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Establishing and validating an HPLC protocol for pralsetinib impurities analysis, coupled with HPLC-MS/MS identification of stress degradation products

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Abstract: This study introduces a novel analytical method for the assessment of pralsetinib impurities and degradation products (DPs), addressing critical gaps in existing methodologies. This research aims to develop a robust HPLC method for impurity analysis, characterize degradation products using LC-MS, and evaluate the environmental impact of the method. The study began by optimizing HPLC conditions with various columns and buffers, ultimately achieving successful separation using an XBridge® RP-C18 column with ethanol as solvent A and 50 mM formic acid at pH 2.9. This setup provided excellent peak resolution and symmetry, essential for reliable stability studies. The developed HPLC method was then adapted for HPLC-MS/MS, enhancing sensitivity and detection efficiency of DPs. Stress degradation studies of pralsetinib under different conditions (acidic, basic, oxidative, thermal, and photolytic) revealed significant degradation under acidic (29.3 %) and basic (21.5 %) conditions, with several DPs identified. Oxidative stress resulted in 19.8 % degradation, while thermal and photolytic conditions caused minimal degradation. HPLC-MS/MS analysis identified structures of five degradation products, providing detailed insights into pralsetinib's stability and degradation pathways. Method validation followed ICH guidelines Q2(R1), confirming method's specificity, selectivity, sensitivity, linearity, accuracy, precision, and robustness. The method exhibited strong linearity with a coefficient of determination (r^2) greater than 0.999 for pralsetinib and its impurities. This method advances impurity detection and DPs characterization, ensuring the quality and safety of pralsetinib. Additionally, method's environmental impact was assessed, aligning with sustainable analytical practices. These findings provide essential data on pralsetinib's stability, guiding storage conditions and ensuring its efficacy and safety in pharmaceutical applications.

Key words: pralsetinib, pharmaceutical impurities, characterization, degradation products, method validation

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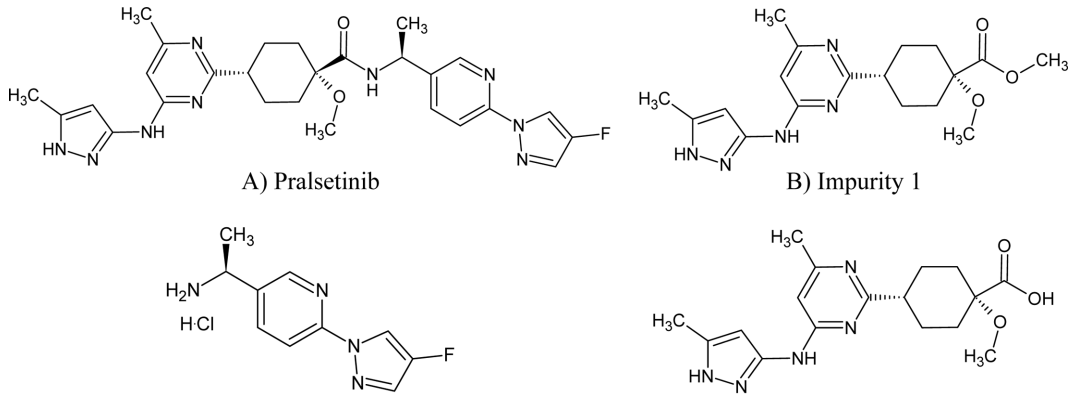

Fig. 1. Structure of pralsetinib and its impurities selected in this study.

1. Introduction

Pralsetinib (*Fig.* 1(A)) is an oral kinase inhibitor specifically targeting rearranged during transfection (RET) gene alterations.[1] It was approved for the treatment of various cancers driven by RET mutations or fusions, such as non-small cell lung cancer and medullary thyroid cancer.[2] Pralsetinib works by inhibiting the RET receptor tyrosine kinase, a protein involved in cell signalling pathways that promote cell proliferation and survival. Mutations or fusions in the RET gene lead to abnormal RET signalling, which can drive cancer development.[3] By selectively targeting and inhibiting RET kinase activity, pralsetinib blocks these signalling pathways, thereby reducing tumor growth and proliferation. Common side effects of pralsetinib include fatigue, constipation, hypertension, musculoskeletal pain and diarrhea. Serious adverse events may include interstitial lung disease/pneumonitis, hepatotoxicity, hypertension, and hemorrhagic events.[4]

Pharmaceutical impurities, including stress degradation impurities, are unwanted chemicals that can arise during the manufacturing, storage, or handling of pharmaceutical products. These impurities can originate from various sources, such as raw materials, synthesis processes, or degradation of the active pharmaceutical ingredient (API) under stress conditions like heat, light, pH changes, or oxidation.[5,6] Stress degradation impurities are particularly concerning as they can form when the API is exposed to extreme conditions during stability testing, mimicking potential real-world scenarios.[7] Identifying, quantifying, and controlling these impurities are crucial to ensure the safety, efficacy, and quality of pharmaceutical products, complying with regulatory standards set by authorities such as the FDA and EMA.[8,9]

A comprehensive literature review indicates that several analytical methods have been established for quantifying pralsetinib in various sample matrixes in single and combinations with other drugs. One HPLC method has been documented for measuring sorafenib in capsule formulations.[10] Additionally, an HPLC-MS/MS method reported to evaluate pralsetinib in conjunction with brigatinib, lorlatinib, and selpercatinib in plasma samples.[11] There is also LC-MS based bioanalytical method available for quantifying pralsetinib in combination with selpercatinib in plasma and tissue homogenates.[12] Furthermore, a UPLC-MS/MS method has been designed to analyze pralsetinib in cerebrospinal fluid and plasma samples.[13] However, no methods have been reported for quantifying both impurities and degradation products of pralsetinib. Therefore, this study aims to fill this gap by developing a green HPLC method for impurity analysis, characterizing degradation products using HPLC-MS/MS, and evaluating the environmental impact of the method. Specifically, impurity 1, 2 and 3 of pralsetinib were selected for analysis due to their availability and

relevance to the research objectives.

2. Experimental

2.1. Chemicals and reagents

A V Pharma, based in New Delhi, India, generously supplied a sample of pralsetinib with purity greater than 98.5% and 100 mg formulation capsules of pralsetinib along with its impurity 1, 2 and 3. For this study, analytical reagent-grade chemicals including hydrogen peroxide, sodium hydroxide, formic acid, hydrochloric acid, and HPLC-grade ethanol were obtained from Fisher Scientific in Mumbai. Milli-Q water, produced using a Millipore water purification system from the USA, was utilized for preparing sample solutions and mobile phases.

2.2. Standard solution preparation

The carefully measured 10 mg of pralsetinib API along with impurities was added to a 10 mL flask separately. In this flask, 5 mL of pure methanol was used as diluent and then thoroughly dissolved them by placing it in sonicator. If there were any particles that did not dissolve were removed by filtering solution through 0.2 μ filter. The final solution volume was adjusted using same diluent to achieve solutions of pralsetinib and impurities each with 1000 μg/mL concentration.

2.3. Sample solution preparation

The pharmaceutical product branded as Gavreto® containing 100 mg of pralsetinib, was utilized to prepare test solution for analysis. In preparation process, sample equivalent to 10 mg of pralsetinib was placed in 10 mL flask which was partially filled with a diluent. The sample was then sonicated to ensure that the active ingredient was completely dissolved. Any excipients that did not dissolve were removed by filtration. The solution was then adjusted to reach a concentration of 1000 μg/mL of pralsetinib and this was further diluted to reach a concentration corresponding to a 100 % precision level. The solution was immediately analyzed after preparation.

2.4. Instruments

For this study, a 2695 series HPLC system (Agilent, USA) with degasser, quaternary gradient pump, UV detector, autosampler, and column compartment was used for experimental analyses. Empower 3 software facilitated data acquisition and processing. To identify degradation products, a 1290 series HPLC-MS/MS system (Agilent, USA) with a quadrupole-time of flight (Q-TOF) mass detector (Agilent 6545 series) and positive mode Electron Spray Ionization (ESI) was employed, with data analyzed using Mass Hunter Workstation software. pH measurements were conducted using a Systronics (India) pH meter. Thermal stability was assessed with a hot air oven (Oswald, India), and photostability studies were carried out in a Newtronic Life Care Sciences photostability chamber, which ensured optimal temperature and humidity control.

2.5. Analytical conditions

The optimal separation of pralsetinib and its impurities, along with DPs, was accomplished using an XBridge® RP-C18 column (250 mm × 4.6 mm, 5 μm) paired with a mobile phase comprising ethanol as solvent A, and 50 mM formic acid at pH 2.9 as solvent B with gradient program of time/% solvent B: 0/20, 4/50, 8/70, 10/80, and 14/20, with a flow rate of 0.7 mL/min, an injection volume of 5 μL, and detection at 254 nm. These optimized chromatographic conditions resulted in enhanced separation, efficiency, and resolution of pralsetinib and its impurities.

For mass spectrometry, specific instrumental parameters were utilized, including operating source conditions, ionization settings, and collision processes. The fragmentation energy was set at 220 V, with a capillary temperature of 340 °C, and a skimmer voltage of 50 V. Nitrogen gas was used for desolvation and nebulization at 45 psi and 360 °C, with a flow rate of 15 L/min. Additionally, ultrahigh-purity nitrogen was used as collision gas, ensuring optimal performance and precision during analytical measurements. These parameters were crucial for achieving accurate and reproducible results in the mass spectrometric analysis performed in this study.

2.6. Forced degradation studies

These studies were performed following the procedures recommended by ICH.[14] These guidelines provide a framework for evaluating the stability and degradation of pharmaceutical substances under accelerated conditions.

2.6.1. Acid and base degradation

To initiate the acid and base degradation study of pralsetinib, 100 mg of the drug was partitioned into two distinct 100 mL volumetric flasks. Into each flask, 50 mL of diluent was added. Following this, 5 mL of 2 N hydrochloric acid was introduced into one flask, while 3 N sodium hydroxide solution was added into the other. The mixtures were then subjected to reflux at 80 °C for duration of 6 hours. Upon completion of the reflux period, either 2 N sodium hydroxide or 3 N hydrochloric acid solution was utilized to neutralize the reaction mixtures. Subsequently, it was diluted to concentration of 1000 µg/mL and for further analysis, 5 mL aliquots of each prepared stock solution were diluted to 20 mL with diluent and thoroughly homogenized. The resultant solutions, 100 % concentration of drug under acid or base stress conditions, were then separately filtered through a 0.22 µm filter prior to analysis.

2.6.2. Oxidative degradation

Sample was prepared by carefully weighed 100 mg of the drug and was combined with 50 mL of a diluent. Next, 5 mL of a 30 % hydrogen peroxide solution was added to initiate the degradation process. The mixture was then left in a flask at room temperature for a period of 3 days, allowing the degradation to progress. Upon completion of the reaction, the volume of the solution was adjusted to 100 mL by adding more diluent resulting 1000 µg/mL stock solution. Before injection into the analysis system, the degraded samples were diluted to achieve a concentration of 100 %, and then filtered through a 0.22 µm filter to eliminate any particulate matter. The filtered samples were then ready for analysis

2.6.3. Thermal and Photo degradation

Pralsetinib, a pharmaceutical compound, was subjected to rigorous testing to assess its stability under different stress conditions. For the thermal stress test, a thin layer of pralsetinib was spread onto a petri dish and then exposed to a constant temperature of 80 °C for a duration of 48 hours within an air oven. Conversely, for the photo stress test, the drug was subjected to UV–Visible light at an intensity of 200-Watt hours/m^2 and 1.2 M Lux hours over a period of 72 hours. Following the stress exposure, 100 mg of the stressed pralsetinib was carefully measured and subsequently diluted to achieve a concentration of 100 % for both the thermal and photo degradation assessments. The resulting degraded samples were then filtered to remove any impurities and prepared for further analysis.

2.7. Method validation

The analytical method underwent validation in accordance with the guidelines delineated in ICH Q2 (R1) to ascertain its dependability and appropriateness for quantifying impurities of pralsetinib.[15-21] The validation encompassed various parameters to ensure the method's efficacy. Firstly, specificity and selectivity were evaluated to discern pralsetinib impurities from potential interferences, thereby confirming the method's capability to differentiate between them. Linearity studies were conducted to establish the correlation between analyte concentration and detector response across a predefined range. Precision and intermediate precision analyses were undertaken to gauge the method's repeatability and reproducibility, respectively, ensuring consistent results over multiple tests. Accuracy studies were conducted to determine the proximity of measured values to the true values, thus validating the precision of the method. Robustness testing was performed to evaluate the method's performance under diverse experimental conditions, ensuring its reliability in varying scenarios. Furthermore, the method's sensitivity was assessed through the determination of LOD and LOQ. These comprehensive evaluations collectively attest to the robustness and reliability of the method for precise and accurate analysis in pharmaceutical research and quality control applications.

3. Results and Discussion

The goal of this study was to propose a chromatographic method suitable for stability studies, specifically designed to achieve optimal resolution of pralsetinib impurities and DPs. The process began with optimizing the HPLC conditions employing Zodiac C18 (100 mm and 250 mm) column paired with various HPLC-MS/MS compatible buffers. Initial experiments focused on enhancing peak shape and resolution by using buffers with formic acid, acetic acid, and ammonium acetate, along with organic modifiers. Unfortunately, these combinations resulted in asymmetric peak shapes, tailing, and poor peak responses for sorafenib and its impurities.

Subsequent efforts involved switching to Waters Spherisorb ODS2 C18 (150 and 250 mm) column and various buffers in different pH range studied. Despite these adjustments, the chromatographic results indicated that none of the tested conditions provided satisfactory separation or peak shapes.

To overcome these challenges, study transitioned to using an XBridge® RP-C18 column (250 mm × 4.6 mm, 5 µm), which significantly improved the symmetry of the drug peak shapes. Successful separation was finally achieved with ethanol as solvent A and 50 mM formic acid at pH 2.9 with gradient program of time/% solvent B: 0/20, 4/50, 8/70, 10/80, and 14/20, with 0.7 mL/min flow, an injection volume of 5 µL and detection at 254 nm. This method provided the desired resolution and peak shapes, meeting the criteria for an effective stability study. This method greatly enhanced chromatographic performance, ensuring clear resolution and well-defined peak symmetry, which are essential for reliable stability studies. The resulting chromatogram, obtained under these optimized conditions, is displayed in *Fig*. 2.

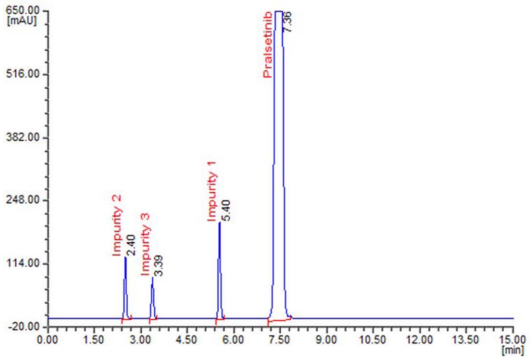

Fig. 2. System suitability chromatogram identified in the method proposed for analysing impurities of pralsetinib..

In this study, the HPLC method was successfully adapted for use with HPLC-MS/MS. Additionally, a thorough optimization of mass spectrometry conditions was carried out to improve sensitivity and ensure effective detection of all DPs. This process included meticulously adjusting various parameters to enhance signal intensity and resolution, allowing for precise identification and quantification of the targeted compounds.

3.1. Degradation study of pralsetinib

The stress degradation study of pralsetinib under various conditions provides insightful details about its stability and the specific degradation products formed (*Table* 1). When subjected to acidic stress, pralsetinib exhibited a significant degradation of 29.3 %. This condition resulted in the formation of three

Table 1. Results noticed while performing stress degradation studies on pralsetinib standard drug

Stress condition	Stressor utilized	Temperature	Time	% degradation	DPs identified
Acid	2 N HCl	80 °C	6 h	29.3	DP 1, 2 and 4
Base	3 N NaOH	80 °C	6 h	21.5	DP 1, 2 and 5
Oxidative	30 % H_2O_2	Room temperature	3 days	19.8	DP 1, 2 and 3
Thermal	Air oven	80 °C	2 days	1.85	No
Photolysis					
Ultraviolet	200 W h/m2	Room temperature	3 days	3.39	No
Florescent	1.2×10^6 Lux. h	Room temperature	3 days	2.78	No

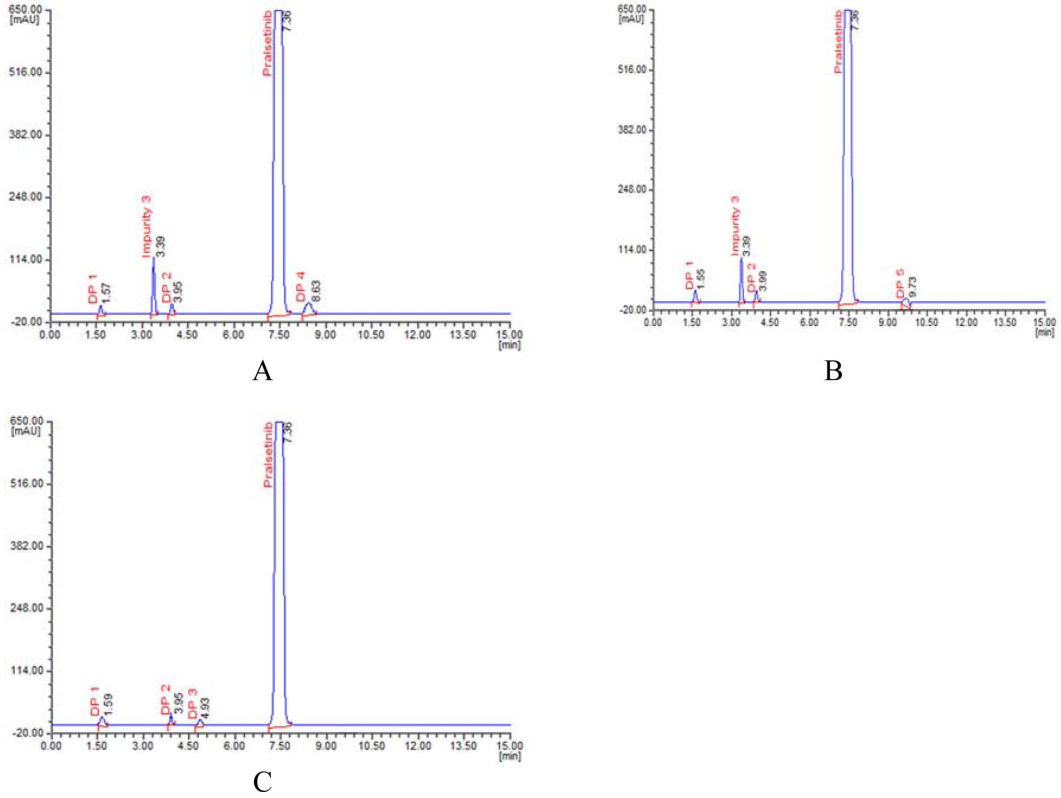

Fig. 3. The stress degradation chromatograms for pralsetinib, generated under different stress conditions, are presented. In chromatogram A, which illustrates the effects of acidic degradation, distinct peaks are observed for pralsetinib, impurity 3 along with DP 1, 2 and 4. The chromatogram B depicts the results of basic degradation, showing clear peaks for pralsetinib, impurity 3 along with DP 1, 2 and 5. Whereas chromatogram C depicts the results of peroxide degradation, showing clear peaks for pralsetinib, along with DP 1, 2 and 3.

distinct DPs which were well resolved and identified in chromatogram (*Fig.* 3A) at retention time of 1.57 min (DP 1), 3.95 min (DP 2) and 8.63 min (DP 4). Similarly, under basic conditions, pralsetinib showed a degradation percentage of 21.5 %, producing degradation products which were retained in the chromatogram (*Fig.* 3B) at 1.55 min (DP 1), 3.99 min (DP 2), and 9.73 min (DP 5). The impurity 3 in the study was also identified in the chromatogram of acid and base stress study. Oxidative stress leading to 19.8 % degradation of pralsetinib with the identification of three DPs (*Fig.* 3C) at 1.59 min (DP 1), 3.95 min (DP 2) and 4.93 min (DP 3). Thermal degradation caused minimal degradation, with only 1.85 % degradation observed, and no degradation products were identified. The photolytic degradation in UV light resulted in a 3.39 % degradation whereas photolytic degradation in fluorescent light showed a 2.78 % degradation. Both UV and fluorescent degradation conditions produces no DPs. These comprehensive results illustrate stability profile of pralsetinib under various stress conditions, highlighting extent of degradation and specific DPs formed under each condition. This result facilitates crucial information for understanding the stability and shelf-life of pralsetinib, guiding appropriate storage conditions, and ensuring its efficacy and safety in pharmaceutical applications.

3.2. HPLC-MS/MS studies of DPs

HPLC-ESI-MS/MS experiments were conducted to identify structures of all the DP (1 to 5) that formed

Table 2. Elemental composition results observed for pralsetinib and its impurities along with DPs formed in the study

Analyte	Retention time	Molecular formula $[M+H]^+$	*m/z* Calculated	*m/z* Observed	Error (Δ ppm)
Pralsetinib	7.36	$C_{27}H_{32}FN_9O_2$	534.6004	534.6001	-0.561
Impurity 1	5.40	$C_{18}H_{25}N_5O_3$	360.4228	360.4230	0.555
Impurity 2	2.40	$C_{10}H_{11}FN_4$	207.2195	207.2188	-3.378
Impurity 3	3.39	$C_{17}H_{23}N_5O_3$	346.3962	346.3960	-0.577
DP 1	1.57	$C_{10}H_{10}FN_3$	192.2049	192.2050	0.520
DP 2	3.95	$C_{17}H_{24}N_6O_2$	345.4114	345.4113	-0.290
DP 3	4.93	$C_{28}H_{33}FN_8O_3$	549.6118	549.6116	-0.364
DP 4	8.63	$C_{24}H_{30}FN_7O_2$	468.5391	468.5392	0.213
DP 5	9.73	$C_{26}H_{30}FN_9O_4$	552.5727	552.5728	0.181

under conditions of various stress studies. The most plausible structures for all these DPs were suggested based on mass-to-charge ratios of their $[M + H]^+$ ions. Additionally, their elemental composition was analyzed using ring double bond equivalents and precise mass measurements. This analysis was further supported by MS/MS data and the elemental compositions obtained from precise mass measurements. *Table* 2 shows the detailed mass characterization information for DPs, pralsetinib, and its impurities. *Table* 3 shows HRMS data DPs achieved during stress study, offering important details about the molecular features and composition of the DPs.

3.2.1. DP 1 at 1.5 min; *m/z* 192 $[M + H]^+$ and DP 2 at 3.9 min; *m/z* 345 $[M+H]^+$

DP 1 and 2 were identified at a retention time of 1.5 min and 3.9 min respectively in the chromatogram of acid, base and peroxide degradation studies. The mass fragmentation spectra confirms 192 $[M+H]^+$ as molecular mass for DP 1 (Supplementary figure S1) and 345 $[M+H]^+$ for DP2 (Supplementary figure S2). The accurate mass data reveals $C_{10}H_{10}FN_3^+$ as molecular formula for DP 1 and $C_{17}H_{24}N_6O_2^+$ for DP2. This means that ethylformamide group in pralsetinib was broken in presence of acid, base and peroxide, results in the formation of DP 1 and DP 2. During the cleavage of C-N bond present in ethyl- formamide moiety of pralsetinib, the 4-fluoropyrazole size of the pralsetinib arise as DP 1 and 5-methyl-3-pyrazolamine side of pralsetinib arise as DP 2. The fragmentation spectra of DP 1 visualize fragments at *m/z* of 179 (lose of -CH from parent ion), 152 (lose of -CHN from *m/z* 179), 138 (lose of $-CH_2$ from *m/z* 152) and 96 (lose of $-C_3HFN$ from *m/z* 138). Whereas DP 2 visualize fragments at *m/z* of 317 (lose of $-CH_2N$ from parent ion), 273 (lose of $-CO_2$ from *m/z* 317), 233 (lose of $-C_3H_4$ from *m/z* 273), 191 (lose of $-C_3H_6$ from *m/z* 233) and 97 (lose of $-C_5H_6N_2$ from *m/z* 191). The attained parent, fragment ions, accurate mass data and possible molecular formulas were well compatible with proposed structure as shown in *Fig.* 4 with name of *5-ethyl-2-(4-fluoro-1H-pyrazol-1-yl)pyridine* as DP 1 and *1-methoxy-4-{4-methyl-6-[(5-methyl-1H-pyrazol-3-yl)amino]pyrimidin-2-yl}cyclohexanecarboxamide* as DP 2.

3.2.2. DP 3 at 4.9 min; *m/z* 549 $[M + H]^+$

DP3, exhibiting a retention time of 4.9 min, emerged under oxidative conditions. Mass difference of 16 units between protonated form of original drug (*m/z* 534) and that of DP (*m/z* 549) indicates addition of an oxygen atom, likely introduced by hydrogen peroxide. The ESI-MS/MS spectrum (Supplementary figure S3) revealed product ions at *m/z* 518 (lose of $-CH_3$ from parent ion), 439 (lose of $-C_5H_3N$ from *m/z* 518), 372 (lose of $-C_2H_5N$ from *m/z* 439), 224 (lose of $-C_8H_8N_2O$ from *m/z* 372), and 133 (lose of $-C_6H_5N$ from *m/z* 244). Among these, ions at m/z 518, 439, 372 and 224 was noticed to be 17 units higher than those found in the protonated form of original drug indicating the presence of N-OH group in the molecule that hinting at the presence of an N-oxide group in DP 3. N-oxides are typically unstable and prone to decomposition

Table 3. HRMS elemental composition results observed for product ions of pralsetinib and its impurities along with DPs formed in the study

Analyte	Elemental composition	*m/z* Calculated	*m/z* Observed	Error (Δ ppm)
Pralsetinib	$C_{27}H_{30}FN_8O$	502.5779	502.5781	0.398
	$C_{22}H_{25}FN_7O$	423.4780	423.4779	0.125
	$C_{18}H_{20}FN_6O$	356.3888	356.3886	-0.561
	$C_{10}H_{12}FN_4$	208.2269	208.2268	-0.480
	$C_4H_7FN_3O$	133.1157	133.1159	1.502
Impurity 1	$C_{16}H_{24}N_5O$	303.3941	303.3943	0.659
	$C_{13}H_{18}N_5$	245.3149	245.3148	-0.408
	$C_{10}H_{12}N_5$	203.2352	203.2351	-0.492
	$C_5H_9N_4$	126.1512	126.1510	-1.585
	$C_3H_6N_3$	85.0992	85.0991	-1.175
Impurity 2	$C_{10}H_8FN_3$	190.1890	190.1889	-0.526
	$C_{10}H_7N_3$	170.1826	170.1827	0.588
	$C_7H_6N_3$	133.1421	133.1420	0.141
	C_7H_4N	103.1128	103.1129	0.970
				-0.317
Impurity 3	$C_{16}H_{20}N_5O_2$	315.3617	315.3616	-0.317
	$C_{15}H_{20}N_5$	271.3522	271.3523	0.369
	$C_9H_{10}N_5$	189.2086	189.2087	0.529
	$C_5H_9N_4$	126.1512	126.1511	-0.793
	$C_3H_6N_3$	85.0992	85.0993	1.175
DP 1	$C_9H_9FN_3$	179.1857	179.1858	0.558
	$C_8H_8FN_2$	152.1603	152.1601	-1.314
	$C_7H_6FN_2$	138.1337	138.1339	1.448
	$C_5H_7N_2$	96.1219	96.1218	-1.040
DP 2	$C_{16}H_{22}N_5O_2$	317.3779	317.3776	0.157
	$C_{15}H_{22}N_5$	273.3681	273.3683	0.732
	$C_{12}H_{18}N_5$	233.3042	233.3041	-0.429
	$C_9H_{12}N_5$	191.2245	191.2244	-0.523
	$C_4H_6N_3$	97.1099	97.1097	-2.060
DP 3	$C_{27}H_{30}FN_8O_2$	518.5773	518.5774	0.193
	$C_{22}H_{25}FN_7O_2$	439.4774	439.4773	-0.228
	$C_{18}H_{20}FN_6O_2$	372.3882	372.3881	-0.269
	$C_{10}H_{12}FN_4O$	224.2263	224.2260	-1.338
	$C_4H_7FN_3O$	133.1157	133.1159	1.502
DP 4	$C_{22}H_{25}FN_7O$	423.4780	423.4781	0.236
	$C_{18}H_{20}FN_6O$	356.3888	356.3887	-0.281
	$C_{17}H_{18}FN_4O$	314.3488	314.3486	-0.636
	$C_{10}H_{12}FN_4$	208.2269	208.2270	0.480
	$C_4H_7FN_3O$	133.1157	133.1156	-0.751
DP 5	$C_{25}H_{27}FN_9O_3$	521.5383	521.5385	0.383
	$C_{23}H_{26}FN_8O_2$	466.5027	466.5026	-0.214
	$C_{22}H_{27}FN_7O$	425.4939	425.4938	-0.235
	$C_{18}H_{20}FN_6O$	356.3888	356.3889	0.281
	$C_{10}H_{11}FN_4$	207.2195	207.2196	0.483

in collision-induced dissociation. Its fragmentation pathway depicted in *Fig*. 4. Based on gathered data, the suggested structure for DP3 is N-oxide of pralsetinib (*4-fluoro-1-(5-(1-(1-methoxy-4-(4-methyl-6-((5-methyl-1H-pyrrol-3-yl)amino)pyrimidin-2-yl)cyclohexanecarboxamido) ethyl)pyridin-2-yl)-1H-pyrazole 2-oxide*).

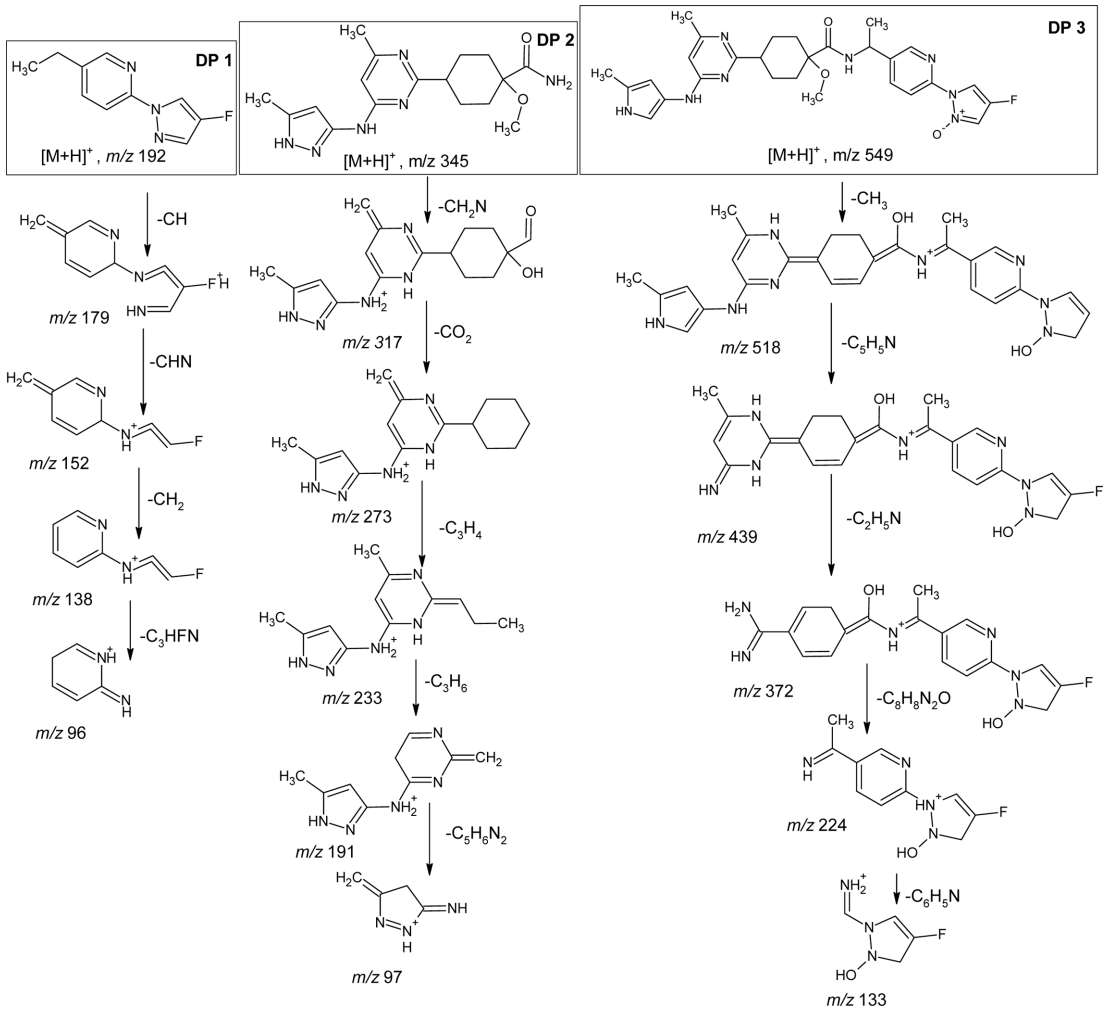

Fig. 4. Suggested fragmentation pathway for DP1, 2 and 3.

The formation mechanism for DP3 likely involves the nucleophilic addition of a hydroperoxide anion to tertiary nitrogen on the *fluoropyrazole* ring, followed by the elimination of a hydroxide ion and the abstraction of hydrogen atoms by hydroxide ions, resulting in the creation of an N-oxide. Elemental compositions of DP 3 and its product ions were verified through precise mass measurements (*Table* 3).

3.2.3. DP 4 at 8.6 min; *m/z* 468 $[M + H]^+$

DP 4 was identified at 8.6 min in acid degradation chromatogram of pralsetinib and exhibit parent ion fragment (Supplementary figure S4) at m/z of 468 $[M+H]^+$ confirms its molecular mass as 467 g/mol. The mass spectral interpretation suggests product ion fragments at m/z of 423, 356, 314, 208 and 133 which corresponds to elemental composition of $C_{22}H_{25}FN_7O$, $C_{18}H_{20}FN_6O$, $C_{17}H_{18}FN_4O$, $C_{10}H_{12}FN_4$ and $C_4H_7FN_3O$ respectively. This product ion was formed by the breakage of C=N bond present in 3-Methyl-1H-pyrazole moiety of pralsetinib followed by the elimination of C_4H_6N in methyl pyrazole moiety. The attained parent, fragment ions, accurate mass data and possible molecular formulas were well correlated to proposed structure (*Fig.* 5). The compound name was finalized as *N-(1-(6-(4-fluoro-1H-pyrazol-1-yl) pyridin-3-yl)ethyl)-1-methoxy-4-(4-methyl-6-(methylamino) pyrimidin-2-yl)cyclohexanecarboxamide*.

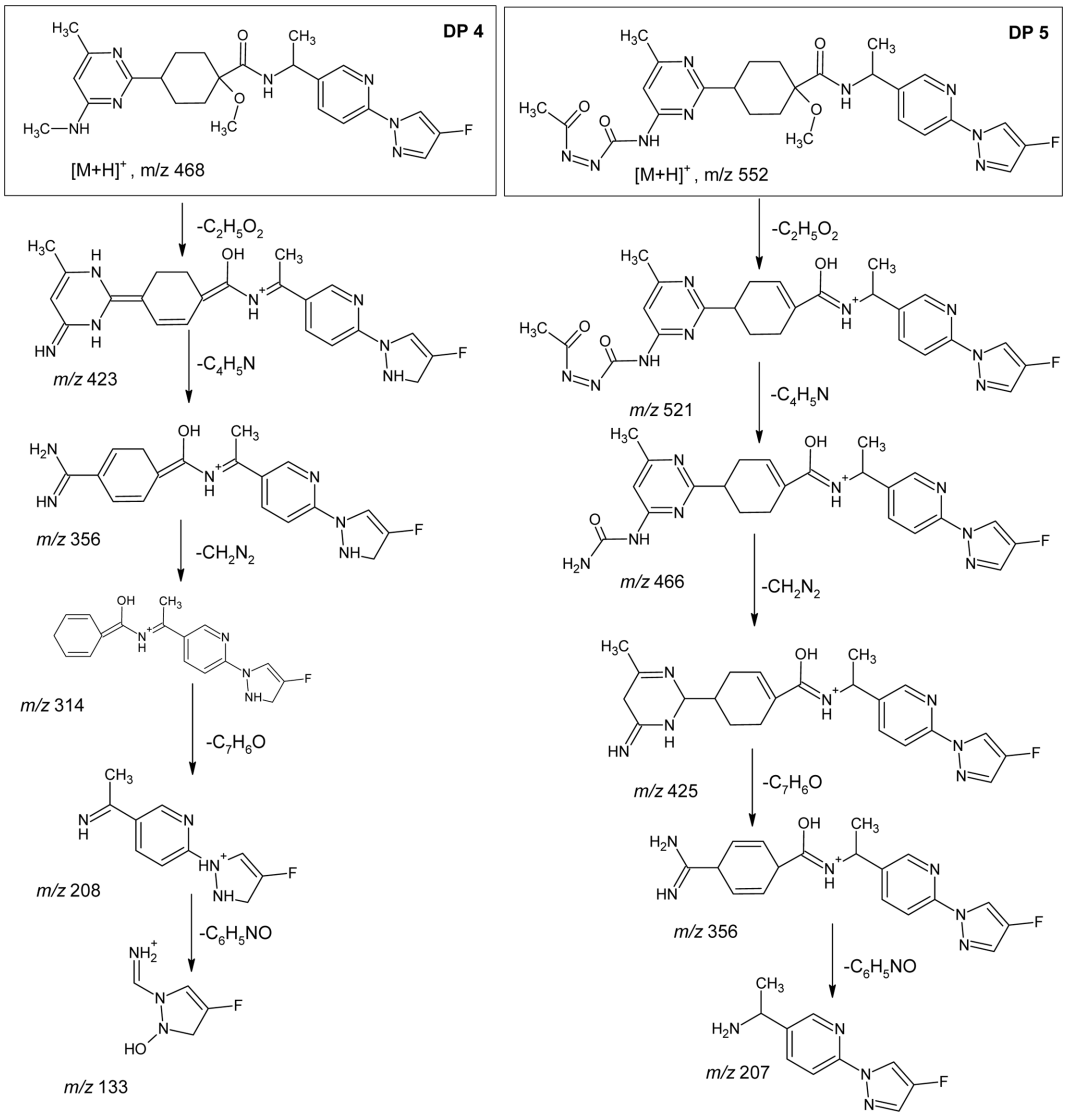

Fig. 5. Suggested fragmentation pathway for DP 4 and 5.

3.2.4. DP 5 at 9.7 min; *m/z* 552 $[M+H]^+$

DP 5 was noticed in base degradation chromatogram at 9.7 min. it exhibit parent ion at *m/z* 552 $[m+1]^+$ corresponds to molecular formula of $C_{26}H_{30}FN_9O_4$. Fragmentation spectra (Supplementary figure S5) shows product ion at *m/z* 521 ($C_{25}H_{27}FN_9O_3$), 466 ($C_{22}H_{27}FN_7O$), 425 ($C_{23}H_{26}FN_8O_2$), 356 ($C_{18}H_{20}FN_6O$) and 207 ($C_{10}H_{11}FN_4$). The acquired parent ions, fragment ions, precise mass measurements, and potential molecular formulas closely matched the proposed structure, as illustrated in *Fig.* 5. The DP 5 was identified as *(Z)-2-acetyl-N-(2-(4-((1-(6-(4-fluoro-1H-pyrazol-1-yl)pyridin-3-yl)ethyl)carbamoyl)-4-methoxycyclohexyl)-6-methylpyrimidin-4-yl)diazenecarboxamide*.

3.3. HPLC method validation

The HPLC technique, refined for the assessment of pralsetinib and its associated impurities, underwent validation in line with the standards outlined in ICH guidelines Q2 (R1). This process confirmed the method's appropriateness for its designated application

and supported its approval for regular implementation in pharmaceutical analysis.

3.3.1. Specificity, selectivity and system suitability

The thorough examination of the method's system suitability, specificity, and selectivity encompassed an in-depth analysis of peak purity for pralsetinib and its impurities. This evaluation entailed determining the purity angle and threshold values to confirm the integrity and distinctiveness of each chromatographic peak. In particular, the peak purity analysis demonstrated that the purity angle of pralsetinib remained below a specified threshold value, indicating the absence of co-eluting substances or impurities that could compromise peak integrity, even in the presence of degradation products. System suitability was assessed by examining parameters such as plate count, tailing factor, and analyte resolution, with results confirming the method's acceptability for analyzing pralsetinib and its impurities. Additionally, the analysis of blank samples showed no interference at the retention times corresponding to pralsetinib, its impurities, and degradation products, validating the method's capability to accurately detect and quantify pralsetinib and its impurities in complex matrices without background interference.

3.3.2. Sensitivity and linearity

LOD refers to smallest concentration of pralsetinib impurities that can be consistently identified, defined by a signal-to-noise (s/n) ratio of 3. On the other hand, LOQ represents the minimum concentration of pralsetinib impurities that can be precisely measured, determined by an S/N ratio of 10. This investigation successfully determined the LOQ values for impurities to be 0.025 μg/mL demonstrating sensitivity and reliability of analytical method employed.

To assess the linearity of the method, a calibration curve was constructed using a standard solution of pralsetinib and its impurities. This curve was generated by analyzing six different concentrations of pralsetinib (ranging from 25 to 150 μg/mL) and impurities (ranging from 0.025 to 0.150 μg/mL), with each concentration being measured in triplicate. The average area obtained from these triplicate analyses at various concentrations, including levels from 25 % to 150 % of the test concentrations, was plotted to form the calibration curve. Statistical analysis involved fitting a linear regression model to the data, which yielded more than 0.999 correlation coefficient for pralsetinib and its impurities, indicates a strong linear relationship within the tested concentration range. These results confirm the method's ability to provide accurate and reliable quantitative measurements of pralsetinib and its impurities within the specified concentration range.

3.3.3. Accuracy

The recovery results for pralsetinib and its impurities demonstrate accuracy and precision of analytical method across different concentration levels. For pralsetinib, the recovery rates at 50 %, 100 %, and 150 % concentration levels are 99.99 %, 99.54 %, and 99.68 % respectively, with %RSD (Relative Standard Deviation) values of 0.56, 0.98, and 0.71. These high recovery percentages and low %RSD values indicate that the method is highly accurate and consistent for pralsetinib across the tested concentration range. Impurity 1 shows recovery rates of 94.67 % at 50 %, 98.00 % at 100 %, and 97.60 % at 150 % concentration levels. The %RSD values for these levels are 0.45, 0.67, and 0.11, respectively. These results suggest that while the recovery rate is slightly lower at the 50 % level, the method maintains good accuracy and precision across all tested levels for Impurity 1. Impurity 2 has recovery rates of 101.33 % at 50 %, 99.00 % at 100 %, and 97.60 % at 150 % concentration levels. The %RSD values are 0.99, 1.04, and 0.38 respectively. These values indicate method accurate and precise with slightly higher variability at the 100 % level but still within acceptable limits. Impurity 3 shows recovery rates of 98.67 % at 50 %, 91.00 % at 100 %, and 96.80 % at 150 % concentration levels, with %RSD values of 0.27, 0.51, and 0.66 respectively. The lower recovery rate at the 100 % level suggests a slight underestimation, but overall the method exhibits good accuracy and precision for Impurity 3 as well. In summary, the recovery results indicate that the analytical method provides reliable and consistent

Table 4. Recovery results of pralsetinib and its impurities in the study method

Analyte	Recovery level	Concentration in μg/mL				% Recovery	% RSD
		Target	Spiked	Final	Recovered		
Pralsetinib	50 %	50	25	75	74.99	99.99	0.56
	100 %	50	50	100	99.54	99.54	0.98
	150 %	50	75	125	124.60	99.68	0.71
Impurity 1	50 %	0.05	0.025	0.075	0.071	94.67	0.45
	100 %	0.05	0.05	0.1	0.098	98.00	0.67
	150 %	0.05	0.075	0.125	0.122	97.60	0.11
Impurity 2	50 %	0.05	0.025	0.075	0.076	101.33	0.99
	100 %	0.05	0.05	0.1	0.099	99.00	1.04
	150 %	0.05	0.075	0.125	0.122	97.60	0.38
Impurity 3	50 %	0.05	0.025	0.075	0.074	98.67	0.27
	100 %	0.05	0.05	0.1	0.091	91.00	0.51
	150 %	0.05	0.075	0.125	0.121	96.80	0.66

n = 3

quantification of pralsetinib and its impurities across various concentration levels, with acceptable accuracy and precision as evidenced by the %Recovery and %RSD values (*Table* 4).

3.3.4. Precision and robustness

The analytical method underwent an extensive assessment of precision, emphasizing both repeatability and inter-day precision. Repeatability was evaluated by injecting the standard solution multiple times within a short period (intraday precision). To ascertain the precision of the method for pralsetinib and its impurities, six replicate measurements were performed at the 100 % sample concentration level (100 μg/mL). The obtained results, expressed as %RSD, were consistently under the permissible limit. This outcome signifies that the method demonstrates excellent reproducibility under the specified conditions and concentration level, ensuring reliable performance in routine analysis.

The robustness of the method was tested by intentionally changing key parameters such as flow rate (0.7 ± 0.05 mL/min), buffer pH (2.8 ± 0.1), and detector wavelength (249 ± 5 °C). These deliberate changes helped evaluate the method's performance under varying conditions. To assess robustness, the method was validated using established system suitability criteria. The performance of the analytical method was closely monitored and analyzed according to these criteria. The results, summarized in *Table* 6, indicate that the method remained robust, showing no significant changes in system suitability parameters or in the assay results for pralsetinib and its impurities.

The method developed in this study introduces several innovative features not found in existing methods for pralsetinib analysis. Previous research has primarily concentrated on measuring pralsetinib in various formulations and biological samples employing various analytical tools. However, these studies have not specifically targeted the quantification of impurities in pralsetinib. The uniqueness of this new method lies in its dedicated focus on detecting impurities of pralsetinib, which are essential for ensuring the drug's quality and safety but have been neglected in prior analyses. Furthermore, the method aims to identify and characterize degradation products using HPLC-MS/MS, providing crucial information on the stability and degradation pathways of pralsetinib under various conditions.

4. Conclusions

This study has successfully developed a novel chromatographic method specifically designed to quantify impurities of pralsetinib along with identifi-

Table 5. Results noticed in precision and ruggedness study of pralsetinib and its impurities in the proposed method

Parameter	Results			
	Pralsetinib	Impurity 1	Impurity 2	Impurity 3
Intraday Precision (n = 3)				
Mean concentration recovered	99.82	0.099	1.01	0.098
Standard deviation	937.82	763.12	1023.99	832.76
% RSD	0.87	0.57	1.23	0.62
Intermediate precision (Day 1, n = 3)				
Mean concentration recovered	99.13	0.100	0.097	0.095
Standard deviation	671.91	843.22	487.23	778.91
% RSD	0.33	0.71	0.67	0.89
Intermediate precision (Day 2, n = 3)				
Mean concentration recovered	99.97	0.098	0.093	0.099
Standard deviation	1085.76	937.88	632.98	1091.23
% RSD	0.23	0.59	0.49	0.47
Intermediate precision (Day 3, n = 3)				
Mean concentration recovered	99.99	0.094	0.097	0.097
Standard deviation	953.98	2408.97	1682.87	2894.45
% RSD	0.88	0.75	0.96	0.88
Intermediate precision (Analyst 1, n = 3)				
Mean concentration recovered	99.87	0.091	0.099	0.094
Standard deviation	1247.87	1480.97	1433.27	1092.98
% RSD	0.54	0.80	0.71	0.56
Intermediate precision (Analyst 2, n = 3)				
Mean concentration recovered	99.67	0.097	0.093	0.092
Standard deviation	952.17	876.88	1062.09	1832.98
% RSD	0.91	0.83	0.56	0.98
Intermediate precision (Analyst 3, n = 3)				
Mean concentration recovered	99.73	0.095	0.091	1.001
Standard deviation	356.90	2367.87	3716.92	9122.98
% RSD	0.95	0.33	0.16	0.36

Table 6. Results noticed in robustness study of pralsetinib and its impurities in the proposed method

Condition	% Assay	Pralsetinib peak plate count	Pralsetinib peak asymmetry	Resolution between Pralsetinib and Impurity 1
0.70 mL/min flow rate (as such)	99.99	18734	0.99	14.89
0.65 mL/min flow rate (Minus)	99.71	18623	0.98	14.62
0.75 mL/min flow rate (Plus)	99.10	19823	1.01	14.72
254 nm detector wavelength (as such)	99.54	17892	0.99	14.55
249 nm detector wavelength (Minus)	100.54	18092	1.00	14.37
259 nm detector wavelength (Plus)	99.01	17891	0.98	14.88
pH of buffer at 2.9 (as such)	100.84	19423	0.99	14.09
pH of buffer at 2.8 (Minus)	99.84	18742	0.99	14.38
pH of buffer at 3.0 (Plus)	99.57	18771	0.98	14.32

cation of degradation impurities. The method was optimized using an XBridge® RP-C18 column (250 mm × 4.6 mm, 5 μm), which significantly improved peak symmetry and resolution. The optimized conditions included a gradient elution with ethanol and 50 mM formic acid at pH 2.9, achieving clear resolution and well-defined peak shapes essential for reliable stability studies. Pralsetinib exhibited varying levels of degradation under different stress conditions, with the most significant degradation (29.3 %) observed under acidic stress. The study identified distinct DPs under each condition: three DPs formed under acidic (DP 1, 2, 4), basic (DP 1, 2, 5), and oxidative (DP 1, 2, 3) conditions, with minimal degradation under thermal and photolytic conditions. The structures of the degradation products were elucidated using LC-ESI-MS/MS, providing detailed mass fragmentation spectra and accurate mass data. DP 1 (m/z 192), DP 2 (m/z 345), DP 3 (m/z 549), DP 4 (m/z 468), and DP 5 (m/z 552) were identified with their respective molecular formulas and fragmentation pathways. The method was validated according to ICH guidelines, demonstrating high specificity, sensitivity, and linearity. The LOD and LOQ for impurities were determined to be 0.025 μg/mL. Unlike previous studies that focused on quantifying pralsetinib in formulations and biological samples, this method uniquely targets the quantification of impurities. The method provides detailed characterization of degradation products using HPLC-MS/MS, offering valuable insights into pralsetinib's stability and degradation pathways.

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