found to be 0.60 µg/mL for nirogacestat and 0.180 µg/mL for all its impurities. The LOQ value indicates the lowest quantifiable concentration with acceptable precision and accuracy and was established as 2.0 µg/mL for nirogacestat and 0.60 µg/mL for all impurities. These results confirm that the method is highly sensitive that allows precise detection and quantification of even trace amounts of nirogacestat and its impurities in pharmaceutical formulations. The low LOD and LOQ values further validate the method's suitability for stability and impurity profiling studies.

The forced degradation study was conducted to evaluate the stability of nirogacestat under different stress conditions. In stress study, the control sample that was not subjected to any stress shows single peak corresponding to nirogacestat with no degradation observed. In acid degradation, the sample exhibits 1.721% degradation whereas in alkali conditions, a % degradation of 1.029% as exhibited. The chromatograms in these conditions display one additional peak corresponding to degradation product at an elution time of 1.3 min (Figure 8A) and 2.6 min (Figure 8B) respectively. The highest degradation was observed under oxidative conditions (peroxide treatment) with 3.023% degradation. The chromatogram shows one additional peak at 1.9 min (Figure 8C) suggesting the formation of one additional product due to peroxide degradation of nirogacestat.

The thermal stress results the formation of one degradation product at 4.4 min with a % degradation of 1.191% (Figure 8D). The reduction stress condition induces 2.0% degradation, photolytic degradation causes 2.311% degradation and the hydrolytic conditions can induce 1.247% degradation of nirogacestat.

The chromatogram in these stress conditions does not show any additional detection of compounds suggesting that these degradations do not form stable degradation products. The purity angle and purity threshold of peak correspond to Nirogacestat in all stress studies ensure that the peak purity remains unaffected and proved that there is no co-elution of degradation products.

In all cases, the purity angle was significantly lower than the purity threshold indicating that the method successfully differentiates between nirogacestat and its degradation products. As a result, the method was found to be stability-indicating, with all degradation conditions meeting the acceptance criteria. The stress study results of nirogacestat are tabulated in table 7.

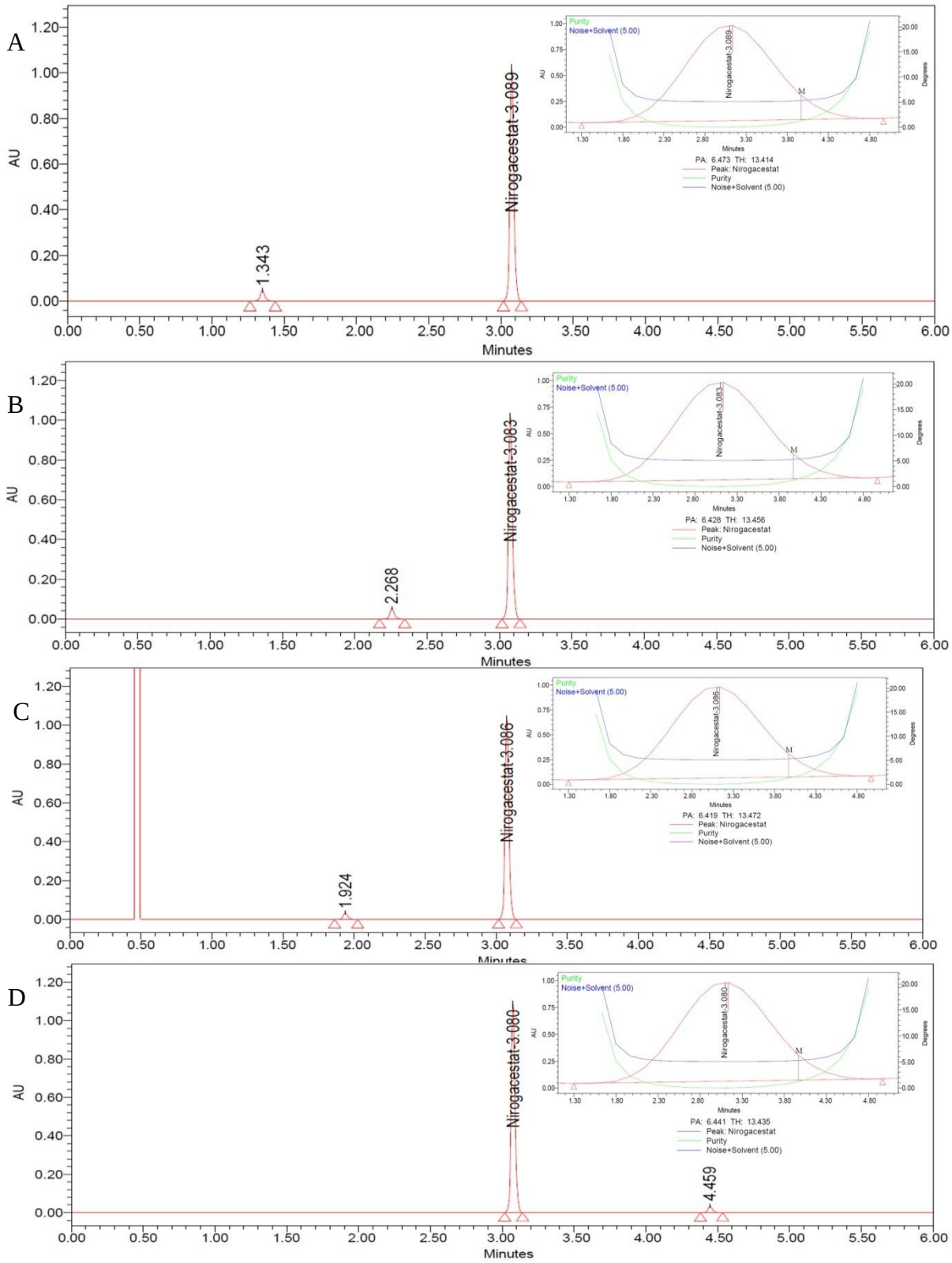
This study established a simple, precise and reliable analytical method for the simultaneous separation and quantification of nirogacestat and its impurities. The proposed method not only enhances sensitivity and specificity but also ensures robust impurity profiling. This study adopted QbD approach that optimizes critical method parameters to achieve superior chromatographic performance by ensuring accurate detection and quantification of impurities.

Conclusion

This study successfully established a robust, precise and reliable UPLC method for the simultaneous separation and quantification of nirogacestat and its impurities. A systematic method development and optimization process was undertaken by incorporating critical chromatographic parameters such as column chemistry, mobile phase composition, flow rate and detection wavelength to achieve high-resolution separation, peak symmetry and enhanced sensitivity. The analytical method was fine-tuned by utilizing QbD approach to ensure optimal performance and robustness.

Table 7
Stress degradation study results noticed for Nirogacestat noticed in the proposed method

S.N.	Degradation condition	% Label Claim	% Degradation	Purity Angle	Purity Threshold	Pass / Fail
1	Control	100	--	6.425	13.427	Pass
2	Acid	98.279	1.721	6.473	13.414	Pass
3	Alkali	98.971	1.029	6.428	13.456	Pass
4	Peroxide	96.977	3.023	6.419	13.472	Pass
5	Reduction	97.974	2.026	6.432	13.469	Pass
6	Photolytic	97.689	2.311	6.496	13.481	Pass
7	Hydrolysis	98.753	1.247	6.404	13.448	Pass
8	Thermal	98.809	1.191	6.441	13.435	Pass



Acid (A); alkali (B); peroxide (C); thermal (D) and the peak purity chromatograms are inserted in all stress study chromatograms

Figure 8: Stress degradation study chromatograms of Nirogacestat noticed in the proposed method

The finalized method employs an X-Bridge C18 column (50 × 4.6 mm, 2.1 µm) with a mobile phase of acetonitrile and 0.1% trifluoroacetic acid (40:60, v/v) at a flow rate of 0.3 mL/min demonstrating to be efficient separation of all analytes with minimal peak tailing and reproducible retention times. The method validation confirms its high specificity, with no interference from excipients and excellent linearity ($R^2 > 0.999$) across a wide concentration

range. The method exhibits strong robustness, with minimal impact from variations in chromatographic conditions and high accuracy, with recovery rates and low variability.

Forced degradation studies confirm the method's stability-indicating capability, effectively differentiating between nirogacestat and its degradation products under various stress conditions. The proposed method is well-suited for routine quality control, impurity profiling and regulatory compliance in pharmaceutical formulations. The integration of the QbD approach enhances the method's reliability, accuracy and precision that make it a superior analytical tool for the quantification of nirogacestat and its impurities.

References

1. Chandrasekar R., Sivagami B., Roshini M., Kumanan R., Sudha S. and Sharma S.K., Design approach in RP-HPLC method development and validation of Nirogacestat in its pure and pharmaceutical dosage form, *J Chem Health Risks*, **14**, 1197 **(2024)**

2. Chen X. et al, Nirogacestat suppresses RANKL-Induced osteoclast formation in vitro and attenuates LPS-Induced bone resorption *in vivo*, *Exp. Cell Res.*, **382**, 111470 **(2019)**

3. FDA, Analytical Procedures and Methods Validation: Chemistry, Manufacturing and Controls, Federal Register (Notices), **65(160)**, 52, 776, 777 **(2000)**

4. Ghos C. et al, Extended progression-free survival and long-term safety of Nirogacestat in patients with desmoid tumors, *J Clin Oncol.*, **40**, https://doi.org/10.1200/JCO.2022.40.16_suppl.11545 **(2022)**

5. Gounder M. et al, Nirogacestat, a gamma-secretase inhibitor for desmoid tumors, *N Engl J Med*., **388**, 898 **(2023)**

6. ICH Guideline, Validation of analytical procedures: text and methodology Q2 (R1), Current Step 4 Version **(2005)**

7. Jayagopal B. and Shivashankar M., Analytical Quality by Design – A Legitimate Paradigm for Pharmaceutical Analytical Method Development and Validation, *Mech Mater Sci Eng J*., Doi: 10.2412/mmse.96.97.276 **(2017)**

8. Murali Krishnam Raju P., Shyamala B., Venkata Narayana, Dantuluri H.S.N.R. and Bhupatiraju R.V., A fast, validated UPLC method coupled with PDA-QDa detectors for impurity profiling in betamethasone acetate and betamethasone phosphate injectable suspension and isolation, identification, characterization of two thermal impurities, *Ann Pharm Fr.*, **80**, 837 **(2022)**

9. Rajesh Varma Bhupatiraju, Bikshal Babu Kasimala, Lavanya Nagamalla and Fathima Sayed, Structural evaluation of degradation products of Loteprednol using LC-MS/MS: Development of an HPLC method for analyzing process-related impurities of Loteprednol, *Anal. Sci. Technol*., **37**, 98 **(2024)**

10. Rajesh Varma Bhupatiraju, Pavani Peddi, Venkata Swamy Tangeti and Battula Sreenivasa Rao, Establishing and validating an HPLC protocol for pralsetinib impurities analysis, coupled with HPLC-MS/MS identification of stress degradation products, *Anal. Sci. Technol.*, **37**, 280 **(2024)**

11. Rajesh Varma Bhupatiraju, Srinivasa Kumar B., Venkata Swamy Tangeti, Kandula Rekha and Fathima Sayed, LC and LC-MS/MS Studies for Identification and Characterisation of Related Substances and Degradation Products of Abrocitinib, *Toxicol. Int.*, **31**, 321 **(2024)**

12. Ravinder Bairam, Hemant Kumar Tatapudi, Vijay Srinivas Pothula, Likhitha Akaram, Sambasiva Rao Tummala and Naveena Gorrepati, Analytical quality by design approach in RP-HPLC method development for the quantification of mirabegron and solifenacin succinate in pharmaceutical formulation, *Lett Appl Nano Bio Science*, **14**, 1 **(2024)**

13. Sambasiva Rao Tummala and Krishnamanjari Pawar Amgoth, LC-MS/MS approach for the quantification of five genotoxic nitrosoimpurities in varenicline, *J Pharm Res*., **26**, 1685 **(2022)**

14. The International Conference on Harmonisation Technical Requirements for Registration of Pharmaceuticals for Human Use on Development and Manufacture of Drug Substances, Chemical Entities and Biotechnological/Biological Entities, Q11, 1 **(2012)**

15. Varma B.H.R. and Rao B.S., Gas Chromatography-Head Space-Mass Spectrometry Sensor based Quality Control of Dobutamine Hydrochloride Bulk Material for a mutagenic impurity, 2-bromopropane, *Res. J. Chem. Environ*., **27**, 54 **(2023)**.

(Received 22nd March 2025, accepted 26th April 2025)