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Original Article

Chromatographic Profiling of Encorafenib Impurities Using HPLC and LC-MS/MS Structural Characterization and In Silico Toxicity Evaluation of Degradation Products

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Abstract

This study systematically evaluated the impurities of Encorafenib along with structural elucidation, and in silico toxicological potential of its degradation products (DPs). The HPLC resolution of Encorafenib and its impurities was achieved on a Waters Symmetry C18 column using an isocratic mobile phase of methanol and 0.1 M ammonium acetate (pH 4.1, 60:40 v/v) at a flow rate of 0.85 mL/min with detection at 231 nm. The method effectively resolves Encorafenib and its three known impurities (Rs > 2) with acceptable ICH validation criteria such as linearity (r^2 > 0.999), sensitivity (LOD: 0.075 µg/mL), precision (RSD < 2%), robustness, and specificity. The base hydrolysis induces 16.31% degradation of Encorafenib with the formation of three new DPs, whereas acid stress produces 8.28% degradation. The LC-MS/MS analysis with fragmentation pattern was utilized for structural elucidation of major DPs. The in silico toxicity assessment proves that acid DP 2 and base DPs 3 and 4 display moderate toxicity (Class 4; LD_{50}: 1000–1300 mg/kg), whereas base DP 1 shows high toxicity (Class 3; LD_{50}: 155 mg/kg). The ADMET prediction using the pkCSM tool indicates favorable absorption characteristics and absence of predicted mutagenicity. Hepatotoxicity was predicted for all DPs; DP3 shows hERG II inhibition, and DP4 displays a broad toxicity profile. The developed method and comprehensive characterization of DPs provide a solid foundation for routine analysis and stability studies of Encorafenib.

Keywords

Encorafenib, impurity analysis, forced degradation, characterization of degradation products, in silico toxicity

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Introduction

Pharmaceutical impurities can originate during synthesis, degradation pathways, or storage conditions. The comprehensive profiling and characterization of these impurities were essential to ensure the safety, efficacy, and stability of pharmaceutical compounds.[1] The regulatory agencies mandate stringent guidelines for the identification and qualification of impurities. Encorafenib is a selective inhibitor of the BRAF V600E mutation and was utilized in the treatment of various malignancies like melanoma and colorectal cancer.[2] In combination with other kinase inhibitors, Encorafenib exhibits high clinical significance in patients suffering from BRAF-mutated tumors. Its mechanism of action involves the inhibition of mutant BRAF kinase activity and thereby interrupts the MAPK/ERK signaling pathway, which is essential for cell proliferation and survival of tumor cells.[3]

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Encorafenib can exhibit significant therapeutic importance and chemical complexity. Despite its therapeutic potential, Encorafenib was prone to degrade under various stress conditions. The stress degradation facilitates the formation of unwanted products, and these unwanted products may compromise both efficacy and safety. Similarly, the synthesis and formulation process also causes the persistence of process-related or synthetic intermediate impurities in the final product.[4] These impurities may impact the pharmacological activity and can induce undesirable toxicological effects. Thus, a thorough assessment of degradation behavior and impurity profile was essential to develop a robust pharmaceutical product with acceptable safety margins.[5]

In this research, comprehensive chromatographic profiling of Encorafenib impurities and its DPs was undertaken through advanced analytical techniques such as HPLC and LC-MS/MS. The forced degradation studies are systematically performed to evaluate the stability of Encorafenib and to induce the formation of potential DPs. Subsequently, LC-MS/MS analysis provides structural elucidation of unknown impurities and DPs that offer insights into the fragmentation patterns and degradation pathways of Encorafenib. The mass spectral data support the proposed fragmentation mechanisms and allow the structural identification of DPs. Meanwhile, the integration of in silico tools for toxicological risk assessment of the identified impurities predicts their potential genotoxic, mutagenic, or carcinogenic risks.[6]

The literature survey proves the presence of several analytical methods for the estimation of Encorafenib alone or in combination with other antineoplastic agents using various analytical techniques. A UV spectrophotometer method is reported for the quantification of Encorafenib in formulations.[7] Analytical formulation assay methods are reported for the quantification of Encorafenib in combination with other antineoplastic agents such as Cetuximab[8,9] and Binimetinib.[10,11] The bio-analytical LC-MS/MS methods reported for quantification of Encorafenib in combination with binimetinib in biological samples.[12,13] These reported methods are applicable for quantitative analysis of Encorafenib in formulation and biological samples. One study reported the environmental degradation of Encorafenib, and the study primarily focuses on environmental degradation pathways under extensive stress conditions.[14] This identified 17 transformation products. However, the literature lacks a comprehensive analytical investigation that integrates impurity profiling and characterization of DPs of Encorafenib. Therefore, the present study was designed to (a) develop and validate a robust HPLC method for routine impurity profiling of Encorafenib, (b) characterize the structural identity of DPs using advanced LC-MS/MS, (c) propose plausible degradation pathways and mechanisms under different forced degradation conditions, and (d) evaluate the in silico toxicity profiles of the identified impurities to ensure drug safety and regulatory compliance. The chemical structure of Encorafenib, along with the proposed structures of its impurities in this study, is illustrated in Figure 1.

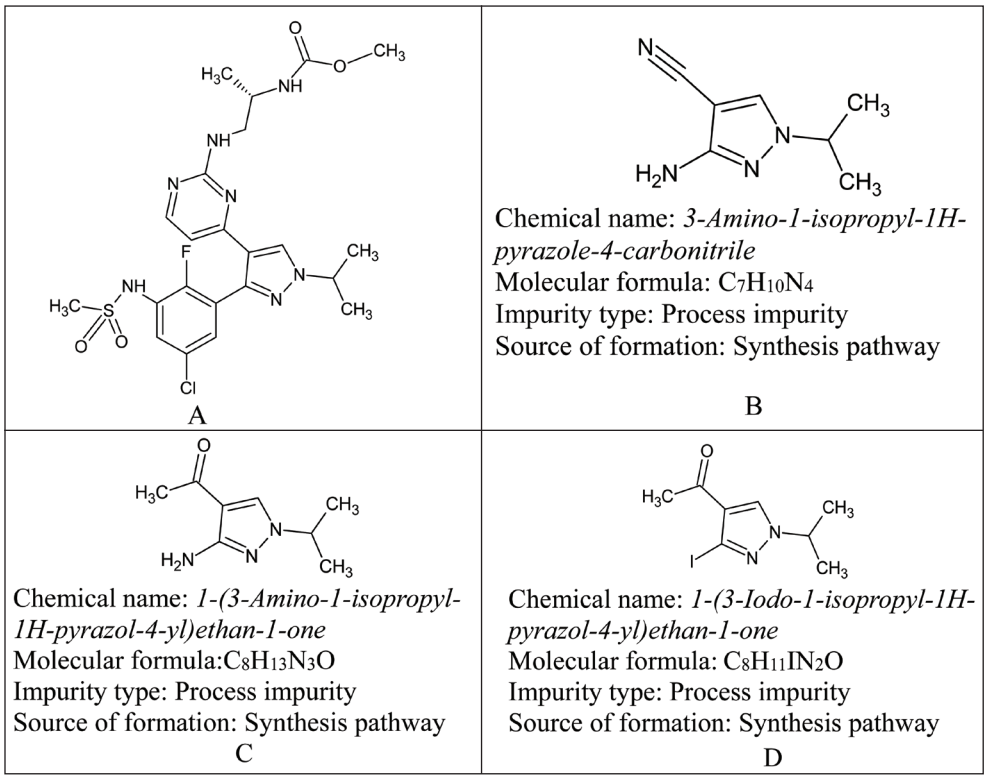

Figure 1. Molecular Structure of Encorafenib (A) and Its Impurities 1 (B), 2 (C), and 3 (D) in the Study.

Materials and Methods

Standards and Reagents

The reference compounds of Encorafenib (purity 98.50%) and its impurities 1, 2, and 3, along with its commercial capsules (Braftovi® – 75 mg), were procured from Speciality Pharma, New Delhi, India. HPLC-grade methanol and water, as well as buffer chemicals along with analytical grade chemicals required for stress degradation study, such as hydrochloric acid (HCl), sodium hydroxide (NaOH), and 30% hydrogen peroxide (H_2O_2), were purchased from Merck Life Science Private Limited.

Apparatus

The HPLC system equipped with UV Detection was utilized for the analysis of Encorafenib and its impurities. An Agilent 1200 series system that includes a quaternary pump, autoinjector, vacuum degasser, and DAD with G1315 C/D and G1365 C/D models was utilized in this study. The Agilent ChemStation software (Agilent Technologies, USA) was utilized to control the instrument and the acquisition and analysis of data.

The HPLC system was coupled to an Xevo G2-XS QToF mass spectrometer (Waters Corporation, Milford, MA, USA). The system was equipped with an electrospray ionization (ESI) source operating in positive ionization mode. Accurate-mass data were acquired in full-scan MS and data-dependent MS/MS modes within a 50–1000 m/z range. The instrument was calibrated using sodium formate, and leucine-enkephalin was employed as a lock-mass reference to ensure mass accuracy within ±5 ppm throughout analysis. Elemental compositions of DPs were proposed based on accurate-mass measurements, isotopic distribution patterns, and high-resolution MS/MS fragmentation data processed through MassLynx software. Typical ESI source conditions, such as capillary voltage, source temperature, desolvation temperature, cone gas flow, and desolvation gas flow, were optimized to obtain maximum ionization efficiency and signal intensity for Encorafenib and its DPs.

Preparation of Solutions

The individual stock solutions of Encorafenib and impurities were prepared by dissolving each reference standard in HPLC-grade methanol to a final concentration of 1000 µg/mL. The solutions were kept in refrigeration at 0°C to maintain their stability during the analysis. Working standard solutions were obtained by dilution of stock solutions using methanol to yield the final concentrations from 25 µg/mL (Level 1) to 150 µg/mL (Level 6) for Encorafenib, and from 0.25 µg/mL (Level 1) to 1.5 µg/mL (Level 6) for impurities. The linearity-level solutions were prepared separately by appropriate dilution from the standard stock solution.

Assay Sample Preparation

The content of five capsules of Braftovi® (each containing 75 mg of Encorafenib) was weighed accurately, and the quantity of powder equal to the average weight of a single capsule was transferred to a 50 mL volumetric flask. 30 mL of methanol was added to this flask, and sonication was conducted for 5 min to dissolve the powder. Then the solution was brought to 50 mL with methanol and was filtered through a membrane filter with a 0.22 µm pore size. This filtrate was again diluted to get a final test solution equivalent to 100 µg/mL of Encorafenib. In order to ensure the method precision, known quantities of pure Encorafenib standard were spiked to individual aliquots of capsule solution. These spiked samples, and the unspiked sample solution, were analyzed in the proposed method.

Forced Degradation

The chemical stability of Encorafenib was assessed, and the possible DPs were detected through forced degradation studies under different stress conditions.[15] These tests assist in establishing the stability-indicating capability of analytical procedures by demonstrating the influence of extreme environments on drug stability. In every stress experiment, 1 mL of a concentrated Encorafenib stock solution (1000 µg/mL) was employed, and this solution was exposed to stress conditions. The strength and volume of stress reagents were selected to achieve controlled degradation (typically 5%–20%), which is recommended for stability-indicating method evaluation according to ICH guidelines. Then, the degraded solutions were neutralized using hydrochloric acid (0.1 M) or sodium hydroxide (0.1 M) solutions wherever necessary, and the final pH of the solutions was adjusted and verified to be within pH 7–7.5 before chromatographic analysis. Subsequently, the solutions were diluted with methanol in 10-mL volumetric flasks to obtain a final concentration of 100 µg/mL before analysis.

The Encorafenib solution was treated separately with 0.1 M HCl solution (1 mL) and 0.1 M NaOH solution (1 mL) for 12 hours at room temperature to induce acid and base stress, respectively. Peroxide stress was performed with 3% (v/v) H_2O_2 (1 mL) at 60°C for 12 hours. These forced degradation conditions were chosen based on commonly reported forced degradation conditions used for stability-indicating method development and recommendations from ICH guidelines. After acid or base stress, the samples were neutralized as described above before dilution and chromatographic analysis. Photolytic stress testing was carried out by exposing the solution to controlled light conditions of approximately

1.2 million lux hours (MLH) of visible light and 200 Wh/m^2 near-UV radiation within the wavelength range of 300–800 nm for 24 h in a photostability chamber. Thermal degradation was evaluated by subjecting the solid drug to dry heat in a hot-air oven maintained at 80°C for 24 h. Neutral degradation was performed with water at 80°C for 30 min.

The percentage recovery of Encorafenib in each stress condition was calculated by comparing the chromatographic peak area of the stressed sample with that of the corresponding unstressed control. The percentage degradation was calculated using the following equation:

$$\% \text{ Degradation} = 100 - \% \text{ Recovery}$$

The calculated degradation values were used to evaluate the relative stability of Encorafenib under different stress conditions.

In Silico Toxicity Studies

The possible toxicity and pharmacokinetic properties of DPs were assessed using in silico prediction tools. The predictions were conducted with the assistance of an internet-based tool, namely ProTox-3.0 (https://tox.charite.de/protox3/) for toxicity assessment and pkCSM (https://biosig.lab.uq.edu.au/pkcsm/) for evaluation of pharmacokinetic properties. These are widely accepted web servers that predict toxicological and pharmacokinetic properties of chemical compounds by analyzing their molecular structures. The prediction model was executed using default parameters, where the SMILES structures of the DPs were provided as input, The SMILES of DPs such as DP 1 Nc2nc(CC(N)c1cc(Cl)cc(N)c1F)ccn2, CC(C)n1cc(c(n1)c2cc(Cl)cc(NS(C)(=O)=O)c2F)c3ccnc(N)n3 for DP 2, CC(C)n1cc(c(n1)c2cc(Cl)cc(N)c2F)c3ccnc(NC[C@H](C)N)n3 for DP 3 and CC(C)n1cc(c(n1)c2cc(Cl)cc(N)c2F)c3ccnc(NC[C@H](C)NC(=O)OC)n3 for DP 4 were generated through ChemSketch 2.0. The results were generated using the platform's machine learning–based classification and regression models.

The ProTox-3.0 provides the LD_{50} value of chemical compounds, which is the quantity of a substance (in milligrams) needed per kilogram of body weight to kill 50% of a test animal group. The LD_{50} value provides the acute toxicity quantitative estimate of DPs. Along with LD_{50}, the software also provides a toxicity class from 1 (most toxic) to 6 (least toxic), which aids in the classification of the possible health hazard due to exposure to the substance. The ADMET (Absorption, Distribution, Metabolism, Excretion, and Toxicity) properties of the identified DPs were predicted using the pkCSM online platform. The pkCSM provides key pharmacokinetic and toxicity parameters such as intestinal absorption, water solubility, blood–brain barrier (BBB) permeability, volume of distribution, cytochrome P450 enzyme interactions, total clearance, and various toxicity endpoints such as hepatotoxicity and AMES mutagenicity. The generated data were analyzed to assess the potential biological behavior and safety profile of the DPs, and the results provide supportive information for risk evaluation.

Results and Discussion

A series of chromatographic parameters was systematically evaluated and optimized to achieve effective and reliable separation of Encorafenib with its known impurities and DPs. Initially, three types of reversed-phase columns, such as Ascentis Express C18, Waters Symmetry RP-C18, and Zorbax Eclipse Plus C18 columns, having dimensions of 250 mm and 150 mm × 4.6 mm with 5 µm particle size. The columns were assessed using equal volume compositions of methanol, acetonitrile, and deionized water to achieve clear and well-resolved peaks for Encorafenib and impurities with minimal tailing. These preliminary experimental trials demonstrate that both columns produce comparable retention behaviors for Encorafenib and impurities. However, the Waters Symmetry C18 column produced superior results in terms of peak shape and symmetry (tailing factor) and signal response (peak area). Hence, the Waters Symmetry C18 column was selected for further method development.

The influence of pH adjustment in the aqueous phase was also explored by adjusting the mobile phase in the initial range of pH values of 3 to 8. The results suggest that the pH value significantly affects the retention time, peak area, and tailing factor of Encorafenib and impurities. Encorafenib and its impurities were identified to be weak basic compounds with functional groups that may be protonated under acidic to neutral pH. The mobile phase composition consisting of methanol and an ammonium acetate buffer was selected to achieve optimal chromatographic performance. The mobile phase pH relative to the pKa of Encorafenib and its impurities will primarily influence their ionization behavior. Hence, ammonium acetate buffer was utilized to maintain a stable pH environment. This study utilized methanol as an organic modifier to improve chromatographic efficiency and peak symmetry. The mobile phase pH of 4.1 was observed to produce improvement in peak shape, reduction in tailing, and enhancement in resolution between the drug and its impurities.

Further, the choice of organic modifiers such as methanol and acetonitrile was evaluated to produce sharper, more symmetrical peaks with very minimal tailing factors. The results prove methanol as an ideal organic component to produce the best resolution of Encorafenib and impurities. An isocratic elution mode was found to be sufficient for the stability-indicating method, even under forced degradation conditions. The method demonstrates a resolution (Rs) greater than two between analytes, which is essential to confirm the purity and stability of the analytes. Based on these findings, the final optimized chromatographic conditions were established as a Waters Symmetry C18

analytical column (250 × 4.6 mm, 5 µm) with an isocratic mobile phase consisting of methanol and 0.1 M ammonium acetate buffer at pH 4.1 (60:40, v/v) at 0.85 mL/min. The detection was carried out at a wavelength of 231 nm, and this wavelength produces maximum absorbance for Encorafenib and impurities. The autosampler temperature of 25°C was proved as ideal to produce consistent sample stability and reproducibility throughout the analytical run. The retention times (t_R) were noticed to be 6.1 min for Encorafenib, 3.6 min for impurity 1, 2.0 min for impurity 2, and 4.9 min for impurity 3 with a total run time of 12 min (Figure 2). The system suitability test result proves the method's reliability. The Encorafenib and impurities eluted within a time frame, and the chromatograms show a high number of theoretical plates that confirm the excellent column efficiency, as well as a good resolution (Rs > 2) between the analytes (Table 1). These results were in compliance with the acceptance criteria and prove that the method was suitable for routine analytical use.

Apart from system suitability, filter compatibility of the method was evaluated using nylon and PVDF membrane filters (0.45 µm). The comparison of chromatographic results observed during the analysis of filtered and centrifuged samples displays no significant difference in peak area or impurity levels (≤2.0%). This indicates that the selected filters were compatible with the analytical method.

Stress Degradation Study

The comprehensive forced degradation of Encorafenib was conducted according to ICH guidelines[15] to determine its stability under various stress conditions. The proposed HPLC method was employed to track the degradation trend of Encorafenib under each condition. In peroxide, thermal, photolytic, and neutral hydrolytic stress conditions, Encorafenib shows only limited degradation of 2.89%–4.73%. In these chromatograms, a slight decrease in the parent peak area of was Encorafenib observed, no distinct DPs peaks were observed. This indicates that the DPs were present above the reporting threshold and hence were not detected in the chromatograms. Therefore, these stress conditions were not subjected to further LC–MS/MS characterization. In contrast, acid and base hydrolysis caused more pronounced degradation, and these conditions result in 8.28% and 16.31% degradation, respectively. Acid stress generates one DP (Figure 3A), whereas base stress includes three DPs (Figure 3B), indicating that Encorafenib was particularly susceptible to hydrolytic degradation under acidic and alkaline conditions. Table 2 displays the stress study results of Encorafenib noticed in this study.

Method Validation

The developed method was primarily intended for the quantification of process-related impurities of Encorafenib, and

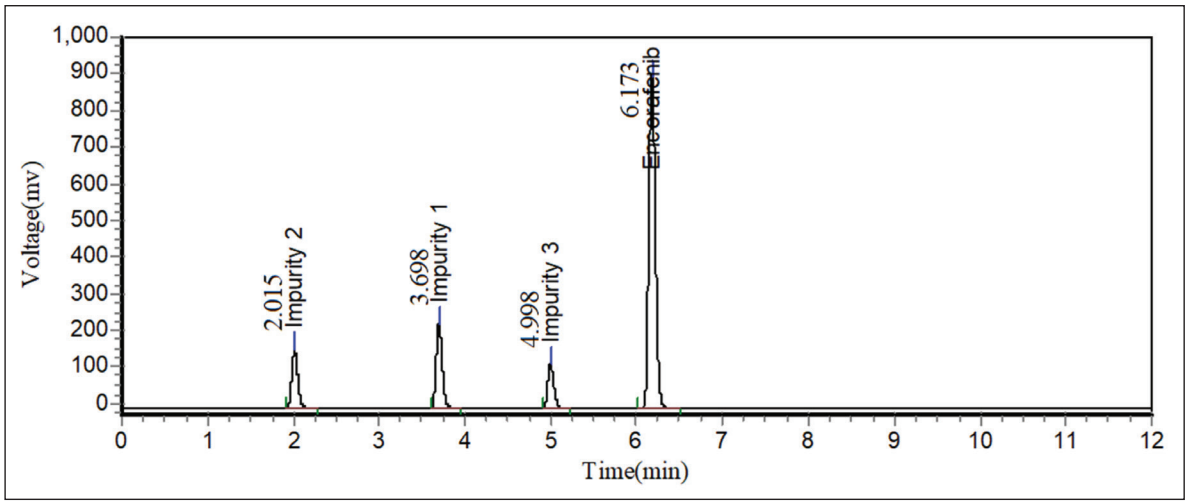

Figure 2. System Suitability Chromatogram of Encorafenib and Impurities in the Optimized Method.

Table 1. System Suitability Results of Encorafenib and Impurities in the Optimized Method.

Parameter	Encorafenib	Impurity 1	Impurity 2	Impurity 3	Acceptance Criteria
t_R in min	6.1	3.6	2.0	4.9	–
Resolution	9.47	14.21	–	11.92	>2
Theoretical plates	13258	7580	5329	9126	>2000
Tailing factor	1.02	0.96	0.98	0.95	<2

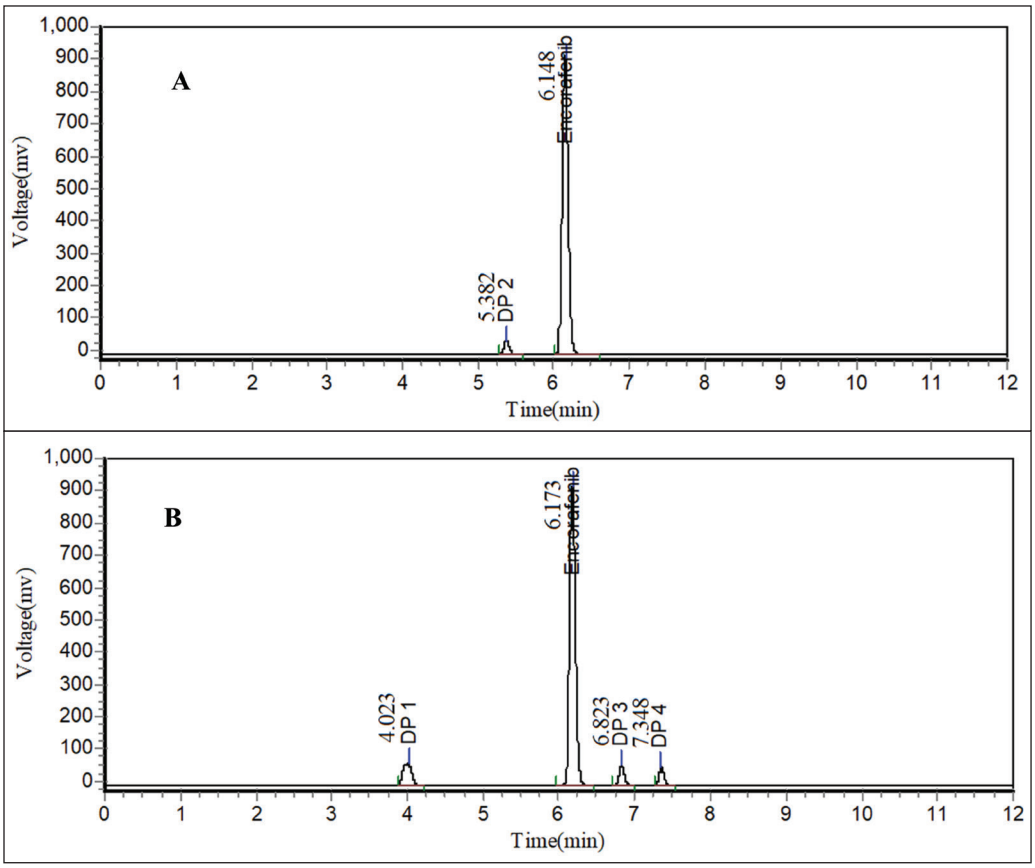

Figure 3. Acid (A) and Base (B) Stress Study Chromatograms of Encorafenib in the Optimized Method.

Table 2. Stress Degradation Study Results of Encorafenib in the Optimized Method *n* = 3.

S No	Condition	Degradation Conditions	% Recovery ± SD	% Degradation ± SD	No. of DPs Formed
1	Acid	0.1 M HCl, RT, 12 hours	91.72 ± 0.352	8.28 ± 0.352	1
2	Base	0.1 M NaOH, RT, 12 hours	83.69 ± 0.095	16.31 ± 0.095	3
3	Peroxide	3% H_2O_2, 60°C for 12 hours	95.87 ± 0.060	4.13 ± 0.060	–
4	Thermal	Dry heat 80°C, 24 hours	97.11 ± 0.230	2.89 ± 0.230	–
5	Photolytic	Visible at 1.2 MLH, UV at 200 Wh/m^2, 24 hours	96.34 ± 0.128	3.66 ± 0.128	–
6	Neutral	H_2O, 80°C, 30 min	95.27 ± 0.179	4.73 ± 0.179	–

therefore, the validation parameters were established according to ICH Q2(R2) validation of analytical procedures requirements for impurity determination.[16] The range of standard and impurity solutions with varying concentrations that exhibit a high correlation linear plot was assessed for linearity. The method shows a strong linear response over the concentration range of 25–150 µg/mL for Encorafenib and 0.25–1.50 µg/mL for impurities. The Coefficient of determination were exceptionally high, with corresponding r^2 values of more than the acceptable value of 0.999, clearly indicating that the method exhibits excellent linearity.

The LOD and LOQ level of impurities in the proposed method was evaluated to prove the sensitivity of the developed method for the determination of process-related impurities. These values were assessed based on signal-to-noise ratio, where a ratio of 3:1 was used for LOD and 10:1 for LOQ. The method can reliably detect and quantify the impurities at low concentrations, suggesting the method's sensitivity. The LOD was noticed to be 0.075 µg/mL, a common LOD of 0.075 µg/mL and LOQ of 0.25 µg/mL were established for the impurity method because the impurities display comparable detector responses under the optimized chromatographic conditions. The validated LOQ of 0.25 µg/mL corresponds to 0.25% relative to the assay concentration (100 µg/mL). This sensitivity was considered appropriate for the intended purpose of impurity profiling, forced degradation studies, and LC-MS/MS characterization of degradation products. The method was not designed as a regulatory release method

for routine impurity control at the ICH Q3B(R2) reporting threshold, and additional optimization would be required if quantification at the 0.10% reporting threshold is required.

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, whereas the same solution was analyzed across three consecutive days to assess the inter-day precision. Ruggedness was analysed same sample by different analyst in three consecutive days. The relative error (Er) and % RSD of the observed results were calculated to assess the method accuracy and precision, respectively. The result shows that the % RSD values of Encorafenib and its impurities were within the acceptable range of below 2%. Additionally, the recovery rates for all tested concentrations were excellent within the acceptable range of 98% and 102%. The % recovery results further prove that the method produces reliable and reproducible results for routine analysis of Encorafenib and its impurities.

The method's robustness was tested by intentionally introducing small variations in key chromatographic parameters, and results were verified to assess the impact of these changes on method performance. In this, the deliberate change in detection wavelength (±2 nm), pH of aqueous phase (±0.2 units), and organic phase composition (±2%) were tested. The results noticed in all changed conditions were noticed to be within acceptable limits (% RSD < 2%). The results suggest that these changes did not significantly impact the results. The Encorafenib and its impurities display consistent peak areas with % RSD values within the acceptable value of less than 2% indicates excellent reproducibility. Additionally, the retention times of the peaks display a very small standard deviation, which proves that the method was reliable and accurate even when minor operational changes.

The interference of common excipients or inactive ingredients in the pharmaceutical formulation with the detection of the active compounds was assessed in a specificity study. The result does not show any interference from the common excipients, indicating that the method was highly specific to the analytes of interest. This was further confirmed with the analysis of the pharmaceutical formulation solution of Encorafenib. The formulation chromatogram shows the presence of Encorafenib peak at its specific retention time, with no additional or overlapping peaks, proving that none of the excipients or inactive ingredients present in the formulation interferes with the analysis. The peak purity analysis using PDA detection confirms that all analyte peaks were spectrally homogeneous with the absence of co-eluting impurities or degradation products (DPs). This indicates that the method was highly selective for the analysis of Encorafenib in complex sample matrixes.

The method reliability was strengthened by performing a standard addition test, and a known quantity of impurities was added to the formulation solution. The observed chromatographic response confirms the successful recovery of the added impurities with no interference from the formulation matrix. This demonstrates that the method was not only accurate but also robust enough for routine quality control analysis. Furthermore, solution stability studies indicate that both standard and sample solutions remain stable for at least 24 hours under laboratory conditions, with no significant change in peak area or retention time (% difference within acceptable limits). The summary of method validation results was tabulated in Table 3.

LC-MS/MS Study of DPs

The HPLC method proposed for the analysis of Encorafenib and its impurities was suitably adapted with the incorporation

Table 3. Method Validation Summary Results Observed for Encorafenib and Its Impurities in the Proposed Method.

Validation Test	Parameter	Results Observed			
		Encorafenib	Impurity 1	Impurity 2	Impurity 3
Linearity	Slope	36987	83207	58453	41332
	Intercept	46852	910.63	1142	163.83
	Coefficient of determination	0.9991	0.9995	0.9994	0.9992
Intra-day precision	% RSD	0.83	0.36	0.31	0.62
Inter-day precision	% RSD	0.55	0.38	0.42	0.67
Ruggedness	% RSD	0.75	0.38	0.51	0.49
50% recovery	% recovery	99.189 ± 0.641	99.981 ± 0.595	99.611 ± 0.708	99.523 ± 0.270
100% recovery	% recovery	101.214 ± 0.563	100.417 ± 0.313	100.212 ± 0.258	99.708 ± 0.364
150% recovery	% recovery	99.549 ± 0.444	101.112 ± 0.923	100.125 ± 0.383	100.043 ± 0.617
Robustness (% change)	+change in MP ratio	0.55	0.08	0.94	0.34
	–change in MP ratio	0.30	0.31	0.57	0.58
	+change in pH of MP	0.89	0.70	0.15	0.55
	–change in pH of MP	0.48	0.66	0.25	0.76
	+change in wavelength	0.79	0.31	0.04	0.51
	–change in wavelength	0.62	0.89	0.25	0.74

of necessary modifications for LC-MS/MS characterization of DPs. Specifically, 0.1 M ammonium acetate (pH 4.1) used in the HPLC method was optimized as 10 mM ammonium acetate (pH 4.1) and mobile phase flow rate was optimized as 0.35 mL/min to achieve better resolution and peak shape for the DPs. The LC-MS/MS proves the formation of four distinct DPs based on their relative retention times (RRTs) of 0.65, 0.88, 1.11, and 1.20. These DPs were designated as DP 1, DP 2, DP 3, and DP 4, respectively, based on retention time. Among these, DP 2 was noticed to be present in the chromatogram of acidic stress, whereas DP 1, DP 3, and DP 4 were formed in basic stress conditions.

Characterization of DPs

In basic stress conditions, Encorafenib underwent significant structural transformation by cleavage of the sulfonyl-methyl group (–SO_2CH_3) present on aromatic nitrogen (Ar–N) moiety. This cleavage leads to the formation of a major DP and was retained at an RRT of 1.20 (designated as DP 4). The mass spectrum of DP 4 reveals a molecular ion peak $[M+H]^+$ at m/z 462.1823 due to mass loss of approximately 77.9773 Da from Encorafenib, which has an $[M+H]^+$ at m/z 540.1596. This mass difference supports des-sulfonylation with net removal of the sulfonyl-methyl functionality from Encorafenib. Mechanistically, it was formed by base-catalyzed nucleophilic attack at the sulfonyl sulfur, and this attack leads to the cleavage of the S–N bond and subsequent elimination of the –SO_2CH_3 moiety. The fragment ion analysis further confirms the proposed structure of DP 4. The key fragment ions were noticed at m/z 406.1319, 386.1425, 361.1214, and others demonstrate stepwise fragmentation of the molecular backbone with retention of the nitrogen-rich heterocyclic core of the parent drug. The presence of ions such as m/z 344.0954 and 318.0795 indicates the stability of electron-deficient aromatic and heterocyclic systems within the degraded molecule. This fragmentation behavior further supports the retention of the core pharmacophore after sulfonyl group removal. The calculated molecular formula was $C_{21}H_{25}ClFN_7O_2$, and the experimental mass data were noticed to align well with the theoretical value, with a minimal mass error of –0.433 ppm. This high mass accuracy strongly validates the identity of DP 4 as the des-sulfonylated analog of Encorafenib. Its fragmentation mechanism was presented in Figure 4, its mass spectrum is given in S1, and MS/MS data were tabulated in Table S1.

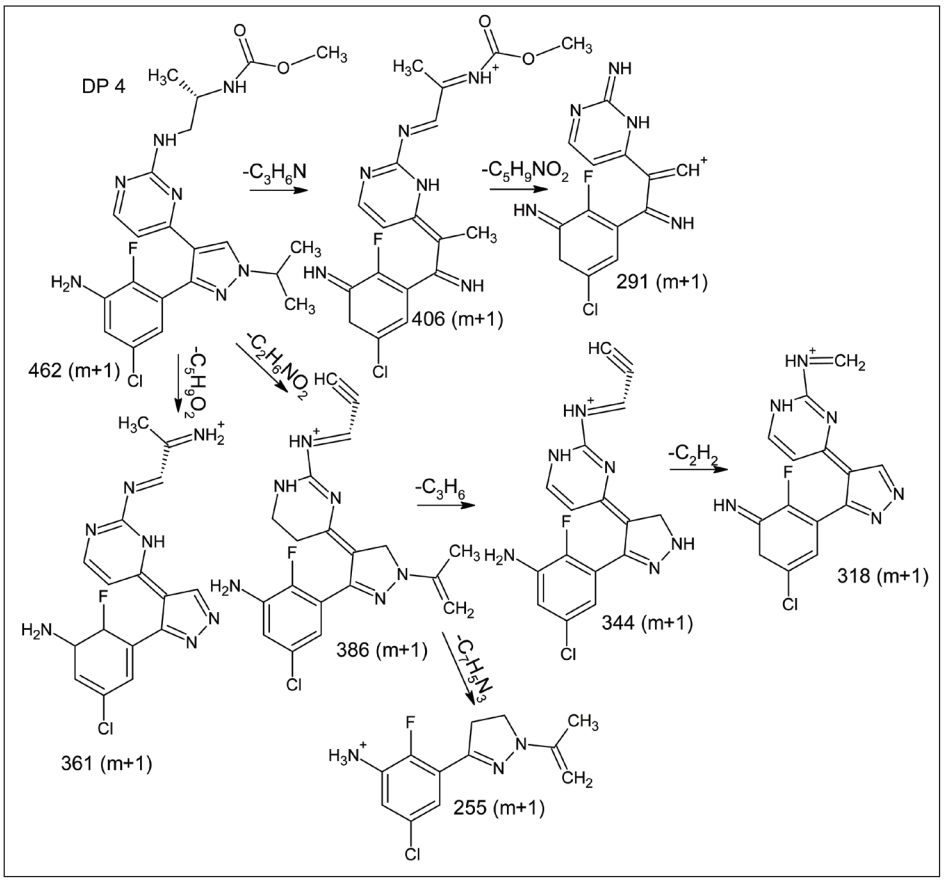

Figure 4. Fragmentation Mechanism Proposed Based on MS/MS Data of DP 4.

The DP 4, which was previously identified as the des-sulfonylated analog of Encorafenib, further undergoes an additional degradation step that involves the cleavage of the amide or carbamate-like moiety – specifically, the N–C bond within the R–NH–$COOCH_3$ side chain to form DP 3. This DP 3 is formed by the nucleophilic attack by hydroxide ions, which leads to cleavage of the carbon–nitrogen bond in the carbamate structure, and the side chain breaks into R–NH_2 (a primary amine group) and $COOCH_3$. This degradation leads to a clear mass difference of 58.0058 Da between DP 4 (m/z 462.1823) and the new DP (m/z 404.1765). The loss of 58.0058 Da supports the proposed mechanism of carbamate cleavage. The new product retains the core pharmacophore of DP 4 but lacks the carbamate side chain and yields a simpler structure with a terminal primary amine (R–NH_2). This cleavage results in the formation of a structurally smaller molecule with high polarity due to the newly formed free amine, and this transformation is in alignment with the identification of a low RRT of 1.11 compared to DP 4 (RRT 1.20). The mass spectrum of DP 3 shows a molecular ion at m/z 404.1765 that corresponds to the molecular formula $C_{19}H_{23}ClFN_7$, with a minimal mass error of 0.247 ppm, indicating high confidence in the accuracy of this assignment. Additional fragment ions (m/z 372.1267, 344.0955, 308.1203, etc.) further confirm the retention of the core heterocyclic framework and provide evidence for stepwise loss of peripheral groups, but the main aromatic and nitrogen-containing scaffold remains intact. Its fragmentation mechanism was presented in Figure 5, its mass spectrum is given in S2, and MS/MS data were tabulated in Table S1.

The prolonged basic hydrolysis can induce the conversion of DP 3 to DP 1. The R–NH bond in R–NH–CH_2–$CH(CH_3)$–NH_2 moiety of DP 3 was cleaved and yielded a free primary amine (R–NH_2) due to the attack of hydroxide ion on the adjacent carbon or nitrogen centers. Additionally, the N-methyl substituted pyrazole ring, which is part of the heterocyclic system in Encorafenib's core structure, was also susceptible to ring-opening or cleavage reactions in prolonged basic environments. The N-methyl pyrazole ring was broken down in DP 1. The transformation from DP 3 (m/z 404.1765) to DP 1 (m/z 282.0919) was due to net mass loss of 122.0846 Da, indicating the elimination of both the ethylamine side chain and N-methyl pyrazole fragment. The resultant structure was noticed to be significantly smaller, more polar in nature, and hence a reduction in retention time (RRT 0.65) was noticed. Its mass spectrum shows the presence of fragmentions at m/z 239.0626, 215.0625, and 200.0511, indicating sequential loss of nitrogenous units and degradation of heterocyclic structures. The low molecular weight fragments noticed at m/z 169.0091, 138.0595, and 98.0531 prove the further fragmentation into stable, halogenated aromatic cores. Based on correlation, the DP 1 was confirmed as 4-[2-amino-2-(3-amino-5-chloro-2-fluorophenyl)ethyl]pyrimidin-2-amine with a molecular mass of 281 g/mol. Its fragmentation mechanism was presented in Figure 6, its mass spectrum is given in S3, and MS/MS data were tabulated in Table S1.

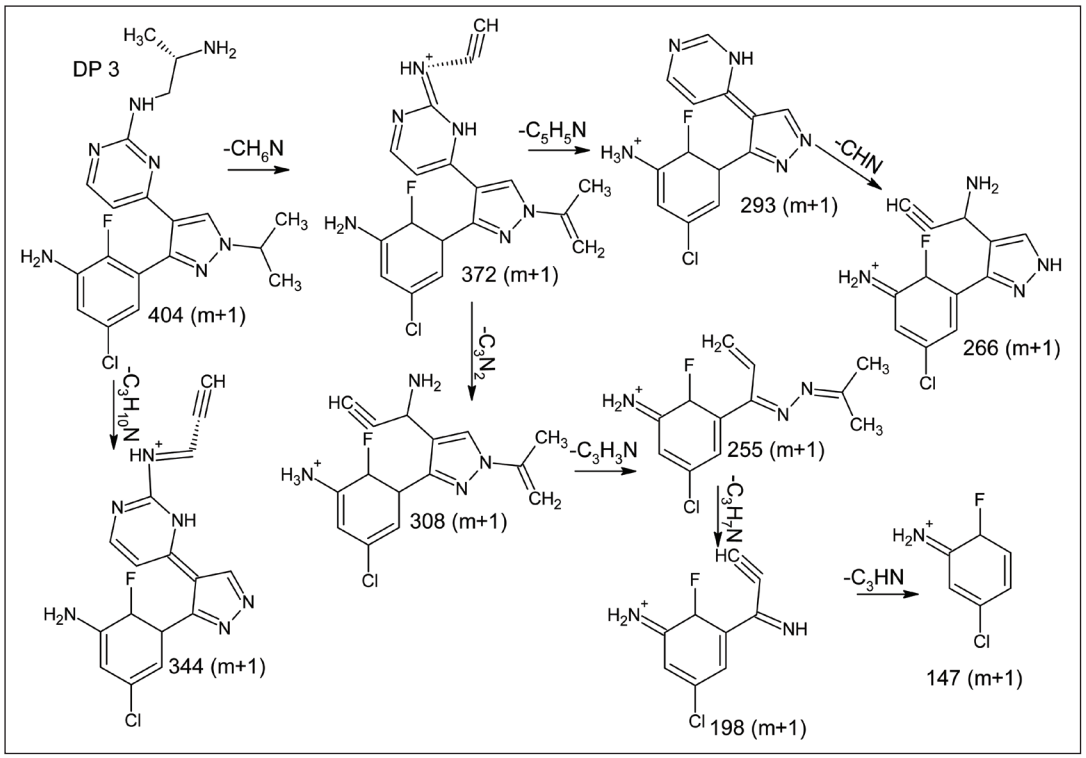

Figure 5. Fragmentation Mechanism Proposed Based on MS/MS Data of DP 3.

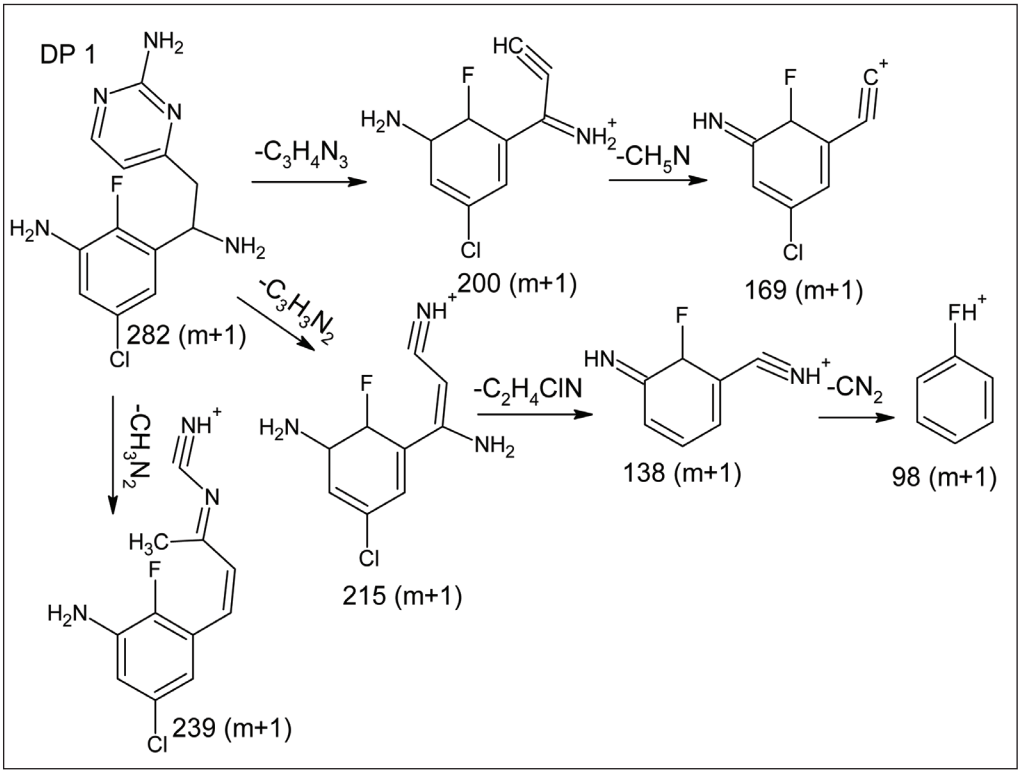

Figure 6. Fragmentation Mechanism Proposed Based on MS/MS Data of DP 1.

DP 2 of Encorafenib was specifically observed under acidic stress conditions and was identified at an RRT of 0.88 in the acid stress chromatogram. It was formed by acid-catalyzed cleavage of the N–C bond in the carbamate side chain attached to the nitrogen linked to the pyridine ring. Protonation of the carbonyl oxygen enhances the electrophilicity of the carbonyl carbon, facilitating nucleophilic attack and subsequent bond scission. Mass spectrometric analysis reveals that DP 2 has a protonated molecular ion $[M+H]^+$ at m/z 425.0965, whereas the parent Encorafenib has an $[M+H]^+$ at m/z 540.1596, indicating a mass loss of 115.0631 Da. This mass reduction corresponds to the loss of the entire carbamate side chain, with retention of the Encorafenib core scaffold, suggesting that the side chain was hydrolyzed due to acidic stress. The decrease in RRT to 0.88 for this DP relative to the parent compound suggests an increase in polarity due to the loss of the nonpolar carbamate chain.

The MS/MS fragmentation data of DP 2 further support the proposed acid-induced cleavage mechanism and structural assignment. The protonated molecular ion of DP 2 was noticed at m/z 425.0965 $[M+H]^+$, with a calculated mass of 425.0963 and a mass error of –0.470 ppm. This molecular mass corresponds to the molecular formula of $C_{17}H_{18}ClFN_6O_2S$. This confirms the structure of DP 2 as the des-carbamate analog of Encorafenib and was formed by the acid-catalyzed removal of the carbamate side chain. The major fragment ion noticed at m/z 384.0577 ($C_{14}H_{13}ClFN_6O_2S$) indicates a neutral loss of 41.0388 Da. This was likely due to the small alkyl fragment, such as the propyl moiety, which further supports the side chain simplification. The appearance of a prominent fragment at m/z 332.0949 ($C_{15}H_{13}ClFN_6$) signifies the loss of sulfone (SO_2) and oxygen functionalities, indicating cleavage around the sulfonamide group. The lowest fragment at m/z 163.0851 ($C_7H_8N_5$) reflects the breakdown of the central heterocyclic ring system. Based on correlation, the DP 2 was confirmed as a des-carbamate analog of Encorafenib with a molecular mass of 424 g/mol. Its fragmentation mechanism was presented in Figure 7. Its mass spectrum is given in S4, and MS/MS data were tabulated in Table S1.

In Silico Assessment of DPs

The in silico toxicity assessment was conducted to assess and compare the toxicological profiles of DPs formed under acid and base stress studies of Encorafenib. The heatmap (Figure S11) representation of the results observed during the toxicity assessment enables the clear visualization of toxicity differences among the four DPs (DP 1 to DP 4). Among them, DP 1 emerges as the most acutely toxic, with the lowest LD_{50} value of 155 mg/kg, and exhibits activity in neurotoxicity, respiratory toxicity, ecotoxicity, clinical toxicity, and BBB penetration. DP 2, with a higher LD_{50} of 1000 mg/kg, shows moderate toxicity and was active in respiratory toxicity, immunotoxicity, carcinogenicity, clinical toxicity, nutritional toxicity, and BBB disruption. DP 3, having a

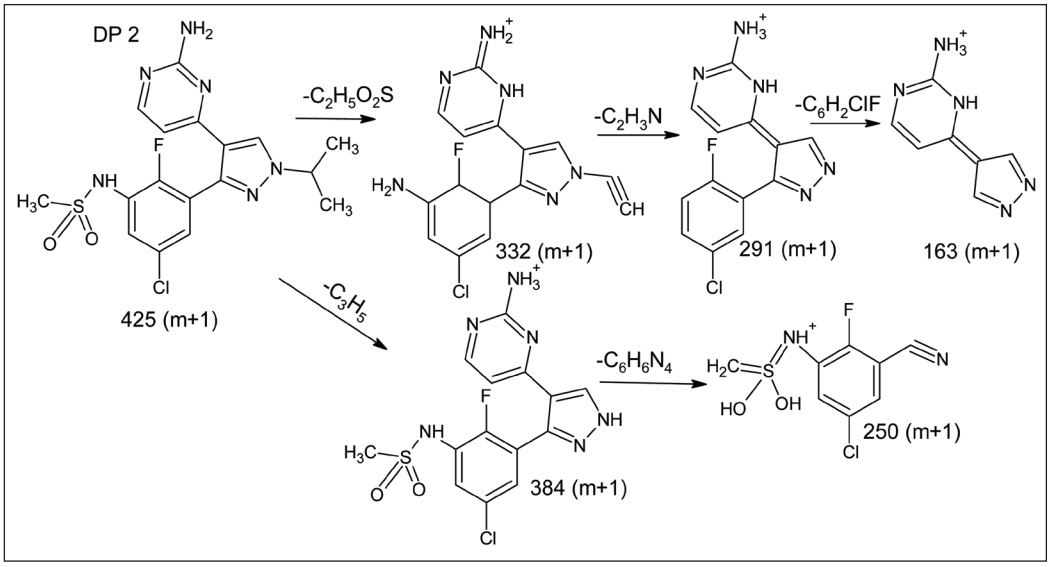

Figure 7. Fragmentation Mechanism Proposed Based on MS/MS Data of DP 2.

similar LD_{50} of 1300 mg/kg, presents a comparable profile to DP 2. In contrast, DP 4, also with an LD_{50} of 1300 mg/kg, demonstrates the widest range of active toxic effects. This study result suggests that DP 1 is the most acutely toxic, and DP 4 represents the greatest overall toxicological risk due to its broad spectrum of toxicities.

The pharmacokinetic and toxicity parameters of identified DPs were assessed through the pkCSM platform, and results were tabulated in Table S2. The results show favorable absorption characteristics, with high intestinal absorption values ranging from 75.97% to 86.75%, despite moderate water solubility. All DPs were identified as P-glycoprotein substrates, suggesting possible efflux-related transport, while limited BBB and CNS permeability indicate low central nervous system exposure. Metabolism studies display minimal interaction with CYP2D6, whereas selective involvement of CYP3A4 and CYP2C9 enzymes was observed for certain DPs. The compounds exhibit moderate clearance values and were not substrates of renal OCT2 transporters. Toxicity predictions reveal that all DPs were non-mutagenic (AMES negative) and non-cardiotoxic (no hERG I inhibition), although DP3 displays hERG II inhibition. All compounds were predicted to be hepatotoxic, with moderate tolerated dose values. Overall, the results suggest that the DPs demonstrated favorable predicted pharmacokinetic properties; however, several toxicity alerts were identified, including predicted hepatotoxicity for all DPs, hERG II inhibition for DP3, and broader toxicity concerns for DP4. These findings are based on computational predictions and may involve uncertainty associated with model assumptions and the applicability domain of the prediction tools. Therefore, the predicted toxicological profiles should be interpreted cautiously and require further experimental validation through in-vitro or in- vivo studies to confirm the actual toxicological behavior of the DPs.

Conclusion

The present study aimed to develop a robust, sensitive, and stability-indicating HPLC method for the simultaneous quantification of Encorafenib and its known impurities. This study also involves the characterization and toxicological profiling of stress DPs. The systematic optimization of chromatographic parameters proves that the Waters Symmetry C18 column with an isocratic mobile phase (methanol: 0.1 M ammonium acetate buffer, pH 4.1) at 0.85 mL/min was suitable for the effective resolution of Encorafenib and its impurities. The method demonstrates excellent linearity ($r^2 > 0.999$), high sensitivity (LOD: 0.075 µg/mL), and precision (% RSD < 2%). The forced degradation studies reveal that Encorafenib was particularly vulnerable under basic and acidic conditions. The influence of these stress conditions leads to the formation of four distinct DPs. Subsequent LC-MS/MS analysis helps the structural elucidation of these DPs. DP 4 was identified as the des-sulfonylated analog of Encorafenib (m/z 462.1823), DP 3 as its carbamate-cleaved derivative (m/z 404.1765), and DP 1 as a further breakdown of the ethylamine side chain and the N-methyl pyrazole ring (m/z 282.0919). The DP 2 (m/z 425.0965) was the only DP observed in acidic conditions and was formed by the loss of the carbamate moiety attached to the pyridine ring of Encorafenib. MS/MS fragmentation data confirms the structures of all DPs with minimal mass error, and provides insight into the degradation pathways. Furthermore, in silico toxicity prediction reveals that all four DPs exhibit different toxicological concerns. DP 1 was observed to be most acutely toxic (LD_{50} = 155 mg/kg), while DP 4 displays the broadest spectrum of toxic effects. The pkCSM-based ADMET analysis indicates that DPs demonstrated favorable predicted pharmacokinetic properties; however, several

toxicity alerts were identified, including predicted hepatotoxicity for all DPs, hERG II inhibition for DP3, and broader toxicity concerns for DP4. In conclusion, the study not only delivers a validated HPLC method for routine quality control of Encorafenib and its impurities but also provides critical insight into the stability and toxicological behavior of its DPs.

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Authors' Contribution

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agreed to be accountable for all aspects of the work. All the authors are eligible to be authors as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

Consent to Participate

Not applicable.

Consent for Publication

Not applicable.

Data Availability Statement

All the data is available with the authors and shall be provided upon request.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical Approval

This study does not involve experiments on animals or human subjects.

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Informed Consent

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Use of Artificial Intelligence-assisted Tools

The authors declare that they have not used artificial intelligence (AI) tools for writing and editing of the manuscript, and no images were manipulated using AI.

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