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LC-MS/MS CHARACTERIZATION OF STRESS DEGRADATION PRODUCTS OF GILTERITINIB AND ESTABLISHMENT OF HPLC METHOD FOR ANALYSIS OF PROCESS RELATED IMPURITIES OF GILTERITINIB

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ABSTRACT

Background: The current investigation entails the characterization of seven degradation products (DPs) formed in different stress conditions of gilteritinib employing liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Methodology: This study developed a stability-indicating reversed-phase high-performance liquid chromatographic (HPLC) method for precisely determining gilteritinib in the presence of its process-related impurities in bulk drug and formulation samples. To explore the stability profile of gilteritinib, it was exposed to forced degradation experiments conducted under various conditions, including acidic, basic, oxidative, photolytic, and thermal stress. These experiments revealed the degradation of gilteritinib under basic, acidic, and photolytic conditions, forming seven distinct DPs.

Result: The chromatographic resolution of gilteritinib and its impurities along with DPs was effectively achieved using a Waters Symmetry C18 (250 mm × 4.6 mm, 5 µm) column using equal volumes of solvent A and B (pH 4.5 phosphate buffer and acetonitrile in 25:75 (v/v) as solvent A, acetonitrile and methanol in 75: 25 (v/v) as solvent B) pumped isocratically at 0.7 mL/min and 230 nm wavelength. The method produces an accurate fit calibration curve in 25-175 µg/mL for gilteritinib and LOQ (0.025 µg/mL) – 0.175 µg/mL for its impurities with acceptable precision, accuracy, and recovery.

Conclusion: The efficacy of this method was validated through LC-MS/MS, which allowed for the verification of the chemical structures of newly generated degradation products of gilteritinib. Hence, this method is appropriate for the resolution and evaluation of process-related impurities of gilteritinib and can also be applied for evaluating stress degradation products.

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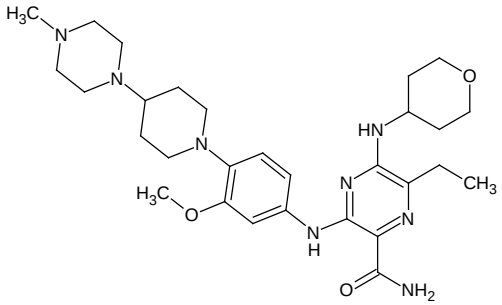
INTRODUCTION

Gilteritinib is a kinase inhibitors class anti-cancer medical drug prescribed for treating relapsed or refractory acute myeloid leukemia. It selectively inhibits the mutations and internal tandem duplication of the FLT3 receptor [1]. It works by inhibiting the phosphorylation of FLT3 and its downstream targets like AKT, ERK, and STAT5 [2]. The more common side effects, such as blurred vision, chest pain, chills, confusion, cough, dizziness, dizziness, faintness, fainting, irregular heartbeat, bloody urine, fever, and headache, are possible during the usage of gilteritinib (figure 1). Pharmaceutical impurities refer to unwanted substances in drug products due to various factors such as synthesis, degradation, or formulation. Among various impurities, degradation impurities specifically result from chemical breakdown or transformation of the drug molecule under conditions like heat, light, or pH extremes. These impurities may be toxic or cause adverse effects, making their identification and quantification essential for patient safety.

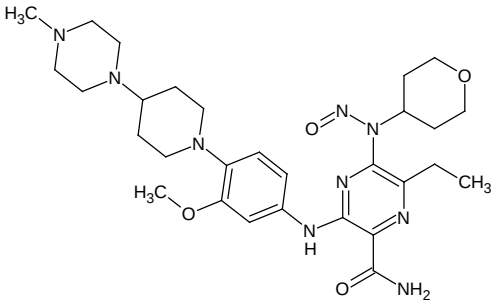
The literature review for available literature proved that only one analytical method was reported for quantifying gilteritinib, and one HPLC and one UPLC method was reported for analyzing gilteritinib in dosage forms. In literature, few bio-analytical methods reported for quantifying gilteritinib in biological samples and reported the pharmacokinetic profile using HPLC [3,4], HPTLC [5], LCMS/MS [6] and UPLCMS/MS [7,8]. No method is available for quantifying process-related impurities of gilteritinib. Hence, this study planned to propose a sensitive HPLC method for quantifying potential process-related gilteritinib impurities and the proposed method's applicability for resolution and structural characterization of forced degradation products of gilteritinib using LC-MS/MS. The impurities such as nitroso impurities 1, 2, and 3 of gilteritinib were selected based on availability to develop analytical methods for quantifying these process-related impurities in gilteritinib bulk drugs and formulations.



A) Gilteritinib

Systemic name: *6-Ethyl-3-[3-methoxy-4-[4-(4-methylpiperazin-1-yl)piperidin-1-yl]anilino]-5-(oxan-4-ylamino)pyrazine-2-carboxamide*

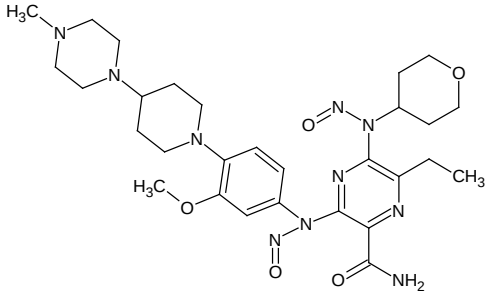
Formula: $C_{29}H_{44}N_8O_3$, ***Mass***: 552.7 g/mol



B) Nitroso impurity 1

Systemic name: *6-Ethyl-3-((3-methoxy-4-(4-(4-methylpiperazin-1-yl)piperidin-1-yl)phenyl)amino)-5-(nitroso(tetrahydro-2H-pyran-4-yl)amino)pyrazine-2-carboxamide*

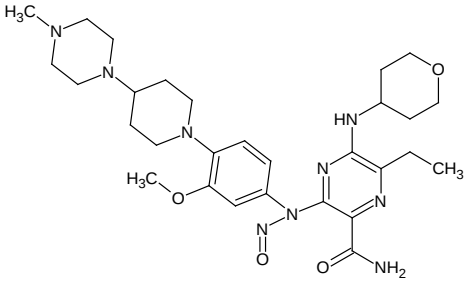
Formula: $C_{29}H_{43}N_9O_4$, ***Mass***: 581.7 g/mol



C) Nitroso impurity 2

Systemic name: *6-Ethyl-3-((3-methoxy-4-(4-(4-methylpiperazin-1-yl)piperidin-1-yl)phenyl)(nitroso)amino)-5-(nitroso(tetrahydro-2H-pyran-4-yl)amino)pyrazine-2-carboxamide*

Formula: $C_{29}H_{42}N_{10}O_5$, ***Mass***: 610.7 g/mol



D) Nitroso impurity 3

Systemic name: *6-Ethyl-3-((3-methoxy-4-(4-(4-methylpiperazin-1-yl)piperidin-1-yl)phenyl)(nitroso)amino)-5-((tetrahydro-2H-pyran-4-yl)amino)pyrazine-2-carboxamide*

Formula: $C_{29}H_{43}N_9O_4$, ***Mass***: 581.7 g/mol

Figure 1: Systemic details of gilteritinib and its impurities in the study

MATERIALS AND METHODS

Instrumentation and Chemicals

The gilteritinib process-related impurities were quantified on the LCMS system (alliance 2695 model, Waters, Japan) connected with the optima ZQ mass analyzer (Waters, Japan) and masslynx 4.2 software. The gilteritinib (98.70 %) and its studied potential process-related impurities, such as nitroso impurities 1, 2, and 3, were obtained from Astellas Pharma India Pvt. Ltd., Mumbai. The 40 mg pharmaceutical tablet formulation of gilteritinib with brand Xospata® was obtained from Singh Traders, Kanpur, Uttar Pradesh. LiChropur™ grade chemicals such as trifluoroacetic acid, ammonium acetate, HPLC grade solvents methanol, and acetonitrile, & 0.2µ filters were brought from Merck Chemicals, Mumbai.

Stock and standard solution preparation

The stock solution and subsequent dilutions of gilteritinib and its process-related impurities were prepared independently with the same diluent. Initially, an appropriate quantity of gilteritinib and impurities was dissolved separately in a diluent to achieve 0.1 mg/mL solution. Then a series of dilutions were made to achieve 25 to 175 µg/mL separately, and an equal volume of the same level concentration of gilteritinib and impurities was mixed to obtain calibration curve concentration [9].

Formulation solution preparation

Xospata® tablets of gilteritinib were utilized to evaluate the efficiency of the method in quantifying genotoxic impurities. Xospata® tablets were finely powdered, and an appropriate quantity of fine powder was dissolved in 100 mL solvent to achieve a 0.1 mg/mL concentration of gilteritinib. The undissolved tablet particles were removed by filtration through a 0.2 µ filter and diluted to a precision-level concentration [10].

Method development

The separation, qualitative, and quantitative evaluation of process-related impurities in gilteritinib followed existing literature [11, 12] and guidelines outlined by ICH [13]. The method optimization process utilizes 100µg/mL of gilteritinib and 0.1 µg/mL its impurities. Various method conditions were systematically altered, and for each change, the analysis included verification of peak area response, symmetry, suitability, and mass pattern to confirm the method [14]. Optimization of method parameters, such as the composition, pH, and flow rate of the mobile phase, as well as column configuration and temperature, was performed. The conditions that yielded acceptable results were deemed suitable and subsequently advanced for further validation [15].

Method validation

The optimized method underwent comprehensive validation, including assessments for sensitivity, analysis range, ruggedness, robustness, and in accordance with ICH guidelines [13] and relevant literature sources [16]. Additionally, the developed method was scrutinized for its suitability in evaluating process related impurities in formulations.

Forced degradation studies

Forced degradation studies were conducted to assess the stability of the drug under various conditions. A known concentration of gilteritinib solution was prepared in HPLC-grade methanol and subjected to different stress conditions. Hydrolytic degradation was evaluated by treating the drug with acidic (0.1 M HCl) and basic (0.0 M NaOH) solutions separately for 24 hours at 70°C. Oxidative degradation was induced using 15% hydrogen peroxide for 2 days [17]. Thermal stress was applied by heating the drug in a sealed ampoule at 70°C for 7 days. Photolytic degradation was assessed by exposing the drug to sunlight and a photostability chamber for 7 days [18]. Samples from each stress condition were analyzed using LC–MS/MS and UV detection to identify and characterize degradation products. The resulting data provided insights into the drug's susceptibility to degradation under different environmental stressors.

RESULTS AND DISCUSSION

This study aimed to develop a robust HPLC method for analyzing gilteritinib and its impurities in pharmaceutical dosages. Initial method development involved determining the optimal detection wavelength using a spectrophotometer, confirming 264 nm as suitable for detection. Several chromatographic conditions were evaluated. Initially, a 50:50 acetonitrile-methanol mobile phase with a C18 column did not adequately separate analytes, showing unresolved peaks and baseline disturbances. Changing to a water-acetonitrile mobile phase with a ProntoSIL ODS C18 column improved separation but did not meet resolution criteria. Subsequent trials with an 80:20 water-acetonitrile composition on a C18 column showed minimal improvement in peak resolution and area responses. Methanol-water (20:80) and methanol-ammonium acetate (50:50) mixtures were also tested but yielded unsatisfactory

resolution and peak area responses. Further optimization using methanol-ammonium acetate (50:50) at different flow rates and column lengths did not achieve sufficient resolution and symmetry for gilteritinib and impurity peaks. Finally, the isocratic 0.7 mL/min flow of pH 4.5 phosphate buffer and acetonitrile in 25:75 (v/v) as solvent A, acetonitrile and methanol in 75: 25 (v/v) as solvent B was finalized to be appropriate for the resolution of nitroso impurities along with gilteritinib. The optimizing a suitable mass detector conditions was very crucial for effective and very sensitive detection of impurities. The electrospray ionization (ESI) source at positive and negative ionization mode was evaluated for effective detection of analytes. In the mass spectra, the intensity of fragments at positive ionization mode was significantly higher than negative mode and hence positive ion mode was finalized as appropriate for sensitive detection of impurities. Optimal ion source parameters were fine-tuned to achieve the desired response, favourable peak shapes, and precise quantitation.

Table 1: optimized mass operating conditions for analysing process related impurities of gilteritinib

Compound	Gilteritinib	NI 1	NI 2	NI 3
Parent ion (m/z)	552	581	610	581
Product ion (m/z)	169	198	249	204
Fragmentor (V)	155	138	135	135
Electron Multiplier Voltage (V)	700	700	700	700
Collision energy (eV)	40	30	25	25
MS1 RES	Wide	Wide	Wide	Wide

NI – Nitroposo impurity

The specificity of fine-tuned method was assessed by analysing a standard solution containing a concentration of 100 µg/mL of gilteritinib and 0.1 µg/mL of its impurities, along with the diluent as a blank. In the chromatogram for the blank (figure 2), no peaks were observed in entire runtime. Conversely, the standard chromatogram displayed well-resolved and symmetric peaks representing gilteritinib, impurities in this study.

In this study, a comprehensive evaluation of chromatographic parameters was conducted to assess the analysis of gilteritinib and its nitroso impurities using an HPLC method. The retention times (tR) for gilteritinib and the three nitroso impurities were determined to be 9.39 minutes, 6.34 minutes, 11.28 minutes, and 2.17 minutes, respectively. Relative retention times (RRT) of the impurities ranged from 0.23 to 1.20, indicating satisfactory separation from the main component. Relative response factors (RRF) for the impurities were low, with values ranging from 0.058 to 0.096, suggesting varying detection sensitivities compared to gilteritinib. Resolution (R_S) values between gilteritinib and each impurity exceeded 2, demonstrating effective separation. Additionally, asymmetry (A_S) values close to 1 indicated symmetrical peak shapes. The number of theoretical plates (N) for all components exceeded 2000, indicating good column efficiency. % RSD of peak area values (ranging from 0.41% to 0.93%) demonstrated high method precision and reproducibility. These findings validate the developed HPLC method's capability to separate and quantify gilteritinib and its nitroso impurities with high sensitivity, resolution, and precision, meeting the requirements for pharmaceutical analysis**.**

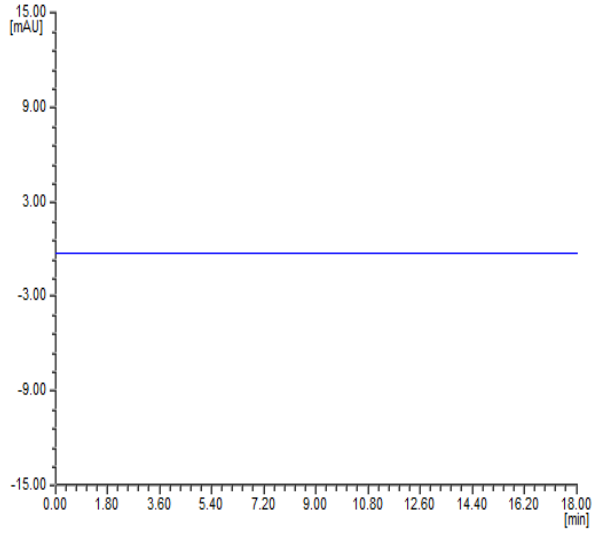


Figure 2 Un-spiked chromatogram in the optimized method that doesn't show any chromatographic detection

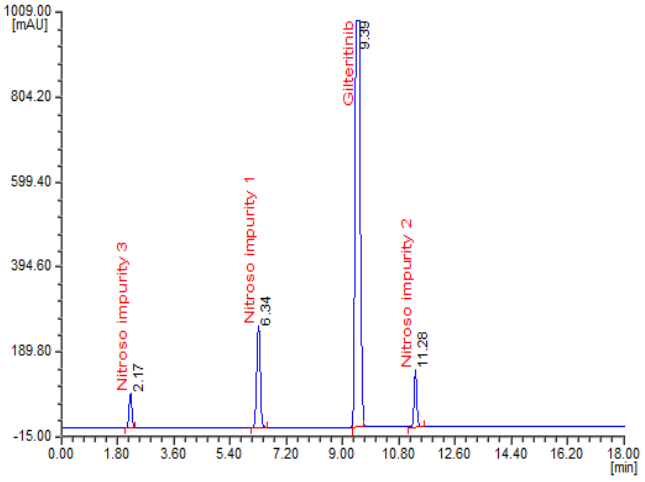


Figure 3 System suitability chromatogram obtained in the optimized method that clearly resolve the analytes

Table 2: System suitability results

Parameter	Experiment results for				AC
	GN	NI 1	NI 2	NI 3	
t_R (min)	9.39	6.34	11.28	2.17	--
RRT	--	0.68	1.20	0.23	< 2
RRF	--	0.096	0.069	0.058	--
R_S	7.15	9.78	5.64	--	> 2
A_S	0.98	1.02	0.93	0.95	< 2
N	5036	7492	9670	13251	> 2000
% RSD	0.34	0.58	0.41	0.93	< 2

GN–gilteritinib, **NI**–Nitroso impurity, **RSD** – RSD of peak area

Method Validation

The developed method involved the analysis of various concentrations of gilteritinib and its impurities. Calibration curve was established by correlating the area response of individual peaks with analyte strength. A highly correlated and accurately fitting linear curve was achieved with in 25 µg/mL to 175 µg/mL concentration level for gilteritinib and LOQ (0.025 µg/mL) – 0.175 µg/mL for its impurities. The calibration parameters including intercept, slope and regression equation was evaluated by performing least-squares linear regression analysis. The linear equations obtained were as follows:

For gilteritinib: $y = 10671x – 15495$, $R^2 = 0.9998$,

For Nitroso impurity 1: $y = 945513x + 4633.6$, $R^2 = 0.9996$

For Nitroso impurity 2: $y = 644616x + 9069.1$, $R^2 = 0.9991$

For Nitroso impurity 3: $y = 533341x + 5391.9$, $R^2 = 0.9995$

These results demonstrating linearity are summarized in Table 3.

Table 3: Linearity results

Concentration (µg/mL)	Gilteritinib	Concentration (µg/mL)	Peak are response obtained		
			Nitroso Impurity 1	Nitroso Impurity 2	Nitroso Impurity 3
25	249694.3	0.025	29053.7	25963.1	18749.8
50	525747.2	0.05	51153.6	42191.7	31689.1
75	789193.5	0.075	74574.2	56491.6	45410.3
100	1041071.3	0.1	100412.8	72267.5	59915.7
125	1306327.6	0.125	121956.7	88876.9	71041.3
150	1594758.7	0.15	147485.4	105654.8	85491.2
175	1854591.3	0.175	169658.1	123269.7	98784.3

Table 4 Accuracy results (n=3 ($))

Parameter	Gilteritinib	Nitroso Impurity 1	Nitroso Impurity 2	Nitroso Impurity 3
Accuracy at 50 % level$ (µg/mL)				
Amount added	75	0.075	0.075	0.075
Recovered	74.45	0.075	0.073	0.075
% Recovery	99.26	100.27	97.64	99.56
% RSD	1.73	1.40	1.53	0.85
Accuracy at 100 % level$ (µg/mL)				
Amount added	100	0.10	0.10	0.10
Recovered	98.50	0.16	0.16	0.16
% Recovery	98.50	100.08	99.10	100.04
% RSD	1.32	0.45	0.79	0.68
Accuracy at 150 % level$ (µg/mL)				
Amount added	125	0.125	0.125	0.125
Recovered	124.17	0.124	0.120	0.125
% Recovery	99.34	99.40	96.24	99.62
% RSD	0.17	0.91	0.22	0.65

The recovery experiment was executed at concentration levels of 75 µg/mL, 100 µg/mL, and 125 µg/mL within the linearity range for gilteritinib and 0.075 µg/mL, 0.1 µg/mL, and 0.125 µg/mL for impurities. The recovery-level solution underwent

triplicate analysis using the optimized method, and the peak area responses for each analyte were compared with the corresponding responses at the calibration level. The analyte strength equivalent to recovery was assessed in this proposed method. The chromatographic response of individual analytes was correlated with equivalent level calibration curve response. The % recovery of gilteritinib and its impurity in each injection was evaluated along with % RSD in every spiked level. According to guidelines, % recovery within the 98-102% range and % RSD of < 2 were deemed acceptable. As indicated in Table 4, the % recovery fell within the range of 98.50 – 99.34, 99.40 – 100.08, 96.24 – 99.10, and 99.56 – 100.04 for gilteritinib, nitroso impurity 1, 2, and 3 respectively. The % RSD at every recovery level was < 2 for gilteritinib and its impurities. The results, meeting the acceptable criteria, affirm the method as recoverable and accurate. The assessment of repeatability and reproducibility of the developed method involved using a standard solution with a concentration of 75 µg/mL for gilteritinib and 0.075 µg/mL for its impurities. The solution underwent six analyses within a day for intraday precision and six analyses over three consecutive days for interday precision. Additionally, three analysts analyzed precision-level solutions in one day (n=6) to evaluate method ruggedness. The peak responses of gilteritinib and its impurities were documented, and % RSD was calculated. Results, summarized in Table 5, revealed % RSD values below 2, confirming the precision and reproducibility of the method.

Table 5: Precision results (n = 6 ($$))

Precision[$$]	**GN**	**NI 1**	**NI 2**	**NI 3**
Intraday	0.40	0.32	0.36	0.39
Interday (day 1)	0.28	0.23	0.85	0.51
Interday (day 2)	0.70	0.68	0.46	0.39

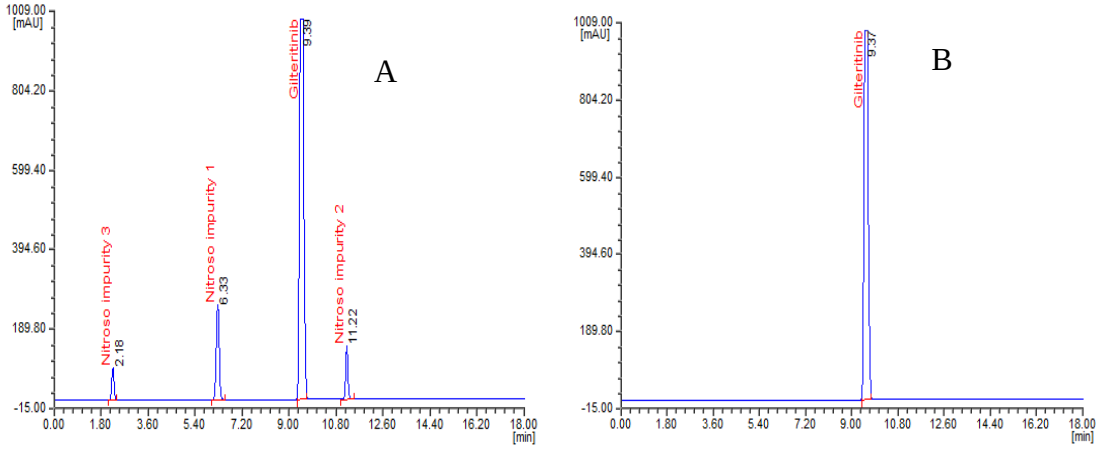
Table 6 Robustness results

S No	**Changed condition**	**Parameter**	**Results observed**			
			Gilteritinib	**Nitroso Impurity 1**	**Nitroso Impurity 2**	**Nitroso Impurity 3**
1	MP 1	% change	0.34	0.54	0.33	0.77
		t_R	9.38	6.35	11.23	2.15
		N	5020	7481	9656	13293
2	MP 2	% change	0.08	1.36	0.33	0.13
		t_R	9.31	6.39	11.25	2.17
		N	5025	7467	9634	13284
3	pH 1	% change	0.65	0.15	0.62	1.75
		t_R	9.32	6.33	11.29	2.16
		N	5052	7732	9619	13316
4	pH 2	% change	0.25	1.69	1.15	0.55
		t_R	9.34	6.37	11.22	2.18
		N	5085	7458	9617	13382
5	WL 1	% change	1.44	0.63	0.68	0.03
		t_R	9.36	6.38	11.24	2.15
		N	5018	7604	9606	13195
6	WL 2	% change	0.83	0.35	0.75	0.78
		t_R	9.37	6.31	11.25	2.19
		N	4993	7564	9533	13179

MP (mobile phase) 1: 45:55 (v/v) of solvent A and B; MP 2: 55:45 (v/v) of solvent A and B; pH 1: 4.4; pH 2: 4.6; WL (wavelength) 1: 235 nm; WL 2: 230 nm

Nominal deviation in proposed conditions, like mobile phase composition (without pH variation) and intentional changes in pH, were introduced to evaluate method robustness. In each altered condition, the standard solution with a concentration of 75 µg/mL for gilteritinib, 0.075 µg/mL for its impurities was analysed. The chromatographic response of individual analyte in every varied condition was correlated with equivalent level calibration curve response. As shown in Table 6, % change

values were below 2 for gilteritinib and its impurities, indicating that the method was rugged with no significant changes observed during minor variations. Method sensitivity was determined by evaluating detection limit (LOD) and quantification limit (LOQ) by following signal-to-noise approach. The established LOD as 0.007µg/mL and LOQ as 0.025 µg/mL respectively confirm the method's high sensitivity for detecting analytes at very low concentrations. Stability testing involves incubating the standard solution for gilteritinib and its impurities in an auto-sampler at 25°C for 48 hours. Analyses were conducted every 6 hours, and % stability was evaluated by correlating area response with calibration curve response at same level. Method stability was proved by observing more than 99 % assay till 24 h. The developed method was successfully applied for the identification and quantification of potential process related impurities in formulations. A formulation solution at a concentration of 100 µg/mL, prepared using Xospata® formulation of gilteritinib, was analysed. The nitroso impurities spiked formulation was also analyzed revealing distinct peaks corresponding to impurities alongside gilteritinib. In contrast, un-spiked sample did not show any peaks at the retention time of nitroso impurities. This demonstrated the method's effectiveness in identifying and quantifying impurities in formulations, making it suitable for quantifying nitroso impurities in gilteritinib bulk drug and formulation dosages.



Chromatogram noticed for formulation solution spiked with impurities (A) and with no impurities spiked (B)

Figure 4: Formulation analysis chromatogram of gilteritinib observed in the developed method

Stability studies

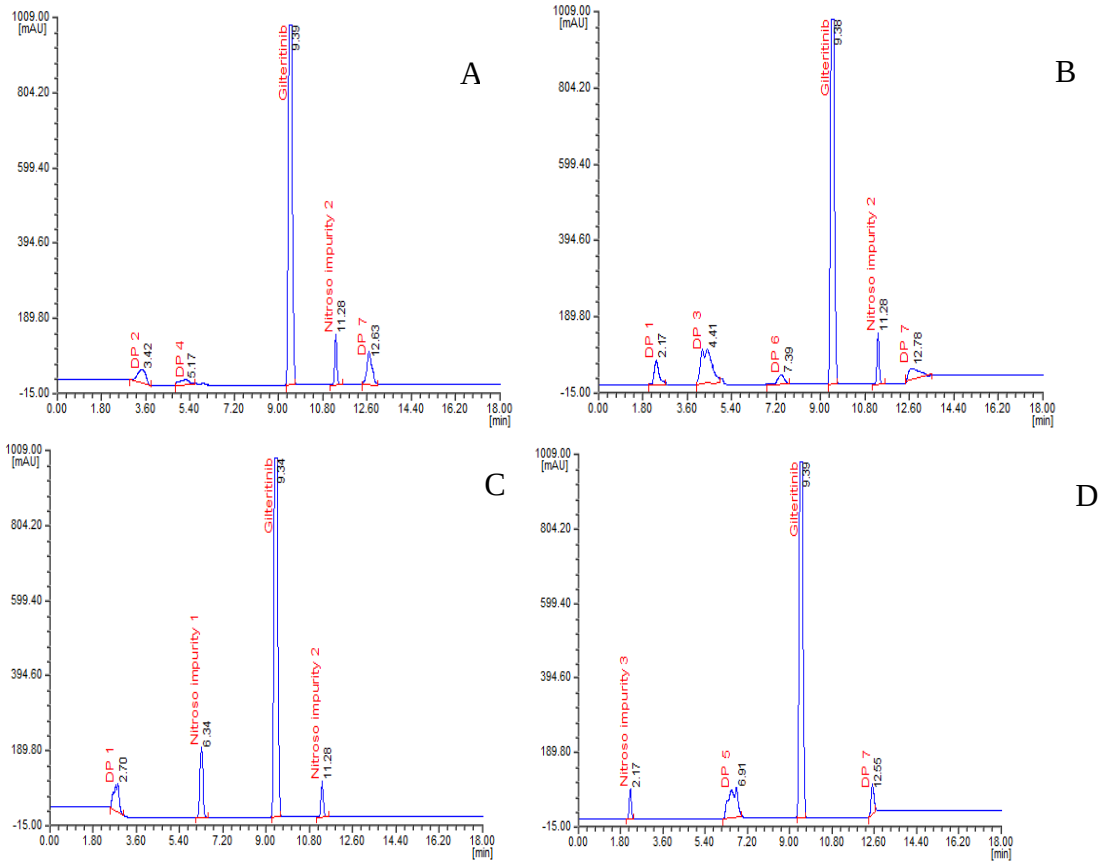
Stability testing is critically significant in pharmaceutical development and manufacturing to ensure drug quality, safety, and efficacy over their shelf-life. It involves evaluating the chemical, physical, and microbiological attributes of a drug substance or product under various conditions (e.g., temperature, humidity, light) to predict its stability and degradation pathways. Stability testing helps establish proper storage conditions, determine shelf life, identify degradation products, and guide formulation improvements. Ultimately, this testing ensures that pharmaceutical products maintain their intended quality throughout their lifecycle, safeguarding patient safety and regulatory compliance. Following the ICH stability Q1A(R2) guidelines, a variety of forced conditions, namely thermal, basic, acidic, oxidative, and photolytic, were employed to conduct degradation studies using the pharmaceutical product of gilteritinib. These studies led to identifying and characterizing seven distinct degradation products, designated as DP1 to DP7, employing HPLC/MS analysis. The outcomes of these investigations have furnished valuable insights into the conditions that render the drug susceptible to degradation, thus facilitating the implementation of appropriate precautionary measures during the formulation process. No significant degradation was observed in thermal degradation conditions, with an assay percentage of 96.26%. Among the various degradation conditions, the most pronounced degradation was noticed in the acid-induced degradation study, where the acid-induced degradation reached 9.67%. Figure 4A depicts the chromatogram from this study, revealing well-separated degradation products with retention times (tR) of 3.42 minutes, 5.17 minutes, and 12.63 minutes, denoted as DP 2, DP 4, and DP 7, respectively. In the base-induced degradation study, as depicted in Figure 4B, four distinct degradation products were resolved at retention times of 2.17 minutes, 4.41 minutes, 7.39 minutes, and 12.78 minutes, designated as DP 1, DP 3, DP 6, and DP 7, respectively. The percentage degradation under base

degradation conditions was measured at 8.97%. Additionally, the chromatogram revealed the presence of nitroso impurity 2 at a retention time of 11.28 minutes. In the peroxide degradation study, the assay percentage for gilteritinib was 5.97%, with a mass balance of 99.13%. The chromatogram for this study delineated a single degradation product with a retention time of 2.70 minutes, labeled as DP 1. The outcomes of the peak purity test, conducted using the PDA detector, provided robust validation of the purity and consistency of the gilteritinib peak across all stress samples under examination. The mass balance for these stressed samples consistently fell within the range of more than 98%. These consistent results from the peak purity tests unequivocally affirmed the uniformity and purity of the gilteritinib peak within the analyzed stress samples. Notably, the gilteritinib assay exhibited remarkable stability even in impurities, further attesting to the specificity and efficacy of the developed method in detecting stability. Table 7 presents the recovery results, and representative chromatograms observed during the forced degradation study are illustrated in Figure 5.

Table 7 Stress degradation results

Condition	% degradation[#] of gilteritinib	% assay[#] of gilteritinib	% Mass balance[$] (assay + total impurities)	Remark
Acidic	9.67	90.33	98.36	DP 2, 4 and 7 were noticed
Basic	8.97	91.03	98.19	DP 1, 3, 6 and 7 were noticed
Peroxide	5.97	94.03	99.03	DP 1 was noticed
Thermal	3.74	96.26	99.75	No degradation was identified
UV light	3.50	92.50	99.13	DP 5 and DP 7 were noticed

[#]average of three replicate experiments



A) Acidic stress study chromatogram of gilteritinib visualizing DP 2, 4 and 7; B) Basic stress study chromatogram of gilteritinib visualizing DP 1, 3, 6 and 7; C) Peroxide degradation chromatogram of gilteritinib visualizing DP 1 D) UV Light degradation chromatogram of gilteritinib visualizing DP 5 and 7

Figure 5: Forced degradation chromatograms of gilteritinib

Characterization of DPs by LCMS/MS:

The stress-induced DPs of gilteritinib were subjected to characterization via LCMS/MS analysis. As optimized in this study, the LC conditions remained unchanged, and the mass operating conditions were fine-tuned to maximize the detection of each mass fragment with minimized or no noise. The collision-induced dissociation spectra of each DP, along with its accurate mass measurements, were noted to evaluate the structure of each DP formed in the stress study.

Figure 6 illustrates the fragmentation mechanism of DP1, with the ESI spectrum (Figure 13A) revealing the most intense [M+H]+ ion at m/z 470 represents the molecular mass of DP 1 as 469 g/mol. The MS/MS spectrum of DP1 exhibited highly intense product ion peaks at m/z-369 (indicating the loss of $C_5H_{13}N_2$), 250 (resulting from the loss of $C_{12}H_{18}N_3O$ from m/z 469), and m/z 180 (loss of $C_{14}H_{20}N_5O_2$ from m/z 273) and the accurate mass measurements suggest the molecular composition of these fragments. The DP 1 was identified as *3-((4-(4-aminopiperidin-1-yl)-3-methoxyphenyl)amino)-6-ethyl-5-((tetrahydro-2H-pyran-4-yl)amino)pyrazine-2-carboxamide* with molecular formula of $C_{24}H_{35}N_7O_3$ and molecular mass of 469 g/mol.

Figure 7 illustrates the fragmentation mechanism of DP 2 of gilteritinib which was identified in acid induced stress study. The fragmentation spectra (Figure 13B) of DP 2 visualizes the parent ion with m/z of 469 (m+1) confirms the molecular mass of DP 2. In the fragmentation spectra, there were notable product ions at m/z 399 (indicative of the loss of C_4H_8N), 290 (resulting from the loss of $C_7H_9N_5O$), 166 (stemming from the loss of $C_{17}H_{27}N_4O$ from parent ion) and m/z 151 (indicative of the loss of $C_{17}H_{28}N_5O$ from parent ion). The MS/MS experiments, in conjunction with accurate mass assessments, provide strong support for the proposed fragmentation scheme. The DP 2 was identified as *5-amino-6-ethyl-3-((3-methoxy-4-(4-(4-methylpiperazin-1-yl)piperidin-1-yl)phenyl)amino)pyrazine-2-carboxamide* with molecular formula of $C_{24}H_{36}N_8O_2$ and molecular mass of 468 g/mol.

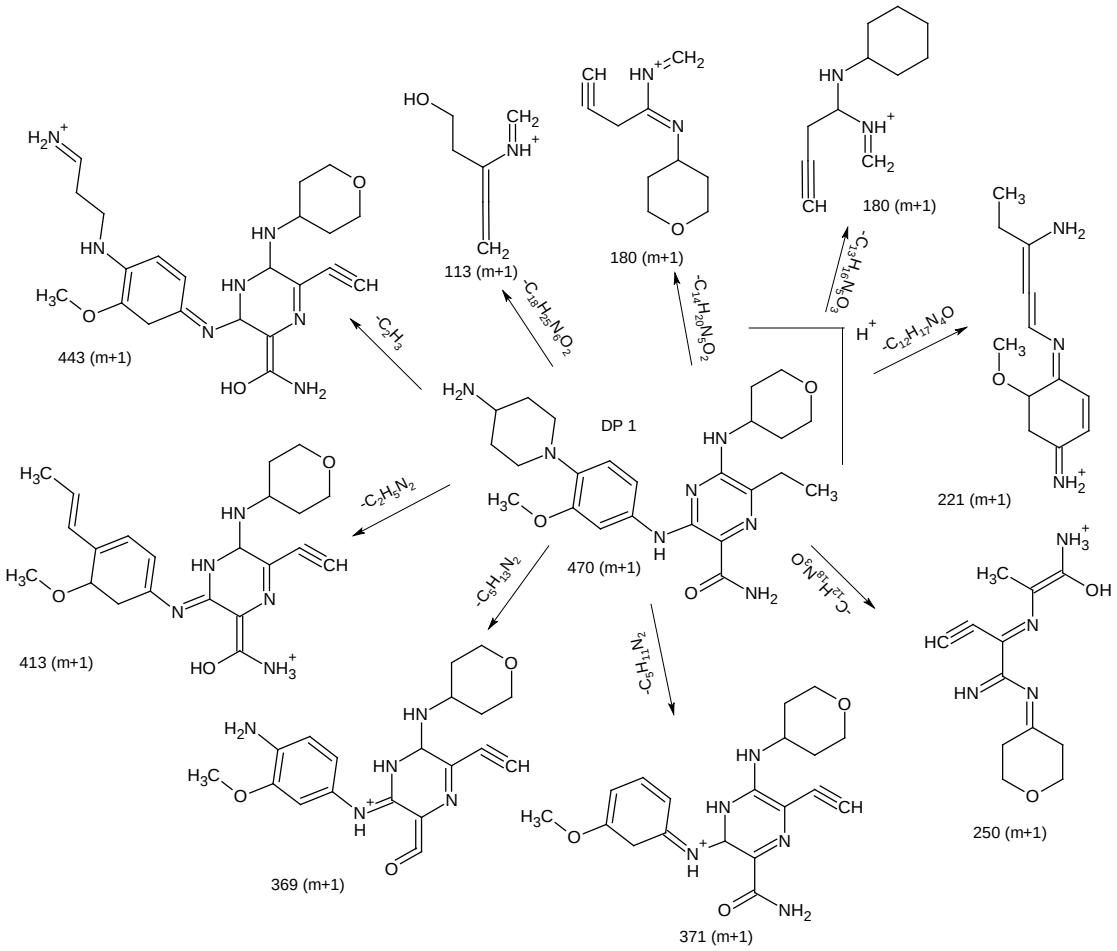


Figure 6: Fragmentation mechanism proposed for DP 1

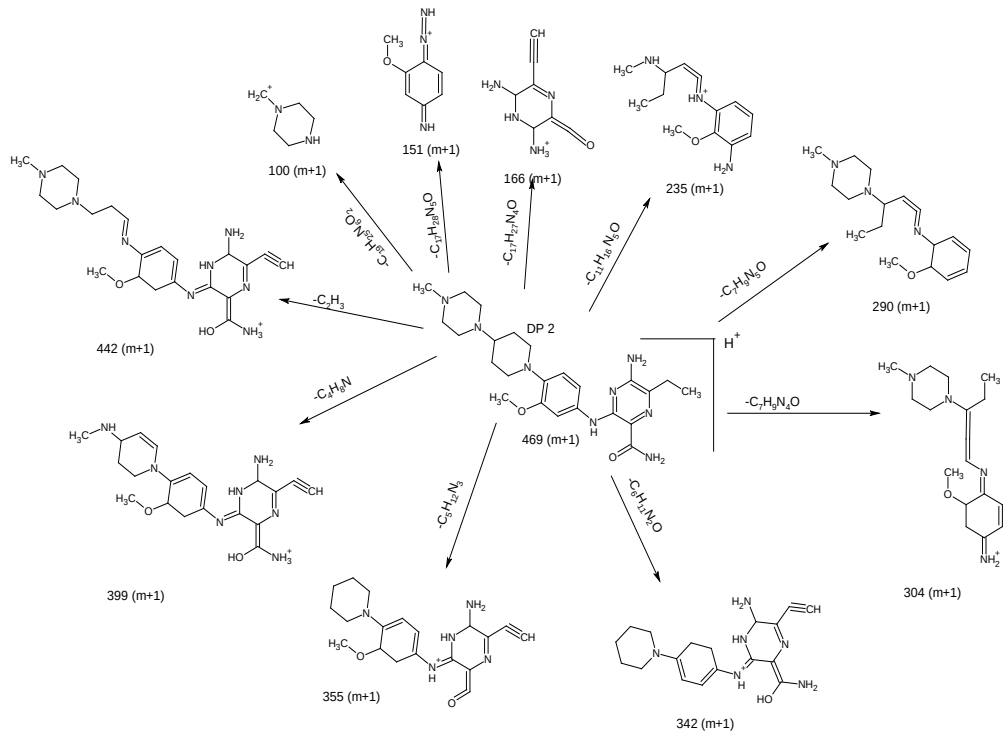


Figure 7: Fragmentation mechanism proposed for DP 2

Figure 8 illustrates the fragmentation mechanism of DP3 of gilteritinib, which was identified in the base-induced stress study chromatogram. The fragmentation spectra of DP 3 as shown in figure 13C visualizes the parent ion with m/z of 412 (m+1) confirms the molecular mass of DP 3. In the fragmentation spectra, there were notable product ions at m/z 259 (indicative of the loss of -$C_{10}H_{17}O$) and m/z 123 (indicative of the loss of $C_{17}H_{25}N_2O_2$ from parent ion). The DP 3 was identified as *3-ethyl-N6-(3-methoxy-4-(piperidin-1-yl)phenyl)-N2-(tetrahydro-2H-pyran-4-yl)pyrazine-2,6-diamine* with molecular formula of $C_{23}H_{33}N_5O_2$ and molecular mass of 411 g/mol.

The ESI-MS spectrum, observed at a t_R of 5.17 minutes (Figure 13D), reveals a parent ion at m/z 386, corresponding to the [M+H]+ ion of DP 4, which is formed under acidic stress conditions. Within this spectrum, there are numerous product ions, notably at m/z 353 (m+1), 325 (m+1), 221 (m+1) and 166 (m+1). The collection of these product ions, in conjunction with the parent ion, serves to confirm that DP 4 as *5-amino-3-((4-(4-aminopiperidin-1-yl)-3-methoxyphenyl)amino)-6-ethylpyrazine-2-carboxamide*, with a molecular formula of $C_{19}H_{27}N_7O_2$. The fragmentation pattern was presented in figure 9. The UV light degradation chromatogram clearly visualizes a peat at 6.91 min and was designated as DP 5 which was not detected in other stress studied performed in the study. The mass fragmentation spectra of DP 5 reveal a prominent parent ion at m/z 455 (m+1) when observed under positive ionization mode. Furthermore, the spectrum exhibits fragment ions at m/z 247 (m+1), which result from the loss of $C_8H_{10}N_5O$. Based on the acquired data (Figure 8), DP 5 has been identified as *5-amino-6-ethyl-3-((4-(4-(4-methylpiperazin-1-yl) piperidin-1-yl)-3-oxocyclohexa-1,4-dien-1-yl)amino)pyrazine-2-carboxamide*, possessing a molecular formula of $C_{23}H_{34}N_8O_2$.

The proposed mass fragmentation pattern of DP 6 is illustrated in Figure 11. The base induced stress degradation chromatogram of gilteritinib visualizes a well resolved and retained peak corresponds to DP 6 at 7.39 min. The mass fragmentation spectra shows parent ion at m/z of 358 corresponds to molecular mass of DP 6. The spectrum visualizes the fragment ion at m/z of 258 formed by losing $C_5H_{10}NO$, 205 formed by losing $C_7H_9N_2O_2$ and 136 formed by losing $C_{11}H_{16}N_3O_2$. The collection of these product ions, in conjunction with the parent ion, serves to confirm that DP 6 as *6-ethyl-3-[(3-oxocyclohexa-1,4-dien-1-yl)amino]-5-(tetrahydro-2H-pyran-4-ylamino)pyrazine-2-carboxamide*, with a molecular formula of $C_{18}H_{23}N_5O_3$.

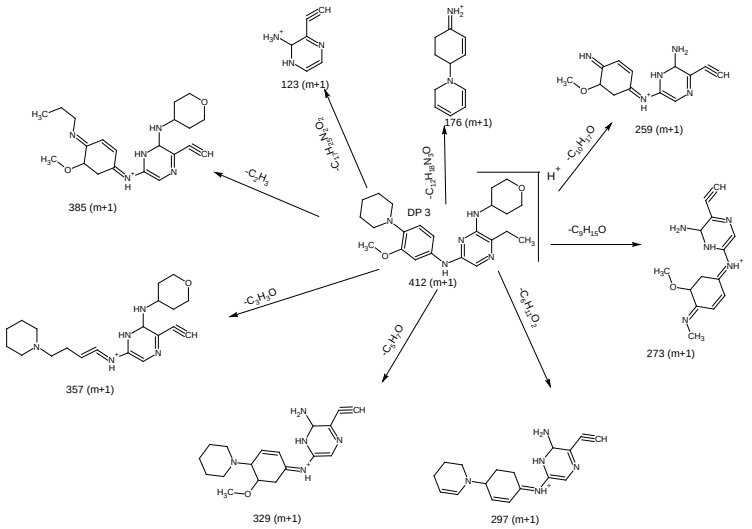


Figure 8: Fragmentation mechanism proposed for DP 3

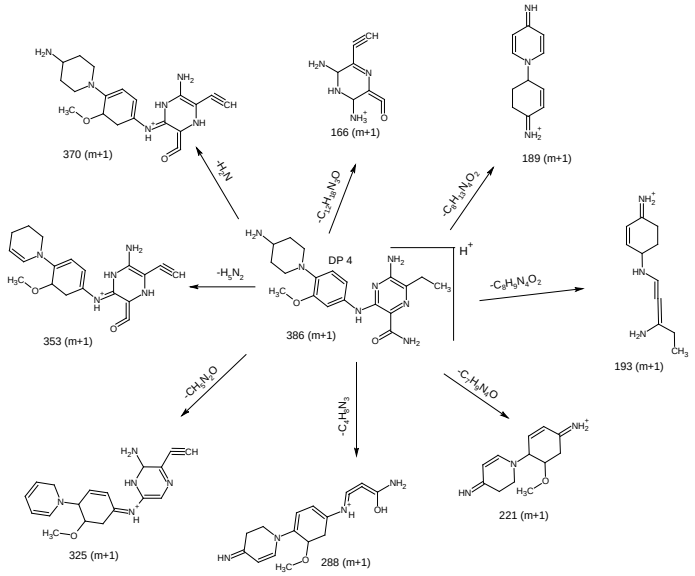


Figure 9: Fragmentation mechanism proposed for DP 4

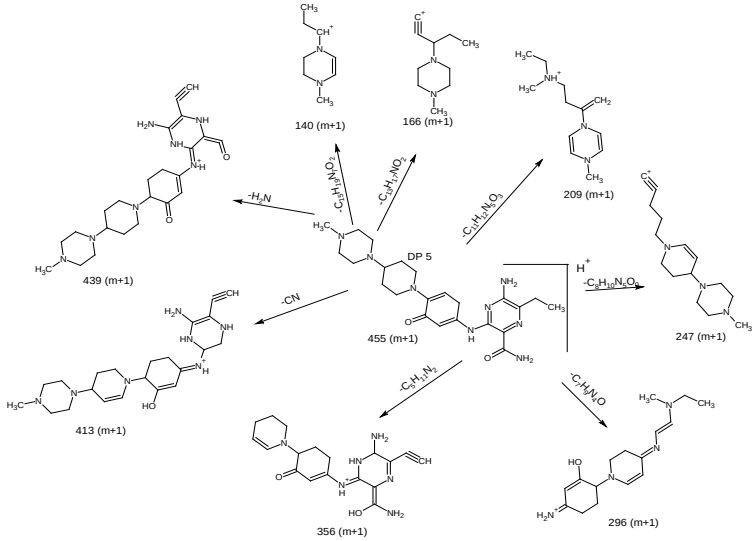


Figure 10: Fragmentation mechanism proposed for DP 5

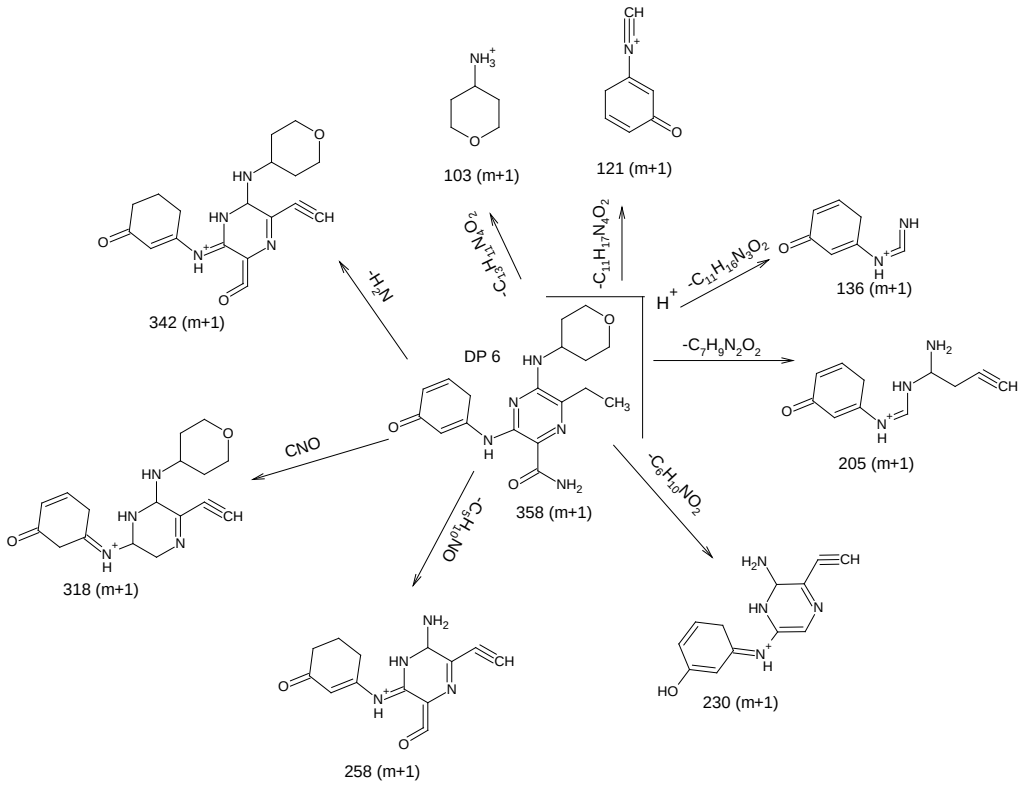


Figure 11: Fragmentation mechanism proposed for DP 6

The DP 7 of gilteritinib was observed in the chromatogram of acid, base and UV light induced stress degradation studies. The proposed mass fragmentation pattern of DP 7 is depicted in Figure 12. The mass fragmentation spectra reveal a parent ion at m/z 511, corresponding to the molecular mass of DP 7. Within the spectrum, there are distinct fragment ions observed at m/z 383 (resulting from the loss of $C_{21}H_{27}N_5O_2$), m/z 258 (resulting from loss of $C_{15}H_{20}N_4$), and m/z 152 (resulting from the loss of $C_9H_{15}N_2$). The combined presence of these product ions, in conjunction with the parent ion, confirms the identity of DP 7 as *3-((4-(4-(5,6-dihydropyrazin-1(2H)-yl)piperidin-1-yl)-3-methoxyphenyl) amino)-5-((tetrahydro-2H-pyran-4-yl)amino)-3,6-dihydropyrazine-2-carboxamide*, having molecular formula of $C_{26}H_{38}N_8O_3$.

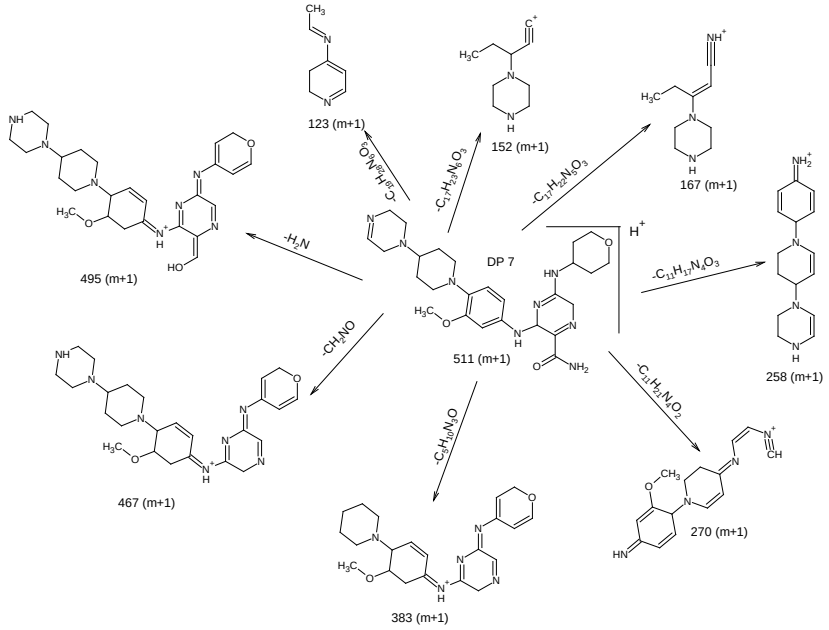
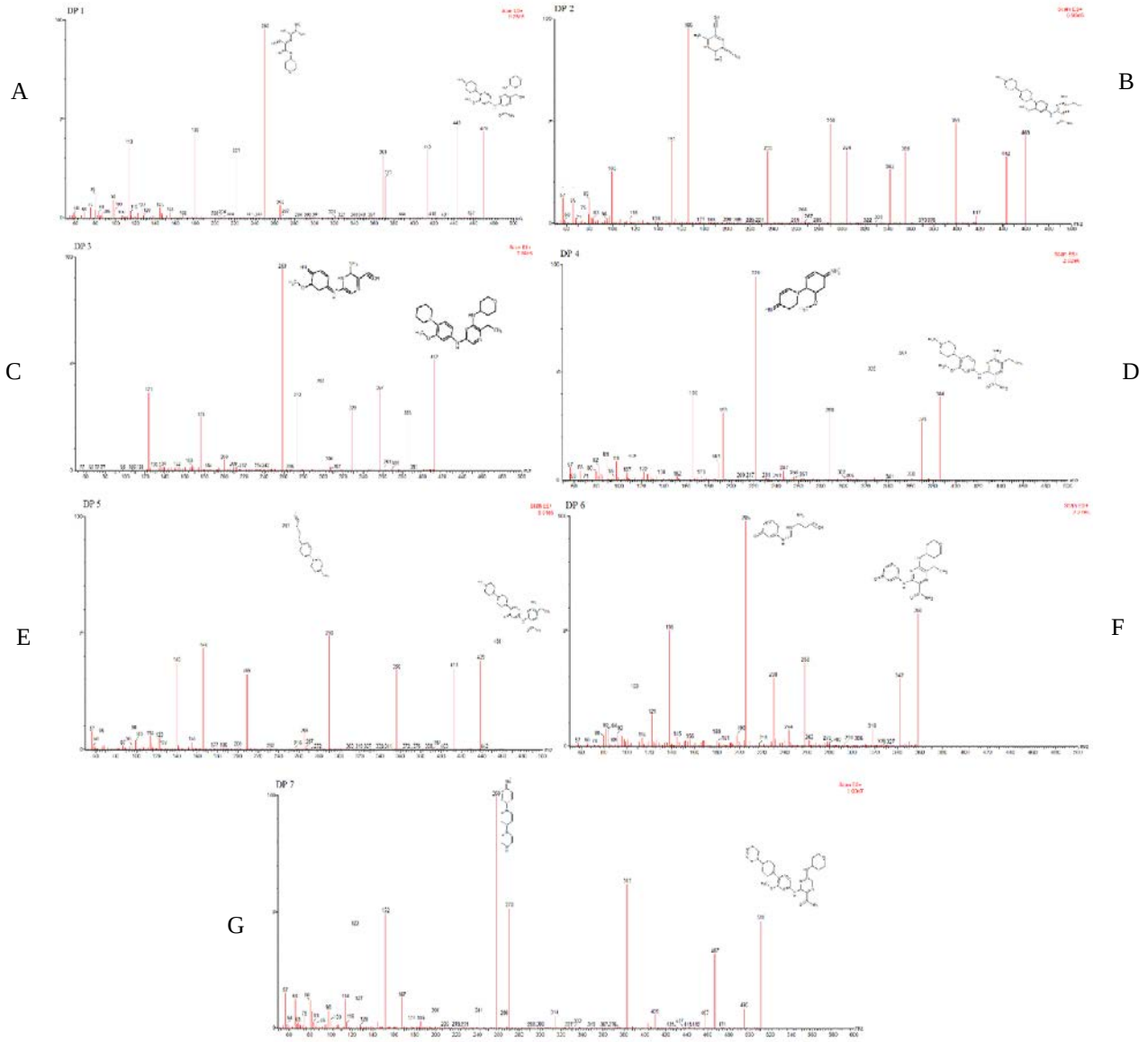


Figure 12: Fragmentation mechanism proposed for DP 7



Mass spectra identified at t_R of 2.17 min for DP 1 (A), 3.42 min for DP 2 (B), 4.41 min for DP 3 (C), 5.17 min for DP 4 (D), 6.91 min for DP 5 (E), 7.39 min for DP 6 (F), 12.63 min for DP 7 (G),

Figure 13: Mass spectra of DPs observed in forced degradation study

DISCUSSION

The preparation of samples plays a crucial role in nitroso impurities analysis, as matrix effects can be amplified, leading to issues like reduced sensitivity, abnormal recovery, and analyte instability. Various diluents were assessed for their extraction efficiency and impact on chromatography. Methanol, acetonitrile, and isopropanol alone and combined demonstrated good solubility for nitroso impurities. Isopropanol as a diluent is deemed unsuitable due to low peak response and poor symmetry of analytes. Using methanol and acetonitrile individually as diluents produces poor peak shape and recovery. Moreover, the equal combination of methanol and acetonitrile with buffer yields the best peak symmetry with significantly high recoveries, suggesting that this solvent composition was utilized as diluent throughout the analysis.

In the process of method development, three columns of different configurations, including the Zorbax SB (100 mm) column, waters C18 (250 mm) column, and YMC-Triart (150 mm) C18 column, were tested for producing the best resolution with high peak symmetry for nitroso impurities. The waters Symmetry C18 (250 × 4.6mm; 5 µm pore size) column at 35°C

performs best among the other columns studied in terms of resolution and symmetry and hence was selected as appropriate for the study. An appropriate mobile phase was finalized by optimizing the different compositions of the mobile phase, including acetonitrile, methanol, and buffers at various strengths. Before finalizing the mobile phase, the elution modes, such as isocratic and gradient, along with flow rate, were tested to achieve the best resolution of nitroso impurities. The nitroso impurities, along with standard gilteritinib, were resolved on waters Symmetry C18 (250 mm) column using 0 pH 4.5 phosphate buffer and acetonitrile in 25:75 (v/v) as solvent A, acetonitrile, and methanol in 75: 25 (v/v) as solvent B as mobile phase. The method produces a sensitive detection limit with characteristic mass fragmentation. This facilitates the trace-level detection and quantification of nitroso impurities of gilteritinib. The findings obtained in this study were correlated with the literature, and no method was reported for quantifying the nitroso impurities of gilteritinib. This study's proposed method can efficiently resolve the gilteritinib impurities studied, suggesting that the method is appropriate for the resolution, identification, and quantification of nitroso impurities in gilteritinib bulk and formulated samples. Finally, this research contributes a reliable analytical method for assessing the quality and stability of selumetinib formulations, enhancing pharmaceutical development and regulatory compliance.

CONCLUSION

In conclusion, we have developed a rapid, cost-effective, highly sensitive HPLC method for quantifying gilteritinib in formulations. The method utilizes a Waters Symmetry C18 column with an isocratic mobile phase consisting of pH 4.5 phosphate buffer, acetonitrile, and methanol, and monitored at 230 nm wavelength. Key findings include the establishment of an accurate calibration curve for gilteritinib ranging from 25-175 µg/mL and a limit of quantification (LOQ) for its impurities between 0.025 µg/mL to 0.175 µg/mL, demonstrating acceptable precision, accuracy, and recovery. This method offers several advantages, including reduced analysis time, cost-effectiveness, accessibility, robustness, sensitivity, and reproducibility. Furthermore, our study investigated the degradation behavior of gilteritinib under various stress conditions, including hydrolysis (acidic, basic, and neutral), oxidation, photolysis, and thermal stress. We observed that the drug exhibited stability under thermal hydrolysis conditions but underwent degradation in photolysis acidic and alkaline environments. The degradation products were identified by observing $[M + H]+$ ions and confirmed via HPLC-MS/MS experiments, with accurate mass measurements supporting structural elucidation. The characterized degradation products include seven compounds (DP 1 to DP 7), each with a distinct chemical structure. Our study effectively elucidated the fragmentation pathways and characterized the degradation products of gilteritinib. Therefore, this developed method not only assesses process-related impurities but also facilitates the identification of stress-induced degradation products, contributing significantly to pharmaceutical analysis and quality control efforts.

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FINANCIAL ASSISTANCE

Nil

CONFLICT OF INTEREST

The authors declare no conflict of interest

AUTHOR CONTRIBUTION

Srinivasa Rao Katta designed the research work. Immani Ramachandra Rao contributed to experimental work and conducted calculations. P Punita, Thota Siva Prasad, and Srinivasa Rao Katta drafted the manuscript. All the authors were involved in editing the draft of the manuscript.

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