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RESEARCH ARTICLE

Evaluation of the Genotoxic Impurities of Selpercatinib Through HPLC and LC-MS/MS Identification of Selpercatinib Stress Degradation Products

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SUMMARY

The current investigation entails the characterization of five degradation products (DPs) formed in different stress conditions of selpercatinib employing liquid chromatography–tandem mass spectrometry (LC-MS/MS). Additionally, high-performance liquid chromatographic (HPLC) method was developed for precise quantifying genotoxic impurities of selpercatinib. To explore the stability profile of selpercatinib, it was subjected to forced degradation experiments including acidic, basic, oxidative, photolytic, and thermal stress. These experiments revealed the degradation of selpercatinib under basic, acidic, and photolytic conditions, resulting in the formation of five distinct DPs. The chromatographic resolution of selpercatinib and its impurities, along with DPs, was effectively attained on a Zorbax C18 (250 mm × 4.6 mm, 5 µm) column using aqueous ammonium acetate and methanol in 70:30 (v/v) at pH 4.5 with 0.1% formic acid as the mobile phase, pumped isocratically at 0.9 mL/min and 226 nm wavelength. The approach generates a precise calibration curve that accurately fits within the 15-120 µg/mL range for selpercatinib and LOQ (0.015 µg/mL) to 0.12 µg/mL for impurities with acceptable precision, accuracy, and recovery. 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 selpercatinib. Hence, this approach can be appropriate for the resolution and quantification of genotoxic impurities in selpercatinib and can also be applicable for the evaluation of stress degradation products.

Key Words: *Selpercatinib, HPLC method development, impurities quantification, forced degradation studies, degradation products, structural characterization.*

Selperkatinibin Genotoksik Safsızlıklarının HPLC ile Değerlendirilmesi ve Selperkatinib Stres Bozulma Ürünlerinin LC-MS/MS Tanımlaması

ÖZ

Mevcut araştırma, sıvı kromatografi-tandem kütle spektrometrisi kullanılarak selperkatinibin farklı stres koşullarında oluşan beş bozunma ürününün (DP'ler) karakterizasyonunu içerir. Ek olarak, selpercatinibin genotoksik safsızlıklarının kesin olarak ölçülmesi için yüksek performanslı sıvı kromatografisi (HPLC) yöntemi geliştirilmiştir. Selperkatinibin stabilite profilini araştırmak için asidik, bazik, oksidatif, fotolitik ve termal stres dahil olmak üzere zorunlu bozunma deneyleri yapılmıştır. Bu deneyler, selpercatinibin bazik, asidik ve fotolitik koşullar altında bozunduğunu ve bunun sonucunda beş farklı DP'nin oluştuğunu ortaya çıkarmıştır. Selperkatinib ve safsızlıklarının DP'lerle birlikte kromatografik ayrımı, mobil faz olarak %0.1 formik asit, izokratik olarak 0,9 mL/dak ve 226 nm dalga boyunda pompalanması ile pH 4.5'te 70:30 (h/h) sulu amonyum asetat ve metanol kullanılarak Zorbax C18 (250 mm x 4.6 mm, 5 µm) kolonunda etkin bir şekilde sağlanmıştır. Yaklaşım, selperkatinib için 15-120 µg/mL aralığına ve safsızlıklar için LOQ (0.015 µg/mL) 0.12 µg/mL aralığına kabul edilebilir hassasiyet, doğruluk ve geri kazanımla doğru bir şekilde uyan kesin bir kalibrasyon eğrisi oluşturmuştur. Bu yöntemin etkinliği, selpercatinibin yeni üretilen bozunma ürünlerinin kimyasal yapılarının doğrulanmasına olanak tanıyan LC-MS/MS aracılığıyla doğrulanmıştır. Dolayısıyla bu yaklaşım, selperkatinibin genotoksik safsızlıklarının ayrımı ve miktarının belirlenmesi için uygun olabilir ve aynı zamanda stres bozunma ürünlerinin değerlendirilmesi için de uygulanabilir.

Anahtar Kelimeler: *Selperkatinib, HPLC yöntemi geliştirme, safsızlıkların ölçümü, zorunlu bozunma çalışmaları, bozunma ürünleri, yapısal karakterizasyon.*

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INTRODUCTION

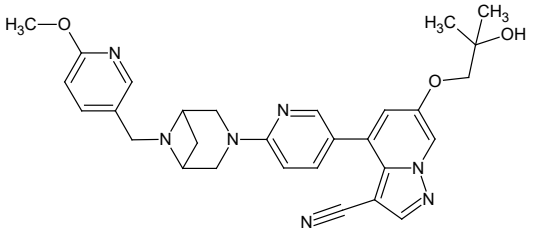
The therapeutic product includes both active pharmaceutical ingredients (API) and excipients. The API is responsible for pharmacological effects upon absorption into the systemic circulation within the living body (Gorog, 2006). However, in specific instances, the active ingredient or the excipients may not be entirely pure, potentially harboring additional substances from various sources, such as synthesis, excipient, residual solvent, or degradation products. These unwanted components, distinct from the API and excipients, are impurities (Gorog, 2018).

In a pharmaceutical product, when the occurrence of impurities are expected, it becomes essential to detect and characterize them using appropriate analytical methods. This systematic procedure is commonly known as impurity profiling. Impurity profiling is a comprehensive strategy aimed at identifying unknown impurities and elucidating their chemical structures. This process is crucial in ensuring that impurities in pharmaceutical substances are identified and quantified within acceptable limits, thus mitigating potential toxicological effects on the human body (Nagpal *et al*., 2011).

Impurity profiling requires analytical techniques that are highly sensitive, selective, and efficient in detecting and quantifying trace amounts of impurities (Murali *et al*., 2022). Traditional methods with lower sensitivity and accuracy are insufficient for quantification due to the nominal quantities in which impurities may exist within drug substances (Patel & Apte, 2016). Furthermore, the structural similarities between many impurities and the parent drug molecule highlight the necessity for advanced hyphenated analytical techniques. HPLC and LC-MS/MS are versatile methods extensively employed for evaluating trace-level impurities in pharmaceutical products (Kiran *et al*., 2017).

Selpercatinib is a targeted cancer therapy designed to treat specific types of cancer associated with mutations in the RET gene. It belongs to the tyrosine kinase inhibitor class and is approved for treating advanced or metastatic non-small cell lung cancer, medullary thyroid cancer, and other RET fusion-positive solid tumors (Li *et al*., 2019). RET gene mutations play a role in the development and progression of certain cancers. Selpercatinib works by specifically inhibiting the activity of the RET protein, which helps to slow down or stop the growth of cancer cells (Russo *et al*., 2020). Selpercatinib can ability to cross the blood-brain barrier, potentially making it effective against tumors that have spread to the brain (Wirth *et al*., 2020).

A review of the literature was conducted to identify various analytical methods reported for the quantification of selpercatinib. One HPLC (Singamsetty *et al*., 2021) was reported for evaluation of selpercatinib in dosage forms, in contrast one HPLC-MS/MS (Gulikers *et al*., 2023) bioanalytical method was reported for analyzing selpercatinib in combination with pralsetinib, brigatinib, and lorlatinib. Existing literature indicates a lack of reported analytical methods for quantifying genotoxic impurities in selpercatinib, and no author has characterized the stress degradation compounds of selpercatinib. Therefore, this paper introduces an optimized HPLC method for quantifying genotoxic impurities of selpercatinib and LC-MS/MS characterization of degradation products (DPs). The study involves genotoxic impurities, namely nitroso and N-oxide impurities based on their availability. Figure 1 provides a comprehensive overview of selpercatinib and its impurities.

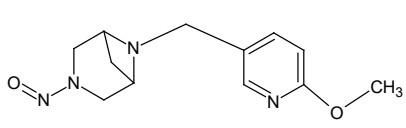


A) Selpercatinib

Systemic name: *6-(2-hydroxy-2-methylpropoxy)-4-(6-(6-((6-methoxypyridin-3-yl)methyl)-3,6-diazabicyclo[3.1.1]heptan-3-yl)pyridin-3- yl) pyrazolo[1,5-a]pyridine-3-carbonitrile*

Formula: $C_{29}H_{31}N_7O_3$

Mass: 525.61 g/mol

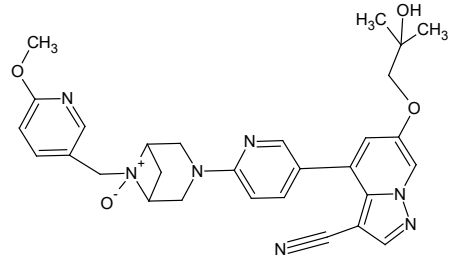


B) Nitroso impurity

Systemic name: *6-((6-Methoxypyridin-3-yl) methyl)-3-nitroso-3,6-diazabicyclo [3.1.1]heptane*

Formula: $C_{12}H_{16}N_4O_2$

Mass: 248.3 g/mol



C) N-Oxide impurity

Systemic name: *3-(5-(3-Cyano-6-(2-hydroxy-2-methylpropoxy)pyrazolo[1,5-a]pyridin-4-yl)pyridin-2-yl)-6-((6-methoxypyridin-3-yl)methyl)-3,6-diazabicyclo[3.1.1]heptane 6-oxide*

Formula: $C_{29}H_{31}N_7O_4$

Mass: 541.6 g/mol

Figure 1. Systemic details of Selpercatinib and its impurities in the study

MATERIAL AND METHOD

Chemicals and instruments

The 98.55 % pure API of Selpercatinib, along with nitroso impurity and N-Oxide impurity, were procured from Eli Lilly and Company (India) Private Limited, Hyderabad. The Retevmo® brand tablet with a 40 mg dose was obtained from the pharmacy. The water (milli-Q®), acetonitrile (HPLC purity), and methanol (HPLC purity), solvents along with filter papers (0.2 µ nylon), were brought from Merck Chemicals, Mumbai. Analytical reagent chemicals, i.e., potassium dihydrogen phosphate (KH_2PO_4), orthophosphoric acid (H_3PO_4), sodium hydroxide (NaOH), hydrogen peroxide (H_2O_2), and hydrochloric acid (HCl), were brought from Fisher Scientific, Mumbai. The HPLC study was performed on a Waters 1100 (Japan) instrument with Agilent ChemStation software whereas as the Waters triple quadrupole LC-MS system (Japan) with MassLynx software was utilized for the LC-MS study.

Selpercatinib and impurity solution preparation

An accurately measured 25 mg of Selpercatinib API and the impurities were then individually placed in 25 mL flasks containing pure methanol as a diluent. Subsequently, the analytes were completely dissolved in the solvent using a sonicator. After dissolution, the

solutions were filtered through a 0.2µm filter. Adjustments to the volume in each flask were made using the same solvent to attain separate solutions of Selpercatinib and its impurities, each with a concentration of 1000 µg/mL. Additional dilutions were prepared from this stock solution whenever necessary.

Formulation solution preparation

The pharmaceutical formulation under the Retevmo® brand, containing 40 mg of Selpercatinib, was employed to prepare the formulation solution. Tablets were finely powdered, and 25 mg of pure drug equivalent powder was placed in a 25 mL flask filled halfway with methanol. The formulation analytes were dissolved in the solvent using a sonicator, and any undissolved formulation excipients were removed by filtration. The final volume was adjusted to achieve a concentration of 1000 µg/mL of Selpercatinib. Additional dilutions were made as necessary to obtain a Selpercatinib concentration equivalent to 100% precision level, and the solutions were analysed immediately after preparation.

Method development

The method development process commenced with the investigating of the optimal wavelength for detecting Selpercatinib and its impurities, utilizing a UV detector. Standard solutions, each containing Selpercatinib and impurities at a concentration of 10 µg/mL, were individually scanned across the range of 200 to 400 nm. Through the overlay of spectra, an iso-absorption wavelength was identified, ensuring accurate detection of both substances. Further, various stationary phase configurations from different manufacturers were systematically evaluated to achieve the highest resolution of analytes. Meticulous adjustments to the composition of the mobile phase were made through systematic experimentation, aiming to fine-tune the method conditions to separate Selpercatinib and its impurities successfully. The finalized conditions, meticulously selected to ensure optimal resolution, were subsequently employed in the following validation procedures, laying the foundation for robust and reliable analytical methodology.

Method Validation

The guidelines prescribed by ICH (ICH 1994; ICH 1996; ICH 2003) and authors reported in literature were utilized for conducting validation study of the proposed method (Varma *et al*., 2022; Rajesh *et al*., 2022; Varma *et al*., 2023; & Rajesh *et al*., 2023).

The method sensitivity for detecting the targeted impurities underwent rigorous assessment employing the signal-to-noise (S/N) ratio procedure. A S/N ratio of 10 and 3 was established as the quantification limit (LOQ) and detection limit (LOD) respectively. To construct a linear plot, each impurity's LOQ was considered the lower limit in the calibration curve. Subsequently, the concentration of Selpercatinib was meticulously adjusted to ensure that the standard solution contained 0.1% of the impurities, facilitating accurate quantification. Utilizing the correlation and regression results obtained from the linear curve, the range of analysis was derived, ensuring comprehensive coverage of analyte concentrations.

Method reproducibility was meticulously confirmed through precision experiments, encompassing interday (n=6), intraday (n=3 for each day), and ruggedness (n=3 for each analyte) assessments. In each precision study, the area response was meticulously recorded, and the percentage relative standard deviation (%RSD) was calculated. A %RSD of less than 2 was deemed adequate, demonstrating the robustness and reliability of the analytical methodology.

The method accuracy was rigorously evaluated through a comprehensive recovery experiment conducted at 50%, 100%, and 150% levels within the calibration range. The %RSD of the area values at each level was meticulously assessed during each analysis. Acceptability criteria were set for recovery results to fall within the range of 98-102%, alongside a %RSD of less than 2%, ensuring precise and reliable quantification. Furthermore, method reliability was systematically examined by varying the proposed method conditions, and analyses were conducted under each altered condition. Changes in the area response were

carefully scrutinized, with a % change of less than 2 deemed acceptable, demonstrating the robustness and consistency of the analytical methodology.

Stability studies

The stability profile of the drug was comprehensively assessed under diverse conditions, encompassing photolytic, dry heat, oxidative, and hydrolytic stress. To ensure a thorough evaluation, absorbance was recorded using an ultraviolet (UV) detector, while the LC–MS/MS technique was utilized to characterize DPs. In examining the effects of hydrolytic, oxidative, and photolytic stress, a solution of Selpercatinib with a precisely known concentration was meticulously prepared in HPLC-grade methanol. This approach facilitated the precise control and reproducibility necessary for accurately assessing the drug's stability under the specified conditions.

Hydrolytic degradation testing was meticulously carried out under both acidic and basic conditions to assess the drug's susceptibility to hydrolytic breakdown. In this evaluation, 1 mL of the Selpercatinib formulation solution was accurately measured and placed into 10 mL flasks. Subsequently, 1 mL of the respective stress-inducing solution (0.1 N NaOH or 0.1 N HCl) was added, and the flasks were left undisturbed for 24 hours. In addition, the samples were subjected to accelerated degradation by placing them in a water bath set at 70°C with the respective stress-inducing solution for 7 hours. Following the degradation period, the samples were neutralized, and the volume was adjusted with the mobile phase up to the mark. Subsequently, the samples were analyzed using appropriate analytical techniques to evaluate any changes or degradation products that may have occurred under the specified conditions.

Oxidative degradation testing was meticulously conducted using 15% hydrogen peroxide as the oxidizing agent. Initially, 1 mL of the Selpercatinib formulation solution was precisely measured and placed into 10 mL flasks. Following this, 1 mL of 15% hydrogen peroxide was added to each flask, and the solutions were allowed to stand undisturbed for 24 hours. Additionally, accelerated degradation was induced by subjecting the samples to a water bath maintained at 70°C for 7 hours. After the degradation period, the samples were neutralized, and the volume was adjusted with the mobile phase up to the mark. Subsequently, the samples were meticulously analyzed using appropriate analytical techniques to assess any degradation products or changes resulting from oxidative stress. For thermal degradation testing, the samples were exposed to a temperature of 70°C for 7 days in an air oven. Conversely, photolytic degradation was performed by exposing Selpercatinib powder to UV light for 7 days within a photo-stability chamber.

The stress-induced Selpercatinib samples were then brought to a standard concentration and assessed using the proposed analytical method. This comprehensive approach ensured a thorough evaluation of the drug's stability under various conditions, providing valuable insights into its formulation and storage requirements.

LC-MS characterization of DPs

DPs formed during exposure to stress conditions were identified and characterized through analysis utilizing LC-MS/MS. Initially, the eluents from the column were detected using a UV detector, providing preliminary information on the compounds present. Subsequently, a portion of the eluents was carefully directed into the mass detector to generate mass spectra. To ensure optimal sensitivity and accuracy, approximately 40% of the eluents were directed into the mass detector with the assistance of a splitter. The resulting mass spectra and fragmentation patterns were meticulously analyzed and summarized to evaluate the nature and extent of degradation observed in the DPs. This comprehensive approach facilitated a detailed understanding of the stress-induced degradation pathways and provided valuable insights into the stability profile of the drug under investigation.

Method applicability

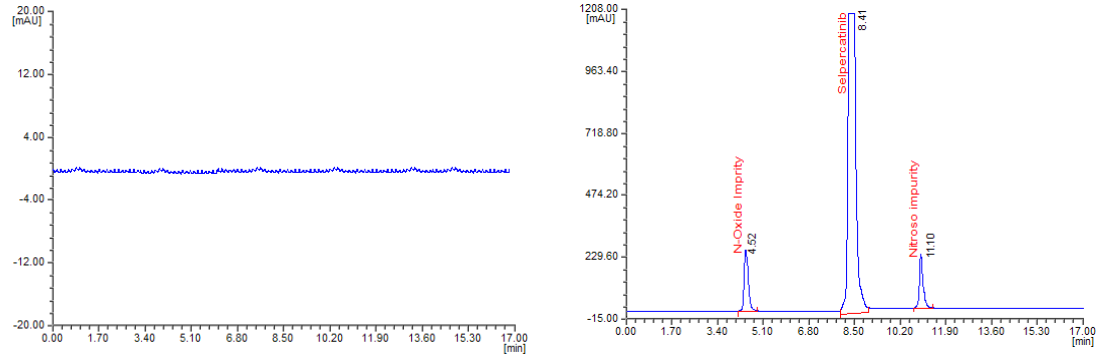
The proposed analytical HPLC method underwent thorough scrutiny to detect and quantify impurities present in Selpercatinib tablet formulations. To validate the method's efficacy, sample solutions derived from Retevmo® tablets were prepared and subjected to analysis. The method's robustness was evaluated through direct analysis of formulation sample solutions, as well as analysis after spiking formulation solution with known concentrations of the target impurities. Subsequent examination of the resulting chromatograms and their corresponding responses allowed for a comprehensive assessment of the method's applicability. The method's accuracy, precision, and sensitivity were thoroughly evaluated by meticulously comparing the peak responses and retention times of impurities in both the unspiked and spiked samples, ensuring its suitability for the intended analytical purpose.

RESULTS AND DISCUSSION

Due to lack of any published analytical method in existing literature for evaluating genotoxic impurities of Selpercatinib, this investigation aimed to develop a straightforward HPLC technique. The purpose of this method was facilitated with nitroso impurity and N-Oxide impurity of Selpercatinib. To achieve the best resolution of analytes, various optimization experiments were conducted using several column configurations. The optimization process also involved fine-tuning pH and composition. Various solvent ratios were explored, including various buffer strengths, to determine the most suitable mobile phase. This enabled us to effectively separate Selpercatinib and its impurities. Consequently, a wide range of buffers with varying pH levels was examined to achieve optimal resolution.

Selpercatinib and its impurities were resolved effectively on Zorbax C18 column (250mm×4.6mm,5µm), at 35°C Column temperature. The chromatographic conditions involved a mobile phase of aqueous ammonium acetate and methanol in 70:30 (v/v) at pH 4.5 with 0.1% formic acid, employing 0.9 mL/min isocratic elution and 226 nm wavelength. The blank and system suitability chromatogram noticed in the developed method is presented in Figure 2.

The blank chromatogram observed while analysing the diluent as sample was presented in Figure 2A, which clearly shows no chromatographic detections throughout the entire run time. The Selpercatinib solution spiked with 0.1 % of each impurity was also analysed. The chromatogram visualizes well-resolved and retained peaks corresponding to Selpercatinib and its impurities. The retention time (tR) was noticed as 8.41 min for Selpercatinib, whereas 4.52 min and 11.10 min respectively for the N-oxide impurity and nitroso impurity. The chromatogram doesn't visualize additional detections throughout the entire run time (Figure 2B), suggesting specificity for analyzing nitroso impurities of Selpercatinib.



A B

Figure 2. Specificity chromatograms in the optimized method

System suitability is a crucial aspect of the chromatographic method validation process, confirming the proposed method's capability to generate well-resolved peaks with high reproducibility consistently. This test is essential for assessing the HPLC system's performance and the procedure's ability to yield high-quality data. The evaluation of system suitability involved the assessment of several parameters, including resolution (Rs), USP tail factor (T), theoretical plates (N), relative standard deviation (RSD) of area, elution time (t_R), and relative retention time (RRT). The calculated RSD values for both t_R and area of Selpercatinib and its impurities were less than 1%, which falls within the acceptance limit. This suggests a high level of reproducibility of peaks representing Selpercatinib and its impurities, with approximately consistent area and retention time during each injection at the fixed concentration. Furthermore, the resolution (Rs) between first eluted peak and subsequent peaks was exceeded 2, indicating a satisfactory resolution of the proposed method. The system suitability results for Selpercatinib and its impurities were compared against regulatory permissible levels and were presented in Table 1. These results affirm that the method successfully meets system suitability criteria.

Table 1. System suitability results of Selpercatinib and its impurities

Parameter	Experiment results for			Acceptance criteria
	Selpercatinib	N-Oxide Impurity	Nitroso Impurity	
t_R (min)	8.41	4.52	11.10	--
RRT	--	0.54	1.32	< 2
RRF	--	0.829	0.038	--
R_S	7.99	--	5.73	> 2
A_S	0.99	0.96	0.91	< 2
N	7568	5234	9682	> 2000

The terms LOD and LOQ signify the method's capacity to accurately detect the smallest analyte amount and quantitate it, respectively. LOD of the impurities was identified as 0.004 µg/mL, and LOQ was finalized as 0.015 µg/mL for impurities. To assess the linearity of the developed method, a linear curve was constructed by plotting the area against various standard concentrations of Selpercatinib and its impurities separately. The standard concentrations range from 15-120 µg/mL for Selpercatinib and 0.015 µg/mL – 0.120 µg/mL for its impurities. The calibration plot, depicting analyte concentration versus peak area, exhibited a linear relationship across the specified concentration range. This linearity was established using the linear simple regression least square method. The regression equation for the line was tabulated in Table 2.

Precision of developed method was evaluated through examination of parameters encompassing both repeatability and intermediate precision. A minimum of six determinations were conducted daily by injecting and analyzing freshly prepared concentrations. This rigorous testing allowed us to evaluate both the within-day (intraday) and between-day (interday) variability, as well as ruggedness. These results indicate acceptable levels of both intraday and interday variability. The mean RSD values for the repeatability and reproducibility study across the studied concentration levels for Selpercatinib and its impurities were found to be acceptable (Table 2), suggesting that the method is reproducible.

To evaluate the method's accuracy, a recovery experiment was conducted. Standard concentrations were intentionally spiked at three levels corresponding to 50%, 100%, and 150% of the target concentration. These spiked samples, prepared in three replicates, were analysed to quantify Selpercatinib and its impurities. The calculated analyte concentrations were subsequently compared to the nominal concen-

trations, allowing us to calculate the % recovery. The results revealed that the mean % recovery values for all three levels, based on three replicates, comfortably fell within the acceptance limit of 98% to 102%, as outlined in regulatory guidelines. These findings affirm the method's accuracy as it consistently delivers results aligned with the expected analyte concentrations. Table 2 presents the summarized results of this study.

Table 2. Summary results noticed in linearity, precision, and accuracy study

Parameter	Results		
	Selpercatinib	**N-oxide impurity**	**Nitroso impurity**
Linearity			
Range (µg/mL)	15-120	0.015-0.120	0.015-0.120
Intercept	10310	605.54	- 1231.5
Slope	13369	741571	683092
r^2	0.9996	0.9996	0.9990
Precision[$$]			
Intraday	0.43	0.61	0.58
Day 1 in interday	0.48	0.46	0.59
Day 2 in interday	0.41	0.12	0.40
Ruggedness	1.16	0.43	0.61
50 % accuracy level[$]			
Amount prepared (µg/mL)	45	0.045	0.045
Amount recovered (µg/mL)	44.70	0.045	0.045
% Recovery	99.33	99.66	99.97
% RSD	1.81	0.58	0.16
100 % accuracy level[$]			
Amount prepared (µg/mL)	60	0.06	0.06
Amount recovered (µg/mL)	59.13	0.060	0.060
% Recovery	98.54	100.10	100.02
% RSD	1.39	0.46	0.28
150 % accuracy level[$]			
Amount prepared (µg/mL)	75	0.075	0.075
Amount recovered (µg/mL)	74.64	0.074	0.075
% Recovery	99.52	99.30	99.63
% RSD	0.99		0.60

n=3 for experiments marked with [$] whereas n = 6 for experiments marked with [$$]

Several critical parameters were investigated to assess the method reliability for analysing impurities of Selpercatinib. These parameters included variations in the pH, mobile phase composition, and detector wavelength. The % change, shifts in retention time, and system suitability were closely monitored as responses to the deliberate changes in method parameters. Pertaining to the mobile phase composition, variations within ± 5%, ± 0.1 % factor variation in pH, and ± 5 nm variation in detector wavelength of the specified method condition was performed. The comprehensive results of this study demonstrate that deliberate changes made within the specified parameter ranges don't affect the elution, retention time as well as system suitability. The mean % change remained stable across all conditions, exhibiting only slight variability within acceptable limits. These findings underscore the robustness of the proposed method, affirming its reliability under variations within the specified parameter ranges, as shown in Table 3.

Table 3. Robustness results of Selpercatinib and its impurities

S No	Changed condition	Parameter	Results observed		
			Selpercatinib	N-Oxide impurity	Nitroso impurity
1	MP 1	% change	0.93	0.95	0.73
		t_R	8.45	4.51	11.10
		N	7639	5129	9871
2	MP 2	% change	-0.08	-1.26	-0.22
		t_R	8.47	4.55	11.18
		N	7756	5276	9743
3	pH 1	% change	-0.25	0.65	-0.42
		t_R	8.44	4.59	11.13
		N	7987	5502	9604
4	pH 2	% change	0.35	-1.29	-0.15
		t_R	8.49	4.56	11.16
		N	8091	5678	9956
5	WL 1	% change	-0.44	-0.43	0.38
		t_R	8.43	4.55	11.11
		N	7566	5233	9612
6	WL 2	% change	-0.43	0.35	0.95
		t_R	8.41	4.52	11.18
		N	7590	5107	9987

MP (mobile phase) 1: 75:25 (v/v) of solvent A and B; MP 2: 65:35 (v/v) of solvent A and B; WL (wavelength) 1: 231 nm; WL 2: 221 nm; pH 1: 4.4; pH 2: 4.6

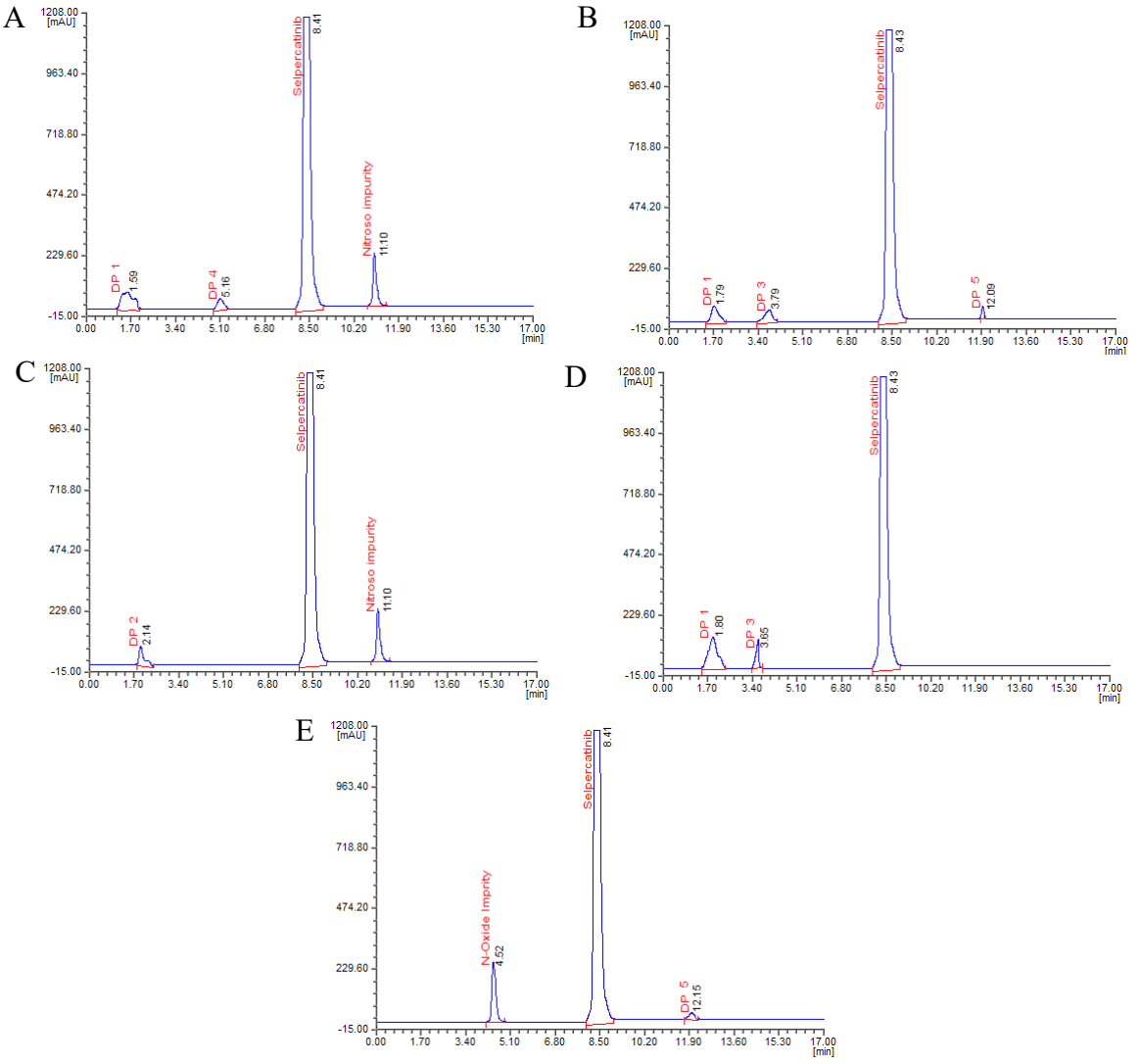
Following the ICH stability guidelines, a variety of forced conditions, namely thermal, basic, acidic, oxidative, and photolytic were employed to conduct degradation studies on the pharmaceutical product Selpercatinib. These studies led to the identification and characterization of five distinct degradation products, designated as DP1 to DP5, using 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 preventive measures during the formulation process.

UV degradation conditions, minimal significant degradation was observed, with an assay percentage of 95.80%. Among the various degradation conditions, the most pronounced degradation was observed in the acid-induced degradation study, the degradation reached 9.51%. Figure 3A depicts the chromatogram from this study, revealing well-separated DPs with retention times of 1.59 minutes and 5.16 minutes, denoted as DP 1 and DP 4, respectively. Additionally, the chromatogram revealed the presence of nitroso impurity at a retention time of 11.10 minutes. In the base-induced degradation study, as depicted in Figure 3B, three distinct degradation products were resolved at retention times of 1.79 minutes, 3.79 minutes and 12.09 minutes, designated respectively as DP 1, DP 3 and DP 5. Percentage degradation under base degradation conditions was measured at 8.74%. In the peroxide degradation study, the assay percentage for Selpercatinib was 6.33%, with a 97.25% mass balance. Figure 3C Chromatogram for this study delineated a single degradation product with a retention time of 2.14 minutes, labelled DP 2. The outcomes of the purity study, conducted using the PDA detector, provided robust validation of the purity and consistency of the Selpercatinib peak across all stress samples under examination. The mass balance for these stressed samples was higher than 98%. These consistent results from the peak purity tests unequivocally affirmed the uniformity and purity of the Selpercatinib peak. Notably, the Selpercatinib assay showed remarkable stability, further attesting to the method's specificity and efficacy in detecting stability. Table 4 presents the recovery results and Figure 3 visualize the stress study chromatograms.

Table 4. Stress degradation results of Selpercatinib and its impurities

Stress	% degradation[#] of Selpercatinib	% assay[#] of Selpercatinib	% Mass balance[$]	Remark
Acidic	9.51	90.49	98.34	DP 1 and 4 were noticed
Basic	8.74	91.26	99.16	DP 1, 3 and 5 were noticed
Peroxide	6.33	93.67	97.25	DP 2 was noticed
Thermal	5.81	94.19	98.01	DP 5 was noticed
UV light	4.2	95.8	97.63	DP 1 and DP 3 were noticed

[#]n = 3; [$]sum of Selpercatinib, impurities and DPs



A) DP 1 and 4 visualized in acid degradation chromatogram; B) DP 1, 3 and 5 visualized in base degradation chromatogram; C) DP 2 visualized in peroxide degradation chromatogram; D) DP 1 and 3 visualized in UV degradation chromatogram; E) DP 5 visualized in thermal degradation chromatogram;

Figure 3. Forced degradation chromatograms of Selpercatinib and its impurities

Characterization of DPs by LC-MS/MS

Stress-induced DPs of Selpercatinib were subjected to characterization via LC-MS/MS analysis. The LC method remained unchanged, and the mass operating conditions were fine-tuned to maximize each mass fragment with minimized or no noise. The collision induced dissociation spectra of each DP along with its accurate mass measurements were noted for evaluated the structure of each DP formed in stress study.

Figure 4 illustrates the fragmentation mechanism of DP1, with the ESI spectrum (Figure 9A) revealing the most intense $[M+H]^+$ ion at m/z 454 representing the molecular mass of DP 1 as 453.49 g/mol. The MS/MS spectrum of DP1 exhibited highly intense product ion peaks at m/z-125 (loss of $C_{19}H_{15}N_5O$), 161 (resulting by loss of $C_{17}H_{17}N_4O$ from m/z 454), and m/z 386 (loss of C_4HN_2O from m/z 454) and the accurate mass measurements suggest the molecular composition of these fragments. The DP 1 was identified as *chloromethyl11,17-dihydroxy-10,13-dimethyl-3-oxo-6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17- dodecahydro- 3H-cyclopenta [a] phenanthrene-17-carboxylate* with molecular formula of $C_{25}H_{23}N_7O_2$ and molecular mass of 453.49 g/mol.

Figure 5 illustrates the fragmentation mechanism of DP 2 of Selpercatinib identified in peroxide induced stress study. The fragmentation spectra (Figure 9B) of DP 2 visualizes the parent ion with m/z of 472 (m+1) confirms molecular mass of DP 2. In the fragmentation spectra, there were notable product ions at m/z 139 (loss of $C_{19}H_{19}N_5O$), 221 (resulting by loss of $C_{14}H_{11}N_4O$), 191 (loss of $C_{15}H_{13}N_4O_2$ from parent ion), and m/z 100 (loss of $C_{21}H_{18}N_5O_2$ 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 *17-(ethoxycarbonyloxy)-11-hydroxy-10,13-dimethyl-3-oxo-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-3H-cyclopenta[a]phenanthrene-17-carboxylic acid* with molecular formula of $C_{26}H_{29}N_7O_2$ and molecular mass of 471 g/mol.

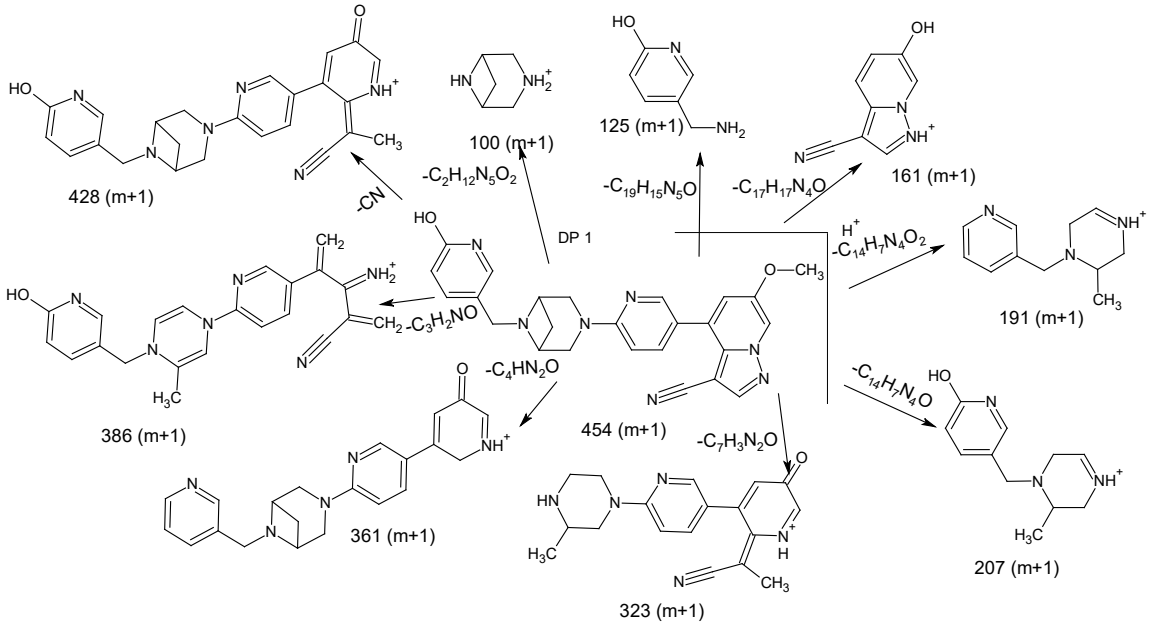


Figure 4. Fragmentation mechanism proposed for DP 1

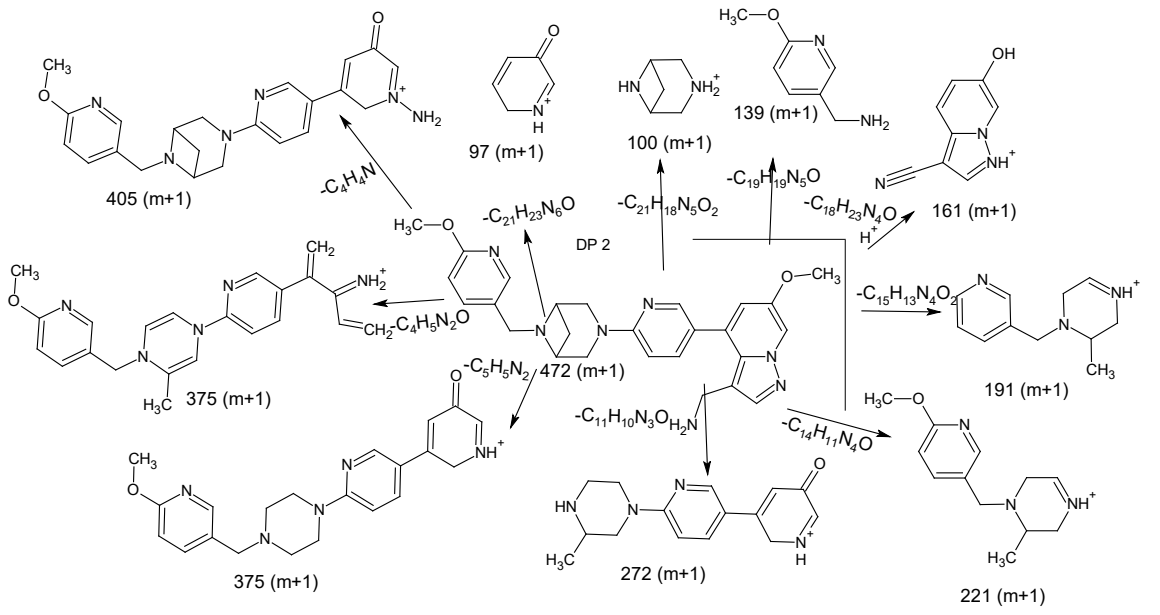


Figure 5. Fragmentation mechanism proposed for DP 2

Figure 6 illustrates the fragmentation mechanism of DP 3 of Selpercatinib which was noticed in base and UV induced stress study. The fragmentation spectra of DP 3 (Figure 9C) visualize parent ion with m/z of 266 (m+1) confirms the molecular mass of DP 3. In the fragmentation spectra, there were notable product ions at m/z 117 (loss of 2 different fragments i.e. $-C_4H_4O$ and $-C_4H_4$) and m/z 250 (loss of H_2N from parent ion). The DP 3 was identified as *1,17-dihydroxy-10,13-dimethyl-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-3H-cyclopenta[a] phenanthren-3-one* with molecular formula of $C_{14}H_{11}N_5O$ with 265 g/mol molecular mass.

The fragmentation spectrum of DP 4 identified at 5.16 minutes (Figure 9D), confirms parent ion at m/z 424 $[M+H]^+$. The spectra show various that can helpful for elucidating the structure of DP 4 was noticed, notably at m/z 145 (m+1), 191 (m+1), 329 (m+1) and 109 (m+1). The pattern of fragmentation in correlation with parent ion suggests DP 4 as *ethyl 11-hydroxy-10,13-dimethyl-3-oxo-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-3H-cyclopenta [a] phenanthren-17-yl carbonate*, with $C_{24}H_{21}N_7O$ as formula. The fragmentation pattern is presented in Figure 7.

The base and thermal degradation chromatogram visualizes a please change as peak at 12.0 min and was designated as DP 5 which was not detected in other stress studied performed in the study. The prominent parent ion at m/z 415 (m+1) identified along with fragment ions at m/z 125 (m+1), which result by losing $C_{17}H_{14}N_4$. The acquired data (Figure 9E) proved DP 5 as *chloromethyl17-(carboxyoxy)-11-hydroxy-10,13-dimethyl-3-oxo-6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17-dodecahydro-3H-cyclopenta[a]phenanthrene-17-carboxylate*, possessing a molecular formula of $C_{23}H_{22}N_6O_2$. The fragmentation pattern is presented in Figure 8.

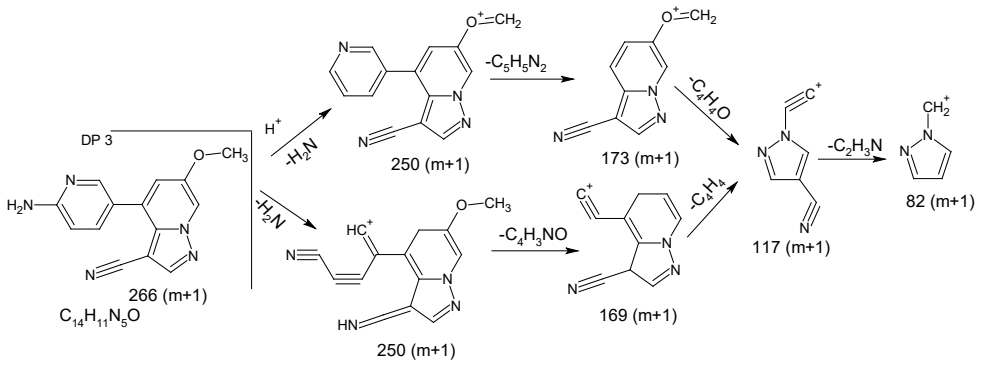


Figure 6. Fragmentation mechanism proposed for DP 3

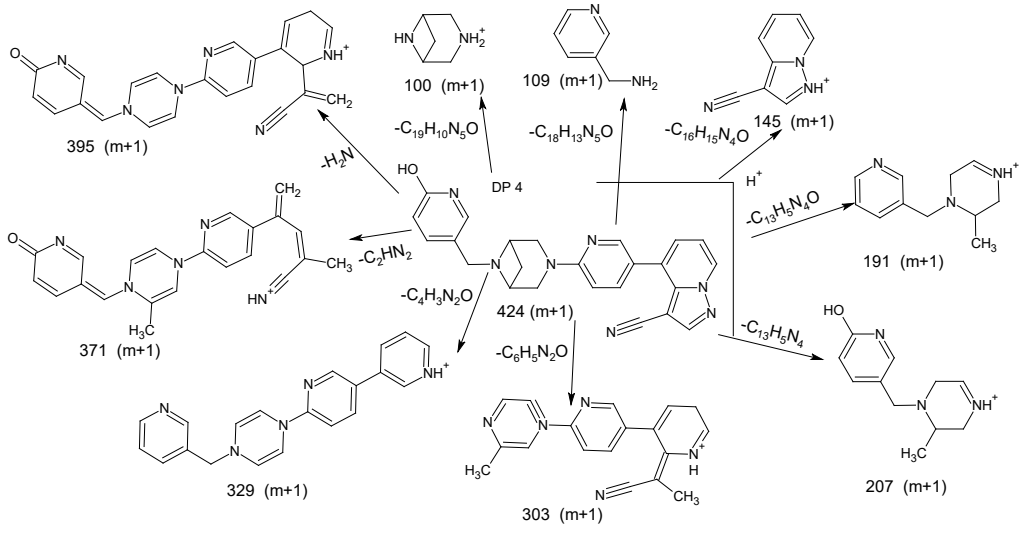


Figure 7. Fragmentation mechanism proposed for DP 4

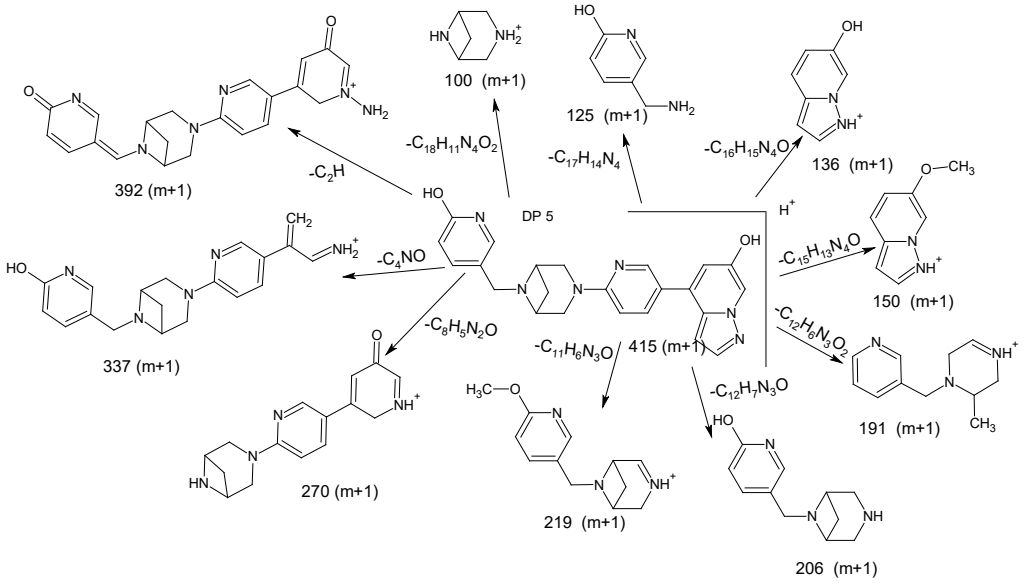
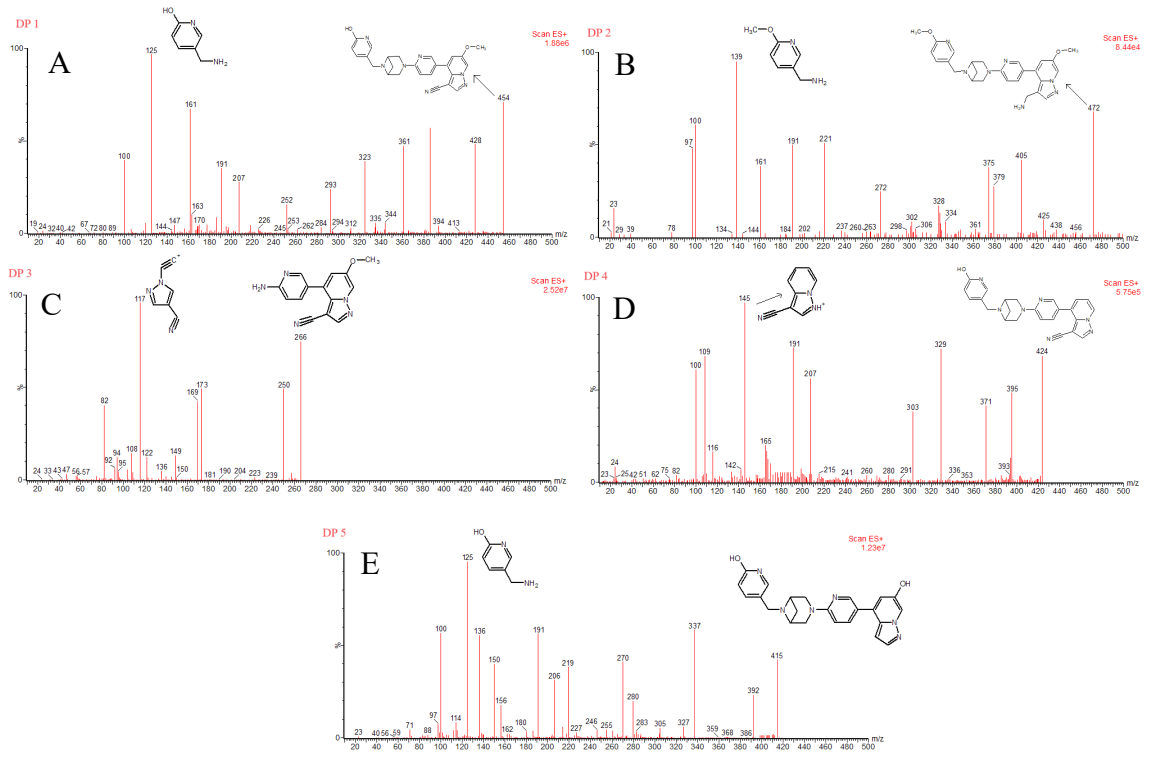


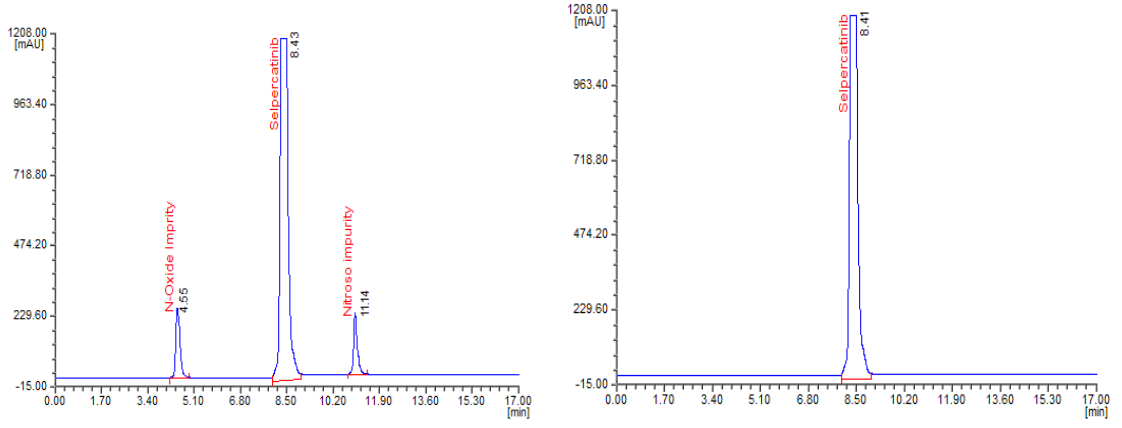
Figure 8. Fragmentation mechanism proposed for DP 5



DP 1 (A); DP 2 (B); DP 3 (C); DP 4 (D); DP 5 (E)

Figure 9. Mass spectra of DPs observed in forced degradation study

The established HPLC technique was employed to quantify the investigated impurities in formulations. The spiked sample analysis chromatogram visualizes well-retained peaks representing impurities and Selpercatinib (Figure 10A). Whereas direct formulation chromatogram doesn't visualize any peak representing impurities (Figure 10B) suggest that the impurities not detected in sample. This highlights that method was adequately fit for analyzing Selpercatinib impurities.



A = spiked formulation; B = Un-spiked formulation

Figure 10. Formulation analysis chromatogram of Selpercatinib

The findings were correlated with literature and observed that the method reported by Singamsetty *et al*., 2021 was applicable for quantification of selpercatinib in dosage forms whereas method published by Gulikers *et al*., 2023 only applicable for quantification of selpercatinib in biological samples. No method is available for the quantification of process-related as well as degradation impurities, and hence this finding offers the best option for quantification of both process-related and degradation impurities in bulk drug and formulations.

CONCLUSION

This study aimed to develop a straightforward HPLC method for evaluating genotoxic impurities of Selpercatinib, addressing the absence of published analytical methods in existing literature. The method was specifically designed to target nitroso and N-oxide impurities of Selpercatinib. Extensive optimization experiments were conducted, including variations in column configurations, pH, and mobile phase composition, to achieve optimal resolution of analytes. The final method, employing a Zorbax C18 column with a mobile phase composed of aqueous ammonium acetate and methanol at a pH of 4.5, demonstrated effective separation of Selpercatinib and its impurities. The robustness and reliability of the method were further validated through system suitability testing, which confirmed consistent peak resolution and reproducibility. Additionally, the method exhibited excellent sensitivity, with LOD and LOQ values well below regulatory limits. Precision and accuracy assessments, including recovery experiments and linearity studies, further demonstrated the method's suitability for quantitative analysis of impurities in Selpercatinib formulations. Moreover, the method's reliability was confirmed through robustness testing, showing consistent performance under deliberate variations in method parameters. The degradation studies conducted under various stress conditions provided valuable insights into the drug's stability profile and susceptibility to degradation, further validating the efficacy and specificity of the developed method. Notably, our findings revealed that the drugs exhibited stability under UV hydrolysis conditions but underwent degradation in photolysis acidic and alkaline environments. This study effectively elucidated the fragmentation pathways and characterized the degradation products of Selpercatinib. Consequently, this developed method can serve not only for assessing the genotoxic impurities of Selpercatinib but also for the identification of stress-induced DPs.

AUTHOR CONTRIBUTION STATEMENT

Concept: BRT; Data Collection or Processing: BD; Analysis: BD, EVC; Interpretation: BD, JMP, Literature Search: BD, EVC; Writing: BD, EVC, JMP; Proof reading: BRT

CONFLICT OF INTEREST

Authors declare that there is no conflict of interest.

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