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CHROMATOGRAPHIC PROFILING OF LENIOLISIB IMPURITIES USING HPLC AND LC-MS/MS: DEGRADATION BEHAVIOUR, STRUCTURAL CHARACTERIZATION, AND IN-SILICO TOXICITY EVALUATION

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ABSTRACT
Background: This study presents a comprehensive analytical investigation of leniolisib, focusing on impurity profiling, degradation kinetics, structural characterization, and in silico toxicity prediction of degradation products (DPs). **Methodology:** A systematic approach was employed to optimize the analytical method for leniolisib and its impurities, along with LC–MS/MS-based identification and *in-silico* toxicity prediction of DPs. **Result and Discussion:** Method optimized as Waters Symmetry C18 column and an isocratic mobile phase (methanol: sodium acetate buffer, 55:45 v/v) at 0.90 mL/min with UV detection at 229 nm. Leniolisib was most susceptible to acid and oxidative stress, resulting in 31.24% and 39.58% degradation, respectively. Pseudo-first-order kinetics was observed with rate constants of 0.0329 h^{-1} (acidic) and 0.0414 h^{-1} (oxidative), with half life of 21.08 h and 16.73 h. LC–MS/MS elucidates the identities of major DPs that enable the proposed degradation pathways. The MS/MS characterization confirms DP 1 with a formula of $C_{13}H_{15}N_5O$ with a mass of 257 g/mol, whereas DP 2, 3, and 4 were identified to have formulas of $C_{20}H_{26}N_6O_2$, $C_{13}H_{12}F_3N_5O$, and $C_{17}H_{19}F_3N_6O$ with masses of 382, 311, and 380 g/mol, respectively. The *In-silico* toxicity predictions show DP 1 (LD_{50} = 500 mg/kg) and DP 2 (729 mg/kg) as moderate toxicity (class 4), DP 4 shows the least toxicity (class 5, LD_{50} = 1750 mg/kg), whereas DP3 shows the highest toxicity (class 3, LD_{50} = 250 mg/kg). **Conclusion:** The developed method and accompanying data provide a critical foundation for routine quality control, stability testing, and regulatory submissions for leniolisib-based formulations.

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INTRODUCTION

Pharmaceutical impurities that may arise from synthetic processes, degradation pathways, or storage conditions can play a critical role in drug development and quality control [1]. The comprehensive profiling and characterization of these impurities are necessary to ensure the safety, efficacy, and stability of pharmaceutical products. The regulatory agencies mandate stringent guidelines for the identification and qualification of impurities. Leniolisib is a selective phosphoinositide 3-kinase delta inhibitor approved for the treatment of activated phosphoinositide 3-kinase delta syndrome. It selectively inhibits the PI3Kδ pathway, restores immune system balance, and reduces symptoms such as recurrent infections, lymphoproliferation, and immune dysregulation that are commonly observed in APDS patients [2].

Leniolisib may hold significant therapeutic potential and chemical complexity. Investigating its stability, degradation behavior, and impurity profile is crucial to ensure its safety and efficacy throughout its shelf life [3]. Despite its clinical significance, leniolisib is prone to chemical degradation under several stress conditions that result in unknown DPs and process-related impurities [4-5]. These impurities can affect the pharmacological activity and can cause unwanted toxicological effects [6]. A comprehensive knowledge of its degradation behavior & impurity profile is therefore critical to create a stable pharmaceutical product with acceptable safety margins. In the current study, a detailed chromatographic profiling of leniolisib impurities and DPs was conducted by employing sophisticated analytical methods like HPLC and LC-MS/MS. HPLC is utilized to create a sensitive, specific, and reproducible assay for the separation and quantification of impurities in normal and stressed conditions. The forced degradation studies are conducted stepwise to assess the stability of leniolisib and also to cause the formation of probable DPs. Then, LC-MS/MS analysis gives structural elucidation of unidentified impurities and DPs, which provides information on the fragmentation patterns and degradation pathways of leniolisib. The mass spectral data confirm the proposed fragmentation mechanisms and facilitate the structural identification of DPs. In the meantime, the combination of in-silico tools, toxicological risk assessment of identified impurities foretells their possible genotoxic, mutagenic, or carcinogenic risks. The studies reported indicate the availability of HPLC [7,8] and UV spectrophotometric procedures [9] for determining leniolisib in pharmaceuticals, facilitating the quantification of leniolisib. However, the literature lacks prior studies that integrate chromatographic, kinetic, and in silico approaches. Furthermore, the degradation kinetics of leniolisib under various ICH-recommended stress conditions have not been thoroughly explored. Therefore, the present study aims to

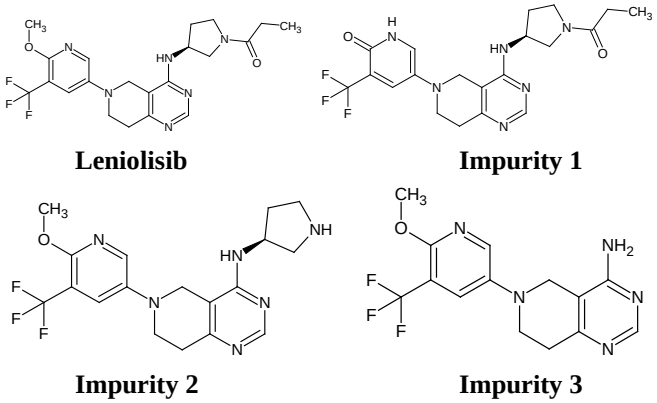
(i) Establish a validated HPLC method for routine impurity profiling of leniolisib,

(ii) Characterize the structural identity of observed impurities and DPs using LC-MS/MS

(iii) Propose possible degradation mechanisms under various stress conditions

(iv) Assess the *in-silico* toxicity profiles of the impurities to ensure patient safety.

The molecular structures of leniolisib and the selected impurities investigated in this study are presented in Figure 1.



Leniolisib **Impurity 1**

Impurity 2 **Impurity 3**

Figure 1: Structure of leniolisib and its impurities

MATERIALS AND METHODS

Standards and reagents

The high-purity reference standards of leniolisib (purity 98.7%) and its impurities 1, 2, and 3, along with commercially available leniolisib tablets (Joenja® 70 mg), were procured from Novartis Pharmaceuticals, HITEC City, Hyderabad, Telangana, India. HPLC-grade methanol and water, as well as buffer chemicals used for HPLC analysis, were obtained from Merck Life Science Private Limited, Mumbai, India. The reagents required for the stress degradation study, which include analytical grade hydrochloric acid (HCl), sodium hydroxide (NaOH), and 30% hydrogen peroxide (H_2O_2), were also sourced from Merck Life Science Private Limited.

Apparatus

The HPLC system equipped with Diode Array Detection (DAD) was utilized for the analysis of leniolisib and its impurities. An

Agilent 1200 series system that includes a quaternary pump, auto-injector, vacuum degasser, and DAD with G1315 C/D and G1365 C/D models was utilized in this study. This setup was connected to a computer operating with Agilent ChemStation software (Agilent Technologies, USA) for instrument control, data acquisition, and analysis. The chromatographic separation was achieved using a Waters Symmetry C18 analytical column with dimensions of 250 × 4.6 mm and a particle size of 5 µm.

The HPLC system was coupled with an API 2000 triple quadrupole mass spectrometer system (Applied Biosystems Sciex, USA), which featured an Electrospray Ionization (ESI) source operating in positive ion mode. The ESI source parameters are as follows: ion spray voltage (+5500 V), source temperature (400°C), nebulizer gas pressure (40–50 psi), heater gas pressure (40–50 psi), and curtain gas pressure (25 psi). The collision gas (nitrogen) was used at a medium pressure to aid fragmentation in the collision cell (Q2).

The mass spectrometric detection was carried out in Multiple Reaction Monitoring (MRM) mode. In this, both the precursor ion (Q1) and product ion (Q3) transitions specific to leniolisib and its impurities were monitored. The Dwell time, collision cell exit potential, collision energy, and declustering potential were individually optimized for each MRM transition to ensure maximum sensitivity and signal-to-noise ratio. The scan range was typically set between m/z 50 and 1000, based on the molecular weights of the analytes. Data acquisition and instrument control were managed through Analyst® software (version 1.6.2).

Preparation of solutions

The individual stock solution of leniolisib and impurities was prepared by dissolving each reference standard in HPLC-grade methanol to a final concentration of 1000 µg/mL. The solutions were stored in a freezer at 0°C to maintain their stability during analysis. Working standard solutions were obtained by dilution of stock solutions using deionized water to yield the final concentrations from 50 µg/mL (level 1) to 300 µg/mL (level 6) for leniolisib, and from 0.5 µg/mL (level 1) to 3.0 µg/mL (level 6) for impurities. Standard calibration solutions were prepared by blending equal volumes of each respective linearity level solution, and 20 µL of each solution was injected into the HPLC–DAD system under optimal chromatographic conditions. Each level of concentration was run in triplicate to maintain precision and accuracy. The resulting peak responses were plotted against their known concentrations to construct calibration curves and determine regression equations for quantitative analysis.

Assay sample preparation

Five Joenja® tablets, each labeled with a content of 70 mg of leniolisib, were opened with care, and their contents were weighed precisely. Tablet powder weighing an amount equivalent to the average tablet weight was transferred to a 50 mL volumetric flask. To facilitate dissolution, approximately 30 mL of ethanol was added, followed by 5 minutes of ultrasonic irradiation to ensure complete dissolution of the drug. Following sonication, ethanol was used to bring the solution to volume. The filtered solution was further filtered through a 0.22 µm membrane filter, after which insoluble excipients or any particulates were removed from the solution. A portion of the filtered product was further diluted to yield the final test solution containing leniolisib with a concentration of 100 µg/mL. To validate the analytical method, including its ruggedness and accuracy, known amounts of pure leniolisib standard were spiked into separate aliquots of the tablet solution using the standard addition method. The spiked samples were processed and analyzed under the same conditions as the test solution to quantify recovery and to confirm that there was no interference from formulation excipients in the measurement.

Forced Degradation

The chemical stability of leniolisib was assessed through a set of forced degradation studies under a variety of ICH-recommended stress conditions to determine possible DPs and to validate the stability-indicating character of the analytical method [10]. Stress tests simulate extreme environmental conditions to determine how the drug substance behaves and degrades, providing valuable information for formulation development and assessing its shelf life. In all experiments, 1 mL of a stock solution of leniolisib at a high concentration (1000 µg/mL) was exposed to specific stress conditions. Following stress exposure, the samples were neutralized where necessary and diluted with deionized water in 10-mL volumetric flasks to obtain a final concentration of 100 µg/mL before analysis.

For acid hydrolysis, 1 mL of 2 M hydrochloric acid was added to the leniolisib solution. The mixture was heated in a water bath at 60 °C for 30 minutes, followed by incubation at room

temperature for 12 hours. In alkaline degradation, 1 mL of 0.1 M sodium hydroxide was added to the drug solution and kept at ambient conditions for 12 hours. The oxidative stress assay consisted of combining 1 mL of leniolisib stock solution with 1 mL of 30% hydrogen peroxide, shaking the mixture at 60°C in a water bath for 2 hours, and then allowing it to reach room temperature. To check degradation by neutral hydrolysis, 1 mL of the standard solution was combined with 1 mL of deionized water and heated at 80°C for 30 minutes in a water bath, followed by cooling. Photolytic degradation was checked by placing 1 mL of leniolisib stock solution in a sealed 10 mL volumetric flask and exposing it to natural sunlight for 4 hours. For thermal degradation, the pure leniolisib powder was subjected to a hot air oven at 100 °C for 2 hours.

Kinetics Investigation

A degradation kinetics study was conducted to investigate the time course degradation of leniolisib under acidic stress conditions [11]. Throughout the test, 1 mL aliquots of the leniolisib stock solution were each subjected to acidic hydrolysis. The samples were left at room temperature, and aliquots were removed at preselected time points to monitor the degradation progress. At each selected time point, the reaction mixture was stopped immediately with neutralization to prevent further degradation. The neutralized solution was then diluted to a final volume of 10 mL with deionized water, making the concentration suitable for HPLC analysis. All samples at time points were then analyzed using the optimized HPLC–DAD method. The concentration of leniolisib at all points in time was determined through the regression formula from the calibration curve. The plot of concentration vs. time was plotted, and the data obtained were analyzed to determine the kinetics of degradation, including reaction order and rate constant. This kinetic study provided valuable insights into the nature of leniolisib degradation, with relevance to predicting its shelf life and informing recommendations for storage or processing conditions.

***In-silico* Toxicity Studies**

The in silico toxicity prediction method was used to assess the potential toxicity of leniolisib DPs. ProTox-II, a well-established web-based tool for predicting the toxicological properties of chemical compounds based on their molecular structure, was utilized in this analysis. ProTox-II calculates the LD_{50} value, i.e., the dose (mg/kg body weight) needed to kill 50% of a population of test animals, used as a quantitative assessment of acute toxicity. Besides LD_{50}, the system also assigns a toxicity class to each compound, ranging from Class 1 (very highly toxic) to Class 6 (non-toxic), thereby contributing to the classification of health hazards related to potential exposure. These forecasts provide helpful information on the safety profile of leniolisib and its breakdown products, particularly in situations involving environmental stress or storage conditions that do not meet specifications, aiding in reducing risks during formulation development and shelf-life evaluation [12,13].

Statistical Analysis

All statistical calculations and data processing were performed using the built-in tools in Microsoft Excel 365. Furthermore, the toxicity of the DPs was assessed using the ProTox-II web server.

RESULTS AND DISCUSSION

To conduct a comprehensive kinetic degradation study, it is necessary to employ a stability-indicating chromatographic technique that can efficiently separate leniolisib from its DPs. The selected technique should not only allow precise quantitation of the parent drug but also permit simultaneous detection and resolution of its impurities and DPs, thereby providing robustness and reliability of the analysis. Such a two-way analytical approach dramatically enhances the suitability of the method in biological matrices and increases its potential for pharmacokinetic and clinical applications. In addition, DPs identified through LC–MS/MS analysis must then undergo extensive structural characterization, which is a critical consideration in terms of determining their chemical nature and potential for transformation routes. To validate these findings, in-silico prediction of toxicity was performed to establish the possible toxicological risks associated with the detected DPs, thereby providing a comprehensive picture of the drug's stability and safety profile.

Optimization of Chromatographic Conditions

A set of chromatographic conditions was optimized in a stepwise manner for the efficient and reproducible separation of leniolisib and its identified impurities and DPs. Two reversed-phase columns, Phenomenex Luna and Waters Symmetry RP-C18 (150 mm × 250 mm × 4.6 mm, 5 µm), were first tested with equal volume mixtures of methanol, acetonitrile, and deionized water. Both columns exhibited similar retention characteristics; however, the Waters Symmetry C18 column provided better

peak symmetry, less tailing, and a more robust signal response, and was therefore chosen for further method development. The influence of the pH of the mobile phase was investigated by setting it at pH levels of 3, 5, 7, and 9. Acidic pH caused poor separation and peak overlap due to the protonation of leniolisib and its impurities, whereas basic pH caused peak distortion and low resolution. The best separation was achieved under moderately acidic to neutral pH conditions, resulting in improved retention and peak symmetry. Organic modifier choice was also examined, where methanol gave enhanced resolution, narrow peaks, and low tailing with respect to acetonitrile. The elution mode isocratic was considered sufficient for maintaining separation under conditions of forced degradation as well. The last optimized technique employed a Waters Symmetry C18 column (250 × 4.6 mm, 5 µm) under an isocratic mobile phase consisting of a 55:45 (v/v) ratio of methanol and 0.01 M sodium acetate buffer (pH ~6.8) at a flow rate of 0.90 mL/min. Detection was performed at 229 nm, the maximum wavelength of absorbance (λmax) of leniolisib and its impurities. The overall run time was 10 minutes, with retention times of 3.848 minutes for leniolisib, 4.957 minutes for impurity 1, 3.215 minutes for impurity 2, and 1.848 minutes for impurity 3. System suitability parameters proved the reliability of the method with high theoretical plate counts (> 2000), tailing factor (< 2), and resolution values (Rs > 2), in accordance with FDA acceptance criteria. These findings confirm the method's robustness and appropriateness for routine stability and quality control analysis of leniolisib.

Table 1: System suitability results of leniolisib and impurities in the optimized method

Parameter	Leniolisib	Impurity 1	Impurity 2	Impurity 3
t_R in min	3.848	4.957	3.215	1.848
Resolution	9.25	11.35	7.54	--
Theoretical plates	13025	15784	8631	6057
Tailing factor	0.99	1.02	1.01	1.02

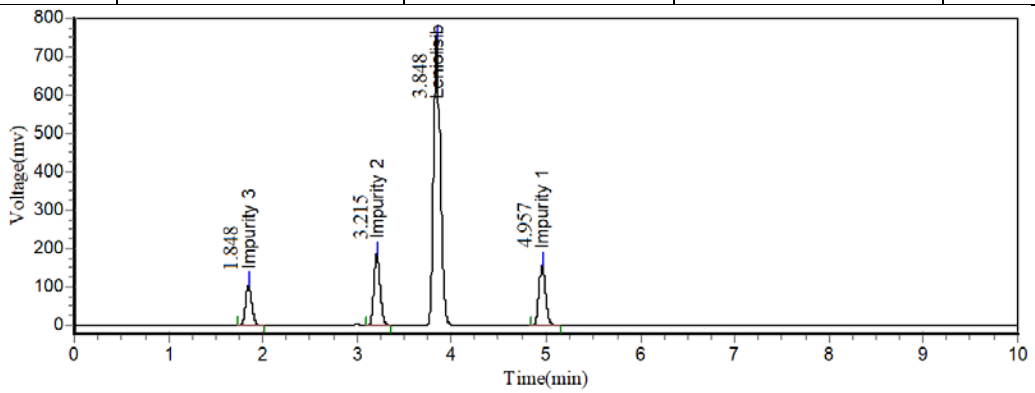


Figure 2: System suitability chromatogram of leniolisib and impurities in the optimized method

Stress degradation study

The comprehensive forced degradation of leniolisib was conducted according to ICH guidelines to determine its stability under various stress conditions. The proposed HPLC–DAD method was employed to track the degradation trend of leniolisib under each condition. Initially, mild conditions, such as base, sunlight, thermal, and neutral water, produce only partial degradation with no DPs in the chromatogram. In contrast, acid and peroxide conditions proved to be highly significant. In acidic conditions, the drug exhibits 68.76% recovery, indicating 31.24 % degradation, and the chromatogram displays well-resolved three new DPs. The known impurities 1, 2 & 3 in the study were also found in the acid stress chromatogram (Figure 3A). In a basic environment, the observed % degradation was 7.41% with a recovery % of 92.59% and the formation of No DPs. The dry heat stress resulted in 88.91% recovery of leniolisib with 11.09% degradation, and this stress also generates a new DP (Figure 3C). On the other hand, leniolisib is more stable under peroxide, photolytic, and neutral hydrolysis conditions with recoveries of 98.35%, 97.54%, and 98.15% respectively. The absence of new DPs under these three conditions indicates that leniolisib is more stable in thermal, photolytic, and neutral conditions than in acidic, basic, and oxidative stress conditions. Table 2 presents the stress study findings of leniolisib observed during this study.

Table 2: Stress degradation study results of leniolisib in the optimized method

S No	Condition	Degradation conditions	% Recovery	% degradation	No of new DPs formed
1	Acid	2 M HCl, RT, 12 hours	68.76	31.24	2
2	Base	0.01 M NaOH, RT, 12 hours	92.59	7.41	-
3	Oxidative	30% H_2O_2, 60 °C for 2 hours	60.42	39.58	3
4	Dry heat	Dry heat 100°C, 2h	98.76	1.24	-
5	Photolytic	Sunlight, 4h (summer)	97.45	2.55	-
6	Neutral	H_2O, 80 °C, 30 min	98.94	1.06	-

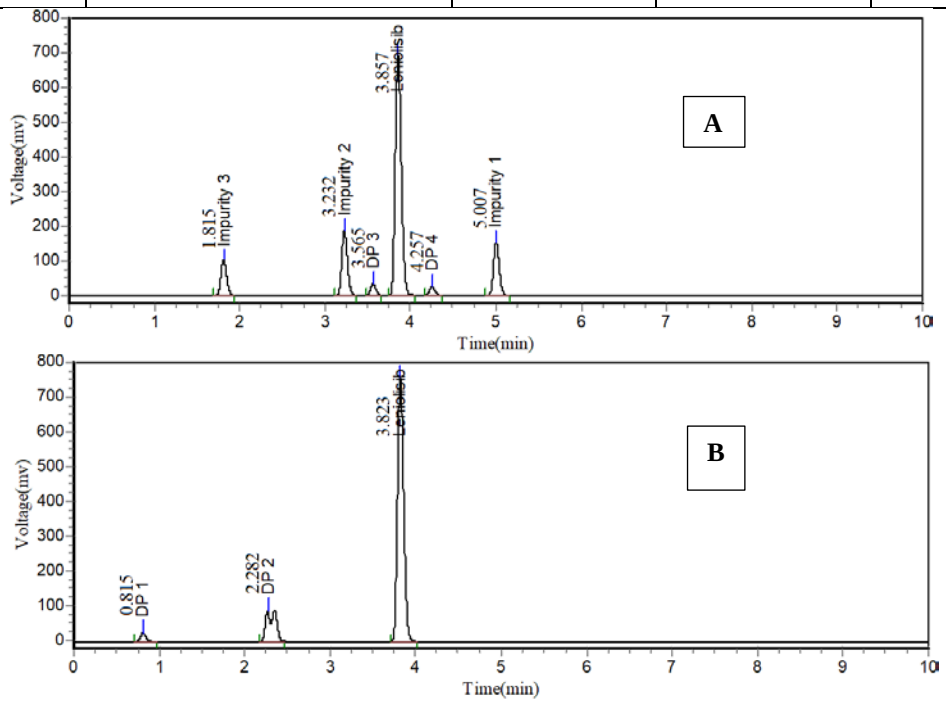


Figure 3: Acid (A), and peroxide (B) stress study chromatograms of leniolisib in the optimized method

Degradation kinetics

The kinetics of degradation of leniolisib was studied extensively to analyze its chemical stability when subjected to oxidative stress & acid conditions, which are essential parts of stability-indicating studies according to ICH guidelines. In acid and oxidative-induced degradation analysis, leniolisib was incubated under controlled acidic and peroxide conditions. The procedure described in the acid and peroxide stress study was used to evaluate the degradation kinetics, as outlined in the stress study experiment. An aliquot was withdrawn at regular time intervals from the reaction mixture, promptly neutralized, and then subjected to the validated HPLC procedure to measure the remaining concentration of the drug (Ct) against its initial concentration (Co). This method enables accurate tracking of the degradation process over time. A progressive and clear reduction in the concentration of leniolisib was observed in both acid and oxidative stress studies, which proves that the compound is prone to degradation under these conditions. The results were graphed as the logarithm of the concentration remaining in the stress reaction versus time. The results yield a linear plot for both acid and oxidative stress studies, validating that the degradation follows pseudo-first-order kinetics. The first-order rate constant (k) for the pathway of acid degradation was found to be 0.0329 h^{-1}, which corresponds to a half-life ($t_{1/2}$) of 21.08 hours. In contrast, the oxidative degradation produces a k value of 0.0414 h^{-1} with a corresponding reduced half-life of 16.73 hours.

These results clearly show that leniolisib is more susceptible to oxidative degradation than acid hydrolysis, which highlights the need to keep the drug from oxidative conditions when formulating and storing. The kinetic profiles observed in both degradations provide essential information about the inherent stability of leniolisib, which will help develop stable formulations and proper packaging solutions. The graphical illustration of the degradation kinetics under acid and peroxide stress conditions is presented in Figures 4A and 5B, respectively.

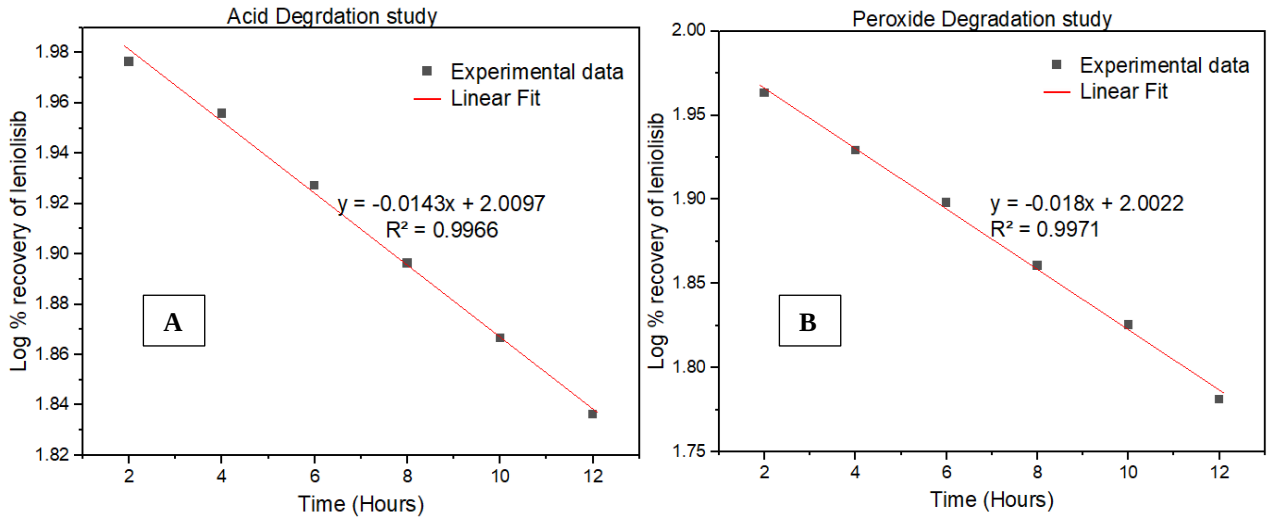


Figure 4: Pseudo first-order kinetic plot observed of leniolisib under acid stress (A) and oxidative stress conditions

Method Validation

The method developed for the quantification of leniolisib, its impurities, and DPs was validated according to the ICH guidelines [14-16]. The range of standard and impurity solutions with varying concentrations that exhibit a highly correlated linear plot was assessed for linearity. The method shows a strong linear response over the concentration range of 50 µg/mL to 300 µg/mL for leniolisib and 0.5 µg/mL to 3.0 µg/mL for impurities. The regression equations obtained were y = 11775x + 42494 for leniolisib, y = 29775x + 787.76 for impurity 1, y = 52541x + 2278.3 for impurity 2 and y = 28287x + 379.21 for impurity 3. The correlation coefficients (r) were exceptionally high with corresponding r^2 values of more than 0.999, clearly indicating that the method exhibits excellent linearity. In addition, the % RSD of the slope was noticed to be well below the acceptable limit of 2% for leniolisib and its impurities, indicating minimal fluctuation in the response factor.

The sensitivity of the method was assessed by determining LOD and LOQ for both leniolisib and its impurities. These values were established based on the signal-to-noise ratio, where a ratio of 3:1 was used for LOD and 10:1 for LOQ. The method proved to be highly sensitive, as evidenced by the very low concentrations that could be reliably detected and quantified. The LOD was 0.15 µg/mL, whereas the LOQ of impurities was 0.5 µg/mL. These findings highlight the method's suitability for detecting and quantifying trace amounts of impurities.

The intra-day and inter-day tests were conducted at three concentration levels to evaluate the accuracy and precision of the method. In intra-day precision, each concentration was analyzed three times on the same day. For inter-day precision, the tests were repeated across three consecutive days. Accuracy was evaluated by calculating the relative error (Er), while precision was assessed using the percentage relative standard deviation (% RSD). The results, summarized in Table 3, indicate that the Er and RSD values of leniolisib and its impurities were consistently within the acceptable range of less than 2%. Additionally, the recovery rates for all tested concentrations were excellent, ranging from 98% to 102%, further confirming that the developed method provides reliable and reproducible results for routine quality control and stability testing of leniolisib and its impurities.

The method's robustness was tested by intentionally introducing small variations in key chromatographic parameters to see if these changes would affect the method's performance. The conditions, such as detection wavelength, pH of the aqueous phase, solvent ratio, mobile phase flow rate, column temperature, and injection volume, were adjusted. These deliberate changes did not significantly impact the results. The leniolisib and its impurities display consistent peak areas with % RSD values below 2% indicating excellent reproducibility. Additionally, the retention times of the peaks show minimal standard deviation values, confirming that the method remains reliable and accurate even when minor operational changes are introduced, thereby proving its robustness. We assessed whether commonly used excipients or inactive ingredients in pharmaceutical formulations interfered with the detection of active compounds in a specificity study. The result doesn't show

any such interference, indicating that the method is highly specific to the analytes of interest. This was further confirmed by analyzing the pharmaceutical formulation solution of leniolisib. The formulation solution displays leniolisib peak at its particular retention time, and no additional or overlapping peaks were detected, confirming that none of the excipients or inactive ingredients present in the formulation interfered with the analysis. This indicates that the method is highly selective for the analysis of leniolisib in complex sample matrices. The reliability of the technique was strengthened by conducting a standard addition test. A known quantity of impurities was added to the tablet solution. The resulting chromatographic analysis confirms the successful recovery of the added impurities with no interference from the formulation matrix, demonstrating that the method is not only accurate but also robust enough for routine quality control analysis.

Table 3: Precision and accuracy results of leniolisib and its impurities in the proposed method

Analyte	Test	Intraday			Interday		
Standard	**% Recovery**	99.63±0.635	99.58±0.591	100.01±0.748	99.44±0.527	98.66±0.598	100.34±0.529
	% RSD	0.25	0.32	0.36	0.65	0.26	0.68
	% error	0.84	0.94	0.59	0.39	0.14	1.15
Impurity 1	**% Recovery**	99.36±0.251	99.62±0.658	99.28±0.547	100.20±0.206	99.82±0.809	98.05±0.281
	% RSD	0.84	0.43	0.28	0.95	0.41	0.99
	% error	0.92	1.23	0.64	0.23	0.87	0.14
Impurity 2	**% Recovery**	99.58±0.590	98.62±0.558	98.96±0.538	99.57±0.581	99.56±0.563	100.25±0.925
	% RSD	1.02	0.62	0.59	1.36	0.58	0.37
	% error	0.74	0.37	0.23	0.87	0.27	0.93
Impurity 3	**% Recovery**	100.01±0.735	99.64±0.748	98.87±0.262	100.95±0.958	100.37±0.359	101.25±1.025
	% RSD	0.56	0.49	0.27	1.05	0.32	0.25
	% error	0.41	0.63	1.35	0.93	0.57	1.39

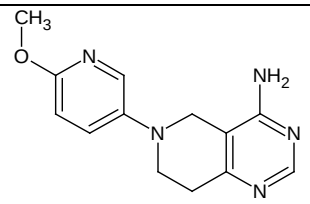
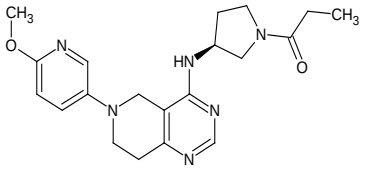
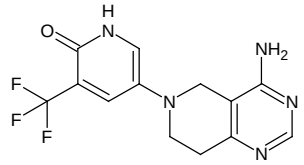
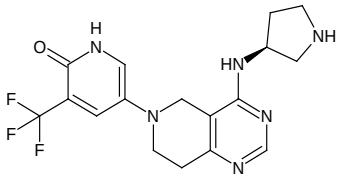
LCMS/MS study of DPs:

The acid, base, and dry heat stress samples were further analyzed through LC-MS/MS, and the results provide adequate information to understand the breakdown of leniolisib under the studied stress conditions. This approach enables the separation and identification of the DPs formed during stress testing. The stress-degraded samples were injected into the system, and the mass-to-charge ratios (m/z) of the resultant peaks were recorded using scan mode. The DP 2 of leniolisib was identified as a major transformation product under oxidative stress conditions induced by hydrogen peroxide. The degradation mechanism involves the oxidative cleavage of the trifluoromethyl (–CF_3) group attached to the pyridine ring of leniolisib. This moiety is very susceptible to nucleophilic or oxidative attack under peroxide-rich environments. This product was detected at an RRT of 0.593 and exhibits a protonated molecular ion $[M+H]^+$ at m/z 383.4594, corresponding to a molecular weight of 382 g/mol with the empirical formula $C_{20}H_{26}N_6O_2$. This mass shift from the parent molecule confirms the elimination of the –CF_3 group (mass ≈ 69 Da), indicative of de-trifluoromethylation followed by structural reorganization and stabilization through protonation or hydroxylation. The HRMS fragmentation analysis further validated the structure of DP 2. Key fragment ions include m/z 328.4035 ($C_{17}H_{23}N_6O$), which represents the core structure following side chain cleavage. Further, the key fragments were noticed at m/z 285.3357 ($C_{15}H_{18}N_5O$), 259.2984 ($C_{13}H_{16}N_5O$), 204.2630 ($C_{11}H_{15}N_4$), and m/z 154.2010 ($C_8H_{13}N_2O$), and these fragments support the presence of substituted aliphatic chains and confirm extensive rearrangement of the molecular framework post CF_3-cleavage. These MS/MS fragments (Table 4), in alignment with the proposed molecular formula, confirm that DP2 is the de-trifluoromethylated oxidative degradation product of leniolisib. The specific process of CF_3 group removal and following structural verification through HRMS fragmentation was demonstrated in Figure 5. The secondary amine functionality (R-NH-R) of DP 2 was oxidatively cleaved, yielding a more polar DP (DP 1) that eluted at an RRT of 0.212. This conversion is due to N-oxidation followed by bond cleavage or rearrangement, resulting in the loss of alkyl substituents from the amine group. The resulting DP has a protonated molecular ion $[M+H]^+$ at m/z 258.2911, corresponding to a neutral molecular weight of ~257

g/mol, indicative of a side chain loss to the leniolisib. The MS/MS fragment spectrum of DP1 shows a series of predominant fragment ions at m/z 242.2679 ($C_{13}H_{13}N_4O$), indicating methyl or hydroxyl loss, at m/z 227.2566 ($C_{12}H_{12}N_5$), indicative of bond cleavage at the heterocyclic core. The fragments observed at m/z 148.1567 ($C_7H_7N_4$), 138.1586 ($C_7H_9N_2O$), and m/z 109.1206 ($C_5H_6N_3$) support the likely structure of DP 1 (Table 4). These fragmentation patterns collectively confirm the structure of DP1 as 6-(6-methoxypyridin-3-yl)-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-amine. The DP 4 was generated through acid-catalyzed degradation by cleavage of the C–O bond of the methoxy (–OCH_3) group that is bound to the pyridine ring of leniolisib. In acidic conditions, the protonation of the methoxy group facilitates the loss of a methyl group, promoting the subsequent oxidation of the pyridine ring and leading to the formation of a carbonyl group (C=O) in place of the methoxy functionality. This chemical transformation was identified by a peak at RRT of 1.106. The experimental mass spectrum shows a protonated molecular ion $[M+H]^+$ at m/z 381.3675, corresponding to a molecular weight of 380 g/mol and a molecular formula of $C_{17}H_{19}F_3N_6O$. The mass difference from the parent molecule and the structural changes are consistent with the loss of a $–CH_3$ group and addition of a carbonyl functionality. The HRMS analysis supports the structural assignment of DP4.

The key fragment ions (Table 4) were observed at m/z 362.3686 ($C_{17}H_{19}F_2N_6O$), which further lose to form fragments at m/z 313.2698 ($C_{13}H_{13}F_3N_5O$), 228.2413 ($C_{12}H_{11}N_4O$), 243.2990 ($C_{13}H_{16}N_5$), 188.2205 ($C_{10}H_{11}N_4$), and 135.1579 ($C_7H_8N_3$). This fragmentation pattern, along with the observed mass data, confirms that DP 4 results from acid-catalyzed hydrolysis and oxidation of the methoxy-substituted pyridine moiety of leniolisib.

Table 4: HRMS data observed for DPs of leniolisib characterized in this study

Name	RRT	Experimental mass $[M+H]^+$	Proposed Molecular formula	Error (ppm)	m/z of major Fragments	Proposed $[M+H]^+$ Formula	Proposed structure
DP 1	0.212	258.2911	$C_{13}H_{15}N_5O$	0.387	$C_{13}H_{13}N_4O$	242.2679	
					$C_{12}H_{12}N_5$	227.2566	
					$C_7H_7N_4$	148.1567	
					$C_7H_9N_2O$	138.1586	
					$C_5H_6N_3$	109.1206	
DP 2	0.593	383.4594	$C_{20}H_{26}N_6O_2$	0.522	$C_{17}H_{23}N_6O$	328.4035	
					$C_{15}H_{18}N_5O$	285.3357	
					$C_{13}H_{16}N_5O$	259.2984	
					$C_{11}H_{15}N_4$	204.2630	
					$C_8H_{13}N_2O$	154.2010	
DP 3	0.927	312.2624	$C_{13}H_{12}F_3N_5O$	0.320	$C_{13}H_{12}F_2N_5O$	293.2635	
					$C_{12}H_{12}F_3N_4O$	286.2445	
					$C_{12}H_{10}N_5O$	241.2401	
					$C_9H_4N_4$	174.1940	
					$C_5H_6N_3$	109.1206	
DP 4	1.106	381.3675	$C_{17}H_{19}F_3N_6O$	0.262	$C_{17}H_{19}F_2N_6O$	362.3686	
					$C_{13}H_{13}F_3N_5O$	313.2698	
					$C_{12}H_{11}N_4O$	228.2413	
					$C_{13}H_{16}N_5$	243.2990	
					$C_{10}H_{11}N_4$	188.2205	
					$C_7H_8N_3$	135.1579	

The R–NH–R moiety of DP4 underwent hydrolytic cleavage under prolonged acidic conditions to form primary amine (R–NH_2). This compound was identified at RRT of 0.927 and was designated as DP3. The HRMS analysis reveals a protonated

molecular ion $[M+H]^+$ at m/z 312.2624, corresponding to a molecular mass of 311 g/mol and molecular formula $C_{13}H_{12}F_3N_5O$ (Table 4).

The structural simplification observed in DP4 is indicates the acid-catalyzed dealkylation and hydrolysis processes that effects the side chain containing the R–NH–R unit. The absence of the additional alkyl substituent and the presence of characteristic fragments consistent with primary amine derivatives support the proposed structural assignment (Figure 5). This stepwise acid hydrolysis, which leads from leniolisib to DP 4 and then to DP 3, highlights the compound's susceptibility to acidic degradation.

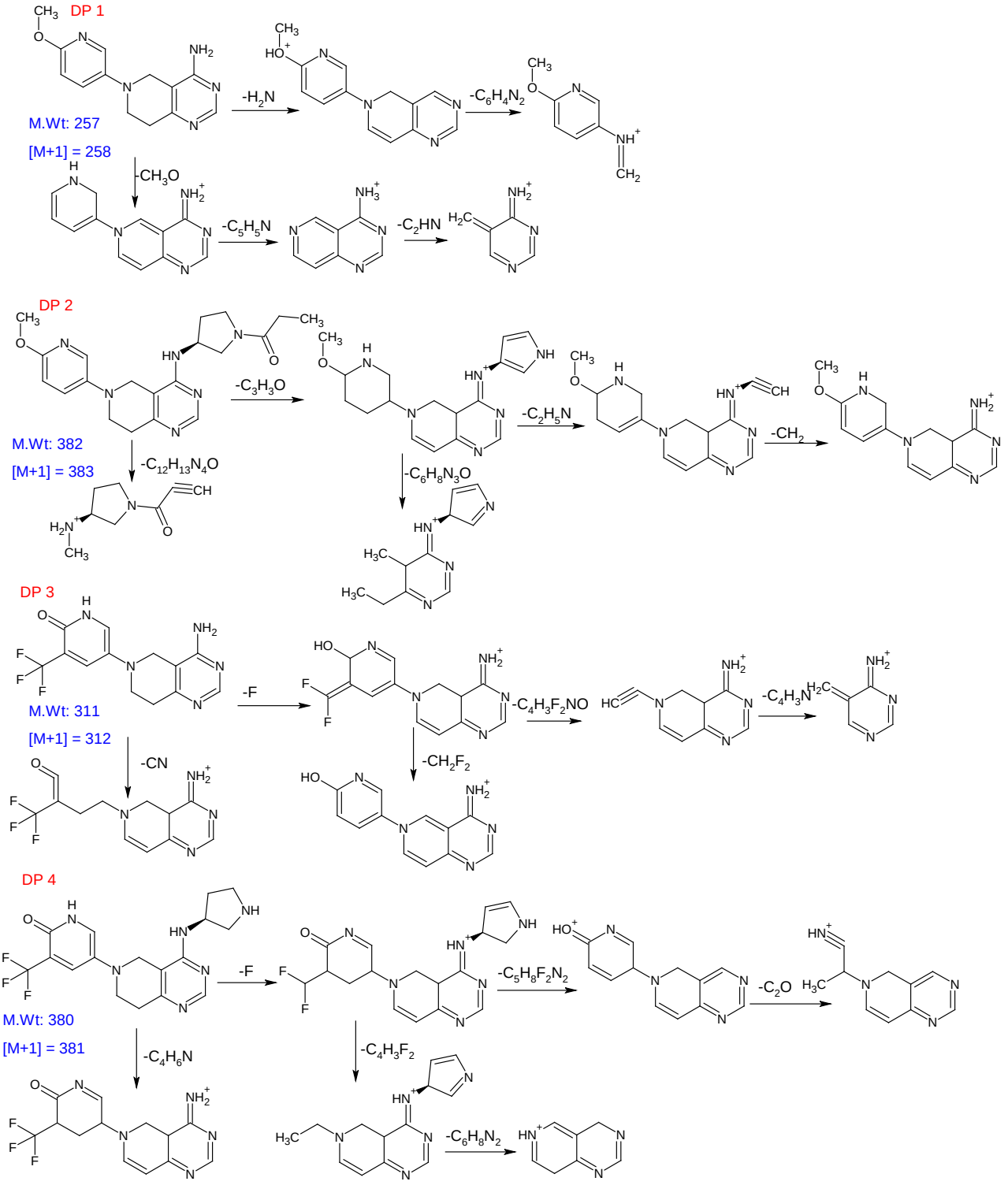


Figure 5: Fragmentation mechanism proposed for DPs of leniolisib characterized in this study

***In-silico* toxicity assessment of DPs**:

The toxicity prediction of DPs was considered essential for evaluating the potential health and environmental risks posed by DPs. It facilitates the early identification of harmful effects, guiding safer drug design, formulation, and regulatory compliance. Hence, the in-silico toxicity studies of DPs characterized in this study were conducted using the ProTox-II software tool. The acute toxicity levels for the DPs were categorized based on the LD_{50} values and the toxicity classes predicted in this study. The DP1 shows an LD_{50} of 500 mg/kg and was classified under toxicity class 4, which indicates its moderate toxic profile. The DP 2 shows a slightly higher LD_{50} of 729 mg/kg and also belongs to class 4. In contrast, DP3 exhibits the lowest LD_{50} of 250 mg/kg and is categorized as class 3, suggesting its relatively higher toxicity among the DPs, whereas DP4, with an LD_{50} of 1750 mg/kg, displays the least acute toxicity.

The four DPs were forecasted to be non-active for most likely hepatotoxicity and nephrotoxicity, which means no significant risk to liver or kidney injury. Neurotoxicity and respiratory toxicity, however, were uniformly forecasted to be active in all DPs, which suggests a possible issue with the central nervous system and respiratory well-being with exposure to these DPs. None of the DPs was linked to cardiotoxic, carcinogenic, or immunotoxic effects, indicating a good safety profile for these particular areas. The DP 1 was found to be mutagenic, but DP 2, DP 3, and DP 4 were inactive for mutagenicity.

The DP 2 and DP 3 were predicted to be ecotoxic, while DP 1 and DP 4 did not indicate a selective risk of environmental harm from certain degradation products. Furthermore, all four DPs were showing clinical toxicity, emphasizing the importance of minimizing their formation or accumulation during formulation and storage. None of the DPs exhibit nutritional toxicity, suggesting no interference with dietary or metabolic functions. The *in-silico* predictions of neurotoxicity and respiratory toxicity observed across all DPs may represent false positives, as these outcomes require experimental validation to confirm their actual clinical relevance. The absence of predicted hepatotoxicity and nephrotoxicity could be a potential false negative; this may overlook underlying organ-specific toxicity risks that might emerge under in vivo conditions. These findings support the need for formulation strategies that limit oxidative and acidic degradation pathways.

CONCLUSION

The present study addresses a critical analytical gap in the literature through a rigorous investigation of impurity profiling, degradation kinetics, structural elucidation of degradants (DPs), and in silico toxicity studies of leniolisib. A stability-indicating & fast chromatographic method was designed and optimized using a Waters Symmetry C18 column with an isocratic mobile phase of methanol and sodium acetate buffer (55:45, v/v). The approach depicted very good resolution (Rs > 2) between leniolisib and impurities and was validated entirely based on the ICH guidelines. Validation results provided better linearity (r^2 > 0.999), high sensitivity, precision, & accuracy as evidenced by low %RSD values & recovery levels of 98–102%, making it fit for routine quality control and stability testing. Forced degradation studies determined that leniolisib is susceptible to degradation under acidic, basic, and thermal stress conditions to generate novel DPs. Three different DPs were formed under acidic stress, and one DP was formed under basic and dry heat stress. Kinetic degradation studies showed leniolisib degradation to be pseudo-first-order kinetics with the highest degradation under oxidative stress ($k = 0.0414\ h^{-1}$, $t_{1/2} = 16.73$ h), followed by acid stress ($k = 0.0329\ h^{-1}$, $t_{1/2} = 21.08$ h), indicating the oxidative instability of the drug. In addition to the above, LC-MS/MS analysis allowed for the identification and structural elucidation of four DPs. DP2 was formed because of oxidative stress-induced detrifluoromethylation of the pyridine ring, whereas N-oxidation and side-chain cleavage led to DP1 formation. DP4 was formed in an acidic pH environment due to the cleavage of the methoxy group and oxidative modifications of the pyridine ring. In contrast, the hydrolytic dealkylation of DP4 resulted in the formation of DP3. In-silico toxicological prediction using the ProTox-II platform provided additional insight into the potential biological effects of such degradation products and assisted in assessing their toxicological relevance.

Overall, this comprehensive analytical strategy not only meets regulatory needs for method validation and impurity profiling but also further elucidates leniolisib's mechanisms of degradation and safety issues. The following validated method and stability data will find application in formulation development, regulatory submissions, and long-term storage planning for drugs containing leniolisib.

FINANCIAL ASSISTANCE

NIL

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTION

Rasheed Babu Shaik collected data and performed experiments. S. Lakshmi Tulasi conducted the analysis. Rahul Gunupati wrote the first draft of the manuscript, and all authors reviewed and revised previous versions. All authors contributed to the study's conception and design and gave final approval.

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