Optimization of a Robust HPLC Method for Abemaciclib and Its Process-Related Impurities; Characterization of Degradation Products Using LCMS/MS

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Abstract:

The current study was undertaken to explore an innovative approach that employs liquid chromatography (LC) and liquid chromatography-mass spectrometry (LCMS/MS) for the resolution, identification, and characterization of minute quantities of degradation products (DPs) of abemaciclib, without the need for their isolation from reaction mixtures. This method successfully separated process-related impurities, including abemaciclib, on Zorbax XDB (250×4.6 mm; 5 µ id) C18 column at room temperature using 0.2 M phosphate buffer with pH 4.2 and methanol in 80:20 (v/v) as mobile phase A, 0.2 M phosphate buffer with pH 4.2 and acetonitrile in the ratio of 30:70 (v/v) as mobile phase B. The mobile phase solvent A and B were mixed at 50:50 (v/v) and the mixture was pumped isocratically at 1.0 mL/min and UV detection at 238 nm. The method demonstrates a sensitive detection limit of 0.09 µg/mL for the studied impurities, with a linear calibration curve spanning the range of 30 – 210 µg/mL for abemaciclib and 0.3 – 0.21 µg/mL for impurities. In accordance with the International Conference on Harmonization (ICH) guidelines, stress studies were conducted on the pure abemaciclib compound. Under various stress conditions, the drug exhibited instability in acidic, alkaline, and UV light environments, while maintaining stability in thermal and peroxide conditions. Six degradation products (DPs) were identified, with their fragmentation patterns and masses elucidated through LCMS/MS. The formed DPs were characterized and confirmed as *5-(piperazin-1-ylmethyl)pyridin-2-amine* (DP 1), *5-fluoro-4-[4-fluoro-2-methyl-1-(propan-2-yl)-1H-benzimidazol-6-yl]pyrimidin-2-amine* (DP 2), *4-(4-amino-3-fluorophenyl)-N-[5-(aminomethyl)pyridin-2-yl]-5-fluoropyrimidin-2-amine* (DP 3), *N-[5-(aminomethyl)pyridin-2-yl]-5-fluoro-4-(4-fluoro-2-methyl-1H-benzimidazol-6-yl)pyrimidin-2-amine* (DP 4), *5-(2-amino-5-fluoropyrimidin-4-yl)-3-fluoro-N1-(propan-2-yl)benzene-1,2-diamine* (DP 5), and 5-fluoro-4-(4-fluoro-2-methyl-1H-benzimidazol-6-

yl)pyrimidin-2-amine (DP 6). The suggested approach proved effective for the routine analysis of abemaciclib and its process-related impurities in both pure drug samples and formulations. Additionally, it demonstrated utility in identifying both known and unknown impurities associated with abemaciclib.

Key words: abemaciclib, process related impurities, HPLC analysis, forced degradation studies, degradation products, LCMS/MS characterization

Introduction:

Abemaciclib is a pharmaceutical compound that belongs to a class of drugs known as cyclin-dependent kinase (CDK) inhibitors [1]. It is used primarily in the treatment of certain types of cancer, with a focus on breast cancer. Abemaciclib works by inhibiting specific enzymes called CDKs, particularly CDK4 and CDK6. These enzymes play a crucial role in cell cycle regulation [2]. By inhibiting CDKs, abemaciclib helps slow down or halt the uncontrolled growth of cancer cells. Common side effects of abemaciclib may include diarrhea, fatigue, nausea, abdominal pain, and decreased appetite. Diarrhea is a notable side effect and is often managed with dose adjustments or anti-diarrheal medications [3].
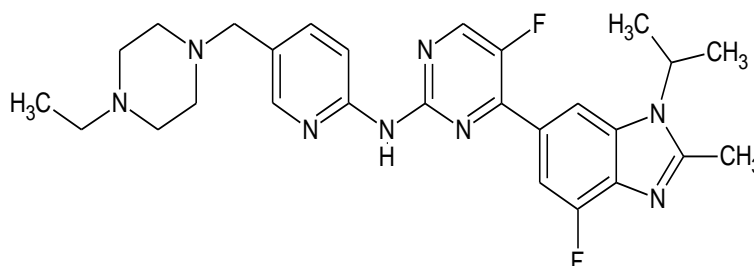

Figure 1: Molecular structure of abemaciclib

HPLC impurity analysis plays a pivotal role in pharmaceutical, chemical, and various other industries due to its significant impact on product quality, safety, and regulatory compliance. Impurity analysis was treated as crucial in ensuring the quality and purity of pharmaceuticals and chemicals. It helps identify and quantify impurities, ensuring that products meet established specifications and regulatory standards. This is especially critical in pharmaceuticals, where even trace impurities can impact a drug's efficacy and safety. The regulatory agencies such as the FDA and EMA mandate stringent impurity analysis as a part of drug approval and post-marketing surveillance. Compliance with these regulations is essential for market approval and ongoing product safety [4]. The impurity profiling helps detect potentially harmful or toxic substances in products. Identifying and controlling impurities ensures the safety of consumers and the efficacy of the product, especially in the case of pharmaceuticals, where impurities can have adverse effects on patients. Impurity

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analysis is vital in stability studies, helping manufacturers determine a product's shelf life and storage conditions. It ensures that a product remains safe and effective throughout its intended lifespan [5].

Various analytical techniques have been documented in the literature for quantifying abemaciclib in samples using various analytical techniques. One HPLC method published for quantification of abemaciclib in formulations [6] and one method reported for resolution of impurities of abemaciclib [7]. One bio-analytical HPLC method reported for quantification of abemaciclib in rat plasma in combination with letrozole [8]. Few HPLC MS/MS bioanalytial methods reported for quantification of abemaciclib in biological samples in single [9,10] or in combination with its metabolites [11,12] or other drugs [13-15]. UPLC/MS bioanalytical method reported for quantification of abemaciclib in biological samples [16,17].

However, upon review of existing literature, it became evident that there is currently no adequate open accesses method available for the comprehensive resolution, identification, and quantification of the process-related impurities of abemaciclib. Consequently, this study was initiated to address the identified gaps in the literature. Specifically, process-related impurities 1, 2, and 3 were selected for investigation. The molecular structures of these process-related impurities are depicted in Figure 2. Further, this study focused to utilize LCMS/MS technique for identification and structural characterization of stress degradation compounds of abemaciclib.
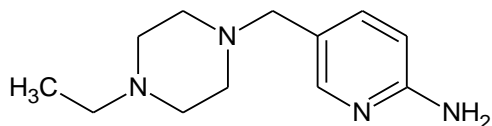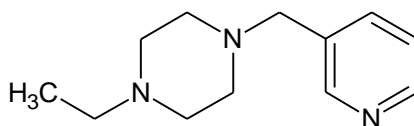

Impurity 1
Name: *5-[(4-ethylpiperazin-1-yl)methyl]pyridin-2-amine*; **MF**: $C_{12}H_{20}N_4$; **MW**: 220.31 g/mol
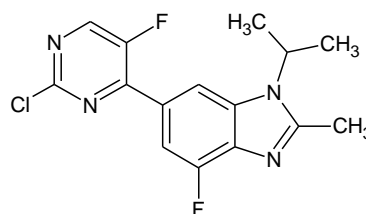
Impurity 2
Name: *1-ethyl-4-(pyridin-3-ylmethyl)piperazine*; **MF**: $C_{15}H_{13}ClF_2N_4$; **MW**: 322.74 g/mol

Impurity 3
Name: *1-ethyl-4-(pyridin-3-ylmethyl)piperazine*; **MF**: $C_{12}H_{19}N_3$; **MW**: 205.29 g/mol
Figure 2: Molecular structure of process related impurities of abemaciclib

The abemaciclib pure drug synthesis route [10] suggests the possible formation of impurities. In the synthesis process boronate ester and 2,4-dichloro-5-fluoropyrimidine underwent Suzuki coupling and produce *1-ethyl-4-(pyridin-3-ylmethyl)piperazine* which was

considered as main structural skeleton of the abemaciclib. The amine side chain was added to main structural skeleton by following Buchwald condensation in presence of xantophos, dioxane, Pd_2 $(dba)_3$ and Cs_2CO_3. The unreacted amine side chain and main skeleton compound were present in the final compound as impurity and were designated as impurity 1 and 2. The impurity 3 was originate as by product in the process of synthesis of amine side chain that involves the amination of 2-bromo-5-formylpiridine and ethylpiperazine followed by Replacement of bromine with amine functional group. These unwanted compounds must be under the permissible limit as per guidelines for producing safe medicine and hence need to be quantified.

Materials and Methods:
Reagents and solvents:

The analytical standard compound abemaciclib with purity of 98.52%, its impurity 1, 2 and 3 were procured from Eli Lilly & Co Pvt. Ltd., Gurgaon, Haryana. The tablet formulation containing 100 mg of abemaciclib with brand VERZENIO® was purchased from local market. HPLC-grade methanol, acetonitrile, and Milli-Q® water were procured from Merck Chemicals, Mumbai. Analytical reagent-grade chemicals, including acetic acid, sodium acetate, hydrochloric acid (HCl), sodium hydroxide (NaOH), and hydrogen peroxide, were sourced from Fisher Scientific, Mumbai.

Instrumentation:

HPLC study was conducted using an Agilent (USA) 1100 instrument equipped with a quaternary pump (G1311 A) for solvent delivery. Analytes were introduced through a temperature-adjustable autosampler (G 1329A) with an injection capacity ranging from 0.1 to 1500 μL. Column eluents were detected using a programmable ultraviolet (UV) detector (G 1314 A), and chromatographic data were integrated using Agilent ChemStation software. LCMS analysis was carried out on a Waters LCMS system (Japan) featuring a triple quadrupole mass detector and MassLynx software

Standard solution preparation:

Standard solutions of abemaciclib and its impurities were prepared at a concentration of 1 mg/mL (1000 μg/mL) individually. This was accomplished by precisely weighing 25 mg of each analyte into separate 25 mL volumetric flasks, each containing 15 mL of methanol. The analytes were then dissolved in the solvent using an ultrasonic bath sonicator. Subsequently, the solutions were filtered through a 0.2 μm membrane filter, and the final volume was adjusted to the mark using the same solvent, resulting in a concentration of 1000 μg/mL for both abemaciclib and its impurities. During the analysis, the required volume of each analyte's specific concentration was used separately.

Test solution preparation:

VERZENIO® tablets with strength of 100 mg were employed in the formulation solution preparation. The tablets were finely powdered using a clean and dry mortar and pestle, and an accurate amount of tablet powder equivalent to 25 mg of abemaciclib was weighed. The measured tablet powder was placed in a 25 mL volumetric flask containing 15 mL of methanol. Subsequently, the drug was completely dissolved in the solvent, and the final volume was adjusted to the mark. The solution was then filtered, diluted to a standard concentration, and the resulting dilute solution was utilized for the assessment of formulation assay.

Method development:

The optimal detector wavelength for the maximum detection of both abemaciclib and its impurities was determined using a UV-visible spectrophotometer. A standard solution containing abemaciclib and its impurities at a concentration of 10 µg/mL was individually scanned using the spectrophotometer, and the resulting absorption spectra were examined to identify the most suitable wavelength. To achieve the best resolution of abemaciclib and its impurities, various stationary phases from different manufacturers were tested. The mobile phase was optimized by experimenting with different solvent compositions encompassing different pH ranges, and variations in flow rates were explored. These conditions that successfully resolved the analytes were subsequently subjected to further validation.

Method Validation:

The method developed in the study was validated for its acceptable performance to ensure suitability of indent purpose. The parameters such as range, linearity, accuracy, precision, specificity, detection limit, quantification limit, ruggedness and robustness experiments were executed ICH guidelines [19].

Forced degradation studies:

The drug's stability was evaluated under various conditions, including photolytic, dry heat, oxidative and hydrolytic (acid, base) stress. An ultraviolet (UV) detector was configured to record absorbance and LC–MS/MS technique was employed to characterize the structure of degradation products (DPs). To investigate the impact of hydrolytic, oxidative, and photolytic conditions, a abemaciclib solution with a known targeted concentration was prepared in HPLC grade methanol. The drug was sealed within a glass ampoule before subjecting it to thermal stress conditions.

Hydrolytic degradation testing was conducted separately under acidic and basic conditions to assess the drug's susceptibility to hydrolytic breakdown. In calibrated 10-mL

volumetric flasks, 1 mL of the gilteritinib stock solution was carefully measured. To this, 0.1 mL of the respective stress inducing solution (0.1 N NaOH or 0.1 N HCl) was added. The samples were exposed to the designated stress conditions for 24 hours. Additionally, the drug was heated at 70℃ in a constant temperature water bath in the presence of respective stress inducing solution for 7 hours to induce degradation. After the stress period, the samples were neutralized using 0.1 N HCl or 0.1 N NaOH as applicable. The volume was adjusted to the mark with the mobile phase, and the samples were injected and analyzed under optimized chromatographic conditions.

Oxidative degradation was carried out using 15% hydrogen peroxide as the oxidizing agent. In calibrated 10-mL volumetric flasks, 1 mL of the gilteritinib stock solution was measured, and 0.1 mL of 15% hydrogen peroxide was added. The samples were exposed to the oxidant for 2 days. For induced oxidative degradation, the drug was heated separately with 15% hydrogen peroxide for 7 hours. After the exposure period, the volume was adjusted to the mark with the mobile phase, and the samples were injected and analyzed under optimized chromatographic conditions.

For thermal degradation, a sealed glass ampoule containing the drug was placed in a hot air oven set at 70 ℃ for 7 days. Following the exposure, the drug sample was injected and analyzed under optimized chromatographic conditions. Photolytic degradation was assessed in both solid and liquid states. The solid drug sample was spread as a thin layer in a Petri dish, and a 10 mL standard stock solution of the drug (1000 µg/mL) was exposed to sunlight for 7 days and subjected to the ICH recommended light dose in a photostability chamber. After exposure, appropriate dilutions were made using the mobile phase to achieve a predetermined drug concentration. These samples were then injected into the HPLC for analysis.

LCMS characterization of DPs:

The DPs generated during stress exposure were identified through analysis using LC-MS/MS. The eluents detected by the UV detector were diverted towards the mass detector for the spectral characterization of these DPs. In this process, it was crucial to allocate 40% of the eluents to the mass detector using a splitter. The resulting mass spectra and mass fragmentation patterns were meticulously compiled to assess the degradation behavior of the DPs.

Method applicability:

The proposed analytical HPLC method was evaluated for the detection and quantification of impurities in abemaciclib tablet formulations. Sample solutions were

prepared from Verzenio® tablets, and the method's performance was assessed by directly analysing these solutions. Additionally, known concentrations of the studied impurities were added to the sample solutions (spiked samples), and the resulting chromatograms and their responses were examined to determine the method's suitability and applicability.

Results and Discussions:

Impurity profiling was conducted to detect both known and unknown impurities in the pure drug, which is essential for ensuring the safety of pharmaceutical products. A review of the existing literature indicated the absence of an analytical method for quantifying the process-related impurities of abemaciclib. Consequently, this study was undertaken to establish a direct HPLC method for the identification and quantification of process-related impurities 1, 2 and 3 in abemaciclib.

Impurities 1, 2, and 3, along with the standard abemaciclib, were characterized as polar compounds, necessitating method optimization on a non-polar stationary phase to achieve optimal analyte retention. To achieve this, abemaciclib standard and its impurities were evaluated using various column configurations, including Octadecylsilane, cyano, phenyl–hexyl, and amino phases, each with varying lengths and particle sizes. Different mobile phase compositions were explored, incorporating buffers such as acetate and phosphate within a pH range of 2-6, along with methanol and acetonitrile as organic modifiers. The presence of ionizable hydrophilic functional groups, it was anticipated that resolving these groups would benefit from mobile phases containing pH buffers. Consequently, the effect of buffer pH on the separation and retention of impurities was examined across a pH range of 2-7.

Successful separation of abemaciclib and its impurities was ultimately accomplished using a zorbax XDB (250×4.6 mm; 5 μm) C18 column at room temperature, which was selected as the stationary phase due to its ability to provide the best resolution and peak symmetry. The mobile phase solvent A and B that comprises 0.2 M phosphate buffer and methanol in 80:20 (v/v) as solvent A and 0.2 M phosphate buffer and acetonitrile in 30:70 (v/v) as mobile phase B which were mixed at 50:50 (v/v) and was pumped at 1.0 mL/min isocratically. The column oven was consistently maintained at 35°C, and a fixed sample injection volume of 20 μL was employed throughout the analysis. A detection wavelength of 238 nm was chosen based on the observation that it provided the optimal detector response when compared to measurements taken at other wavelengths for all analytes.

Under these conditions, it was observed that the peaks corresponding to abemaciclib and its impurities exhibited symmetrical shapes, and the resolution between adjacent peaks

exceeded 2. Identification of the compounds in the standard solution was achieved by injecting individual standard solutions and comparing their retention times with the standard. The retention times were as follows: 4.73 min for abemaciclib, 6.12 min for impurity 1, 9.22 min for impurity 2, and 2.67 min for impurity 3. Column efficiency assessments for abemaciclib and all its impurities revealed values that were within acceptable limits for theoretical plates and resolution, while exceeding the limit for tail factor. These results are presented in Table 1. Chromatograms of the placebo and the standard abemaciclib solution spiked with impurities are depicted in Figure 3. Based on these chromatograms, it was confirmed that the method was specific for the separation and detection of process-related impurities of abemaciclib.
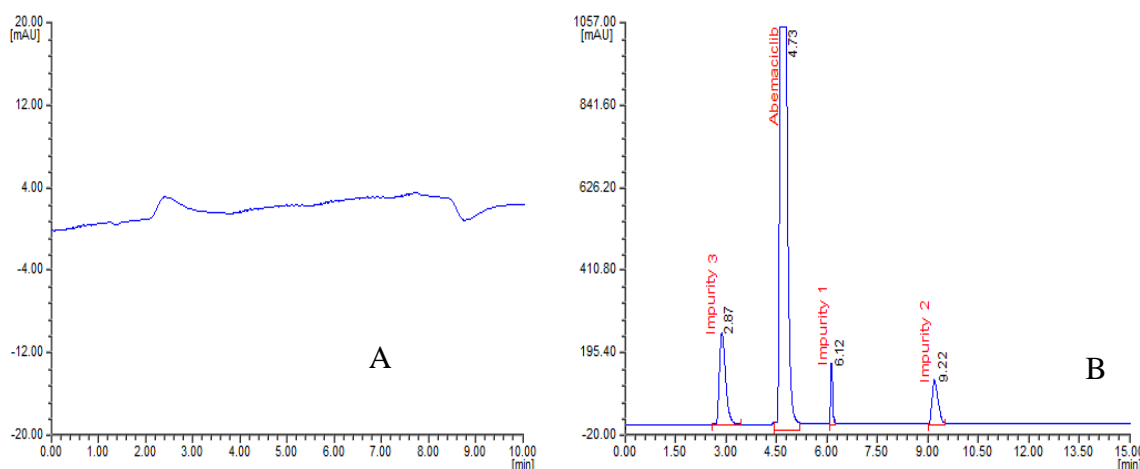
A) placebo chromatogram; B) standard chromatogram

Figure 3: Specificity chromatograms in the proposed method

In every method validation run, the system suitability of the peaks representing abemaciclib and its impurities was confirmed, and the acceptance criteria for each validation run included the following: theoretical plates (N) exceeding 2000, resolution (R_S) between adjacent peaks greater than 2, and a tailing factor (A_S) of no more than 2.0. The values obtained for N, R_S, and A_S for the peaks associated with abemaciclib and its impurities fell within the acceptable limits (Table 1), indicating that the optimized method is suitable for the analysis of these compounds.

Parameter	Results			
	Abemaciclib	**Impurity 1**	**Impurity 2**	**Impurity 3**
System suitability[$]				
t_R (min)	4.73	6.12	9.22	2.87
RRT	--	1.29	1.95	0.61
R_S	7.46	6.91	12.28	--
A_S	1.03	0.95	0.91	1.07
N	6401	8325	12043	5834

Table 1: System suitability results in the proposed method

The method's sensitivity for detecting impurities was assessed by determining the Limit of Detection (LOD) and Limit of Quantification (LOQ) of the impurities using the signal-to-noise (s/n) ratio method. The obtained results established LOD as 0.010 µg/mL and LOQ at 0.03 µg/mL for impurities, affirm the method's high sensitivity and method shows capability to detect analytes at extremely low concentrations.

For the calibration curve dilutions of the impurities, concentrations were selected based on the high sensitivity observed for the impurities, and the quantification limit was defined as the minimum concentration within the calibration range. To ensure that the solution contained 0.1% of each impurity, the concentration of abemaciclib was adjusted accordingly. Two separate calibration curve ranges, each consisting of six data points, were established for abemaciclib and its impurities. This was achieved by analysing calibration standards of abemaciclib prepared as described earlier and plotting the analyte peak area (A) against analyte concentration. The equations of the calibration curves were determined through linear least squares regression analysis, and the results are presented in Table 2. The calibration curves exhibited linearity over the concentration range of 30 – 210 µg/mL for abemaciclib and 0.3 – 0.21 µg/mL for its studied impurities. These findings demonstrate excellent linearity, affirming the method's suitability for quantifying impurities even at very low concentrations.

Method precision and reproducibility were evaluated through intra-day and inter-day precision experiments, as well as ruggedness assessments. Control samples were employed to assess the method's suitability for the quantification of abemaciclib and its impurities. The % relative standard deviation (% RSD) for both abemaciclib and its impurities was determined and found to be within the acceptable limit of less than 2% (Table 2), indicating that the method exhibited precision and ruggedness for the analysis of abemaciclib and its impurities.

The accuracy of the method was determined based on the recovery (R %) of known amounts of analyte in a placebo sample and was calculated using the following formula

$$R\% = C_{found} \times 100 / C_{taken}$$

The accuracy of the method was assessed by performing three consecutive replicate injections of control samples at concentrations of 90 µg/mL, 120 µg/mL, and 150 µg/mL for abemaciclib, each spiked with 0.1% of the studied impurities. The acceptable % recovery fell within the range of 98-102% for both abemaciclib and the studied impurities. Additionally,

the % RSD at each spiked concentration level was found to be less than 2%, which is well within the acceptable limit, thus confirming the accuracy of the method. A summary of the results obtained from system suitability, linearity, precision, accuracy, and sensitivity studies in the proposed method is presented in Table 2

Parameter	Results			
	Abemaciclib	Impurity 1	Impurity 2	Impurity 3
Linearity				
Range in µg/mL	30-210	0.03-0.21	0.03-0.21	0.03-0.21
Slope	8691.5	346699	276680	441013
Intercept	1921.6	- 168.2	211.67	1890.5
r^2	0.9991	0.999	0.9992	0.9992
Precision (n=6)				
Intraday	0.28	0.19	0.30	1.17
Interday (day 1)	1.09	0.30	0.46	1.32
Interday (day 2)	0.21	0.40	0.32	0.76
Accuracy at 50 % level (n=3)				
Amount added (µg/mL)	90	0.090	0.090	0.090
Recovered (µg/mL)	88.557	0.089	0.089	0.089
% Recovery	98.40	99.00	98.65	98.71
% RSD	0.38	0.41	0.36	0.65
Accuracy at 100 % level (n=3)				
Amount added (µg/mL)	120	0.120	0.120	0.120
Recovered (µg/mL)	118.608	0.118	0.119	0.120
% Recovery	98.84	98.69	99.31	100.18
% RSD	0.56	0.20	0.56	0.72
Accuracy at 150 % level (n=3)				
Amount added (µg/mL)	150	0.150	0.150	0.150
Recovered (µg/mL)	148.690	0.150	0.150	0.150
% Recovery	99.13	99.91	100.12	99.79
% RSD	0.71	1.15	0.66	1.00

Table 2: Linearity, precision and accuracy results in the proposed method

Robustness of the method was evaluated by deliberately introducing minor modifications to the proposed method conditions to assess its effectiveness in resolving and quantifying abemaciclib and its impurities. Various parameters were adjusted during this study, including alterations to the mobile phase composition (45:55 and 55:45, v/v, for mobile phases A and B in MP 1 and MP 2, respectively), changes in mobile phase pH (4.2 in pH 1 and 4.3 in pH 2), modification of detector wavelength (243 nm in WL 1 and 233 nm in WL 2), and adjustments to the column temperature (30°C in CT 1 and 40°C in CT 2). The % change in peak area response and system suitability for abemaciclib and its impurities were summarized for all the altered conditions in Table 3. Notably, no significant changes were observed under any of the modified conditions, affirming the robustness of the method

S No	Changed condition	Parameter	Results observed			
			Abemaciclib	**Impurity 1**	**Impurity 2**	**Impurity 3**
1	MP 1	% change	0.90	0.25	0.37	0.92
		t_R	4.72	6.11	9.25	2.89
		N	6398	8150	1119	5163
2	MP 2	% change	0.25	0.17	0.53	0.68
		t_R	4.78	6.13	9.22	2.88
		N	6347	8198	1209	5298
3	pH 1	% change	0.14	0.68	0.87	0.26
		t_R	4.76	6.15	9.23	2.89
		N	6429	8209	1196	5182
4	pH 2	% change	0.24	0.40	0.17	0.54
		t_R	4.73	6.12	9.25	2.82
		N	6281	8165	1238	5132
5	WL 1	% change	0.36	0.35	0.34	0.42
		t_R	4.79	6.18	9.28	2.85
		N	6290	8215	1167	5392
6	WL 2	% change	0.37	0.48	0.54	0.39
		t_R	4.77	6.16	9.21	2.87
		N	6234	8139	1265	5288
7	CT 1	% change	0.14	0.16	0.78	0.36
		t_R	4.73	6.13	9.22	2.82
		N	6408	8203	1177	5270
8	CT 2	% change	0.01	0.23	0.56	0.12
		t_R	4.76	6.15	9.27	2.86
		N	6492	8277	1209	5318

**Table 3: Robustness study results in the proposed method**

Forced degradation studies were conducted to identify potential degradation products, aiding in the establishment of the degradation pathway and the inherent stability of the molecule. The ability of an analytical method to indicate stability is a valuable tool for determining the shelf life of products. As such, the stability-indicating capability of the proposed method was evaluated, and the DPs generated during the study were characterized using LCMS/MS analysis. The % degradation was calculated to be 9.52 % in acidic, 7.68 base, 4.89 % in peroxide, 2.76% in thermal and 4.25 % in UV light degradation study. According to the degradation findings, it was noted that there was no significant degradation observed in the thermal degradation study, and no degradation products were detected in the chromatogram. The chromatogram observed in acid degradation study resolve three DPs at the retention time of 3.13 min (DP 3) and 7.50 min (DP 4) and 10.12 min (DP 6). In the base degradation study, the chromatogram shows three well resolved degradation products (DP 1, 2 & 5) at the retention time of 0.95 min (DP 1), 1.54 min (DP 2) and 9.95 min (DP5) along with impurity 3 and 1. DP 2 & 5 was retained at 1.54 min & 9.95 min in the chromatogram of
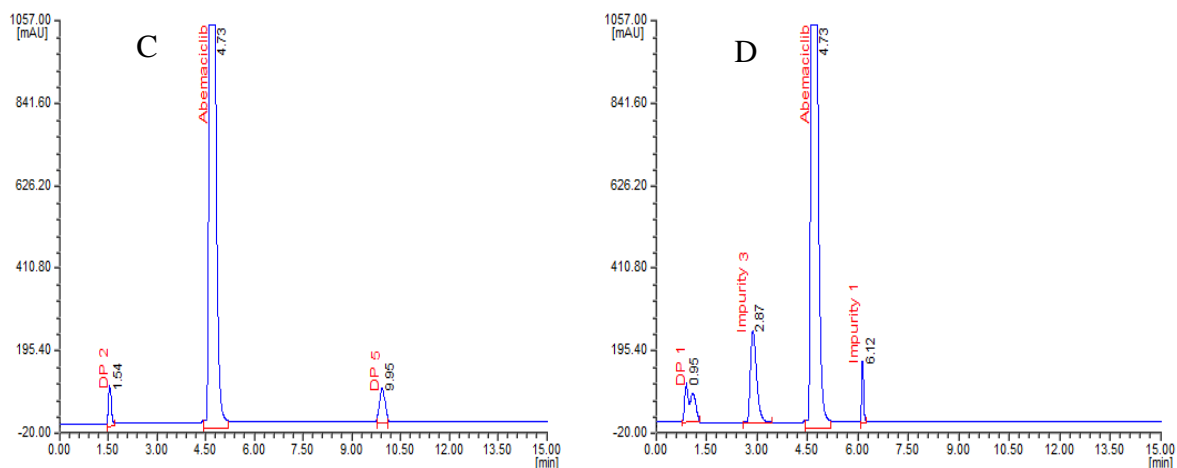
peroxide degradation and the chromatogram also shows no peak corresponds to impurities. The chromatogram of UV light degradation study show peak corresponds to DP 1 at the retention time of 0.95 min and the chromatogram also shows the peaks corresponds to DP 1, impurity 1 and 3. The results of the Peak Purity test obtained from the PDA detector unequivocally affirmed that the abemaciclib peak remained pure and uniform in all the analyzed stress samples. The mass balance for the stressed samples fell within the range of 97.96 - 99.03 %. These Peak Purity test results consistently confirmed the homogeneity and purity of the abemaciclib peak in all the stress samples investigated. The insignificant change in the abemaciclib assay in the presence of impurities, coupled with the Peak Purity test results for the stress samples, serves to substantiate the specificity and stability-indicating capability of the developed method. The results are presented in Table 4, and representative chromatograms observed during the forced degradation study are depicted in Figure 4

Stress condition	% degradation$^\$$ of abemaciclib	% assay$^\$$ of abemaciclib	% Mass balance$^\$$ (assay + total impurities	Remark
Acid	9.52	90.48	98.24	DP 3,4 and 6 were identified
Base	7.68	92.32	99.03	DP 1, 2 and 5 were identified
Peroxide	4.89	95.11	98.57	DP 2 & 5 was identified
Thermal	2.76	97.24	97.96	No degradation products separated
UV light	4.25	95.75	99.73	DP 1 were identified

$^\$$n = 3

Table 3: Stress degradation results
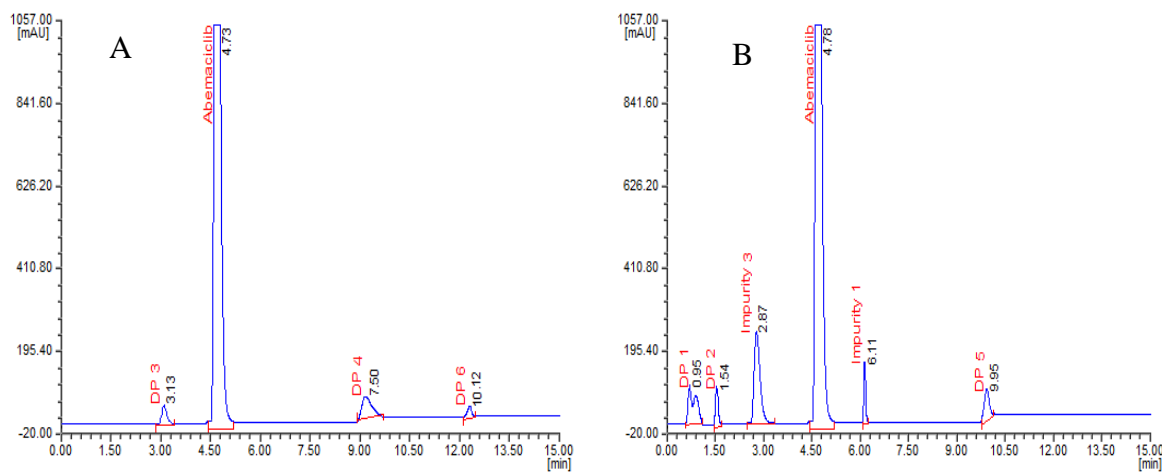
A) Acid degradation chromatogram showing DP 3, 4 and 6 of abemaciclib; B) Base degradation chromatogram showing DP 1, 2 & 5 of abemaciclib; C) Peroxide degradation chromatogram showing DP 2 & 5 of abemaciclib; D) UV light degradation chromatogram showing DP 1 of abemaciclib

Figure 4: Forced degradation chromatograms of lasmiditan

LCMS/MS Characterization of DPs:

The characterizing degradation products is a crucial step in pharmaceutical analysis, as it helps identify and understand the stability and potential safety concerns of a drug substance. LCMS/MS is a powerful analytical technique for characterizing degradation products due to its structural elucidation capabilities, sensitivity, specificity, and ability to provide quantitative data. It is a valuable tool in pharmaceutical development, quality control, and research, as well as in environmental and chemical analysis. Hence, the DPs generated during forced degradation of abemaciclib were characterized through LCMS/MS analysis. By observing the retention times of DPs in the forced degradation chromatogram, it was ascertained that six distinct DPs were formed and were designated as DP 1 to 6 by following the elution sequence observed in the chromatogram.

The DP 1 was generated through based as well as UV light degradation study and was identified at 0.95 min. The molecular ion peak for DP I was detected at an m/z value of 193 (m+1) confirm the molecular weight of DP 1 as 192 g/mol. The initial fragment was generated at an m/z of 108 (m+1) by lose of NH_2 group and then the second abundant fragment was noticed at an m/z of 108 due to lose of $C_4H_9N_2$ from the parent ion. A third fragment with m/z 153 was produced by the loss of C2H2N and another fragment was noticed at m/z of 112 with a total loss of $C_4H_5N_2$ from the parent ion. Based on the date, the DP 1 was identified as *5-(piperazin-1-ylmethyl)pyridin-2-amine*. The proposed fragmentation pathway is illustrated in the figure 6 and mass fragmentation spectra were presented in figure 5A.

The base and peroxide degradation chromatogram visualizes the peak at 1.5 min and was marked as DP 2 which possess a molecular weight of 303 g/mol which was confirmed by observing parent ion fragment at m/z of 304 (m+1) in the mass fragmentation spectrum (Figure 5B). The spectrum clearly shows the high intense fragment ion at an m/z of 150 (m+1) corresponds to *4-fluoro-2-methylidene-2H-benzimidazol-1-ium* ion. This abundant fragment was formed by the loss of $C_7H_9FN_3$ from the parent ion. Apart from this, high intense and characteristic fragments were noticed at m/s of 247, 224 and 114 in positive ion mode. Based on the correlation and interpretation, the DP 2 was identified as *5-fluoro-4-[4-fluoro-2-methyl-1-(propan-2-yl)-1H-benzimidazol-6-yl]pyrimidin-2-amine*. The molecular structure and fragmentation mechanism was presented in figure 6.

The acid degradation chromatogram only visualizes the peak corresponds to DP 3 and 4 at 1.1 min and 7.5 min with a molecular mass of 328 and 368 g/mol. The fragmentation spectra of DP 3 (Figure 5C) and DP 4 (Figure 5D) suggest that DP 3 was derived from DP 4. The DP 3 shows intense fragment at m/z of 297, 228, 182 and 106 in positive ion mode which suggest the molecular formula as $C_{16}H_{13}FN_5$, $C_{12}H_8FN_4$, $C_9H_7F_2N_2$ and $C_6H_5N_2$ respectively. Whereas the fragmentation spectra of DP 4 shows abundant product ion at m/z of 330 (formed by losing C_2N), 276 (lose of $C_4H_6FN_2$), 235 (lose of $C_6H_2N_4$) and 133 (lose of $C_{10}H_7F_2N_5$). Based on the data, compound 3 and 4 were finalized as *4-(4-amino-3-fluorophenyl)-N-[5-(aminomethyl)pyridin-2-yl]-5-fluoropyrimidin-2-amine* and *N-[5-(aminomethyl)pyridin-2-yl]-5-fluoro-4-(4-fluoro-2-methyl-1H-benzimidazol-6-yl)pyrimidin-2-amine*. The fragmentation mechanism of DP 3 and 4 was summarized in figure 6.

The DP 5 and 6 were identified to be having a molecular mass of 279 g/mol (Figure 5E) and 261 g/mol (Figure 5F) with a molecular formula of $C_{13}H_{15}F_2N_5$ and $C_{12}H_9F_2N_5$ respectively. The DP 5 was identified by observing high abundance fragment ions at m/z of 236 (loss of C_3H_8), 185 (loss of $C_5H_7N_2$) and 137 (loss of $C_5H_8FN_4$) whereas DP 6 shows high intense fragments at m/z of 149 corresponds to $C_8H_5FN_2$ and 137 corresponds to $C_6H_3FN_3$. The structure of DP 5 and 6 along with fragmentation mechanism was presented in figure 6.
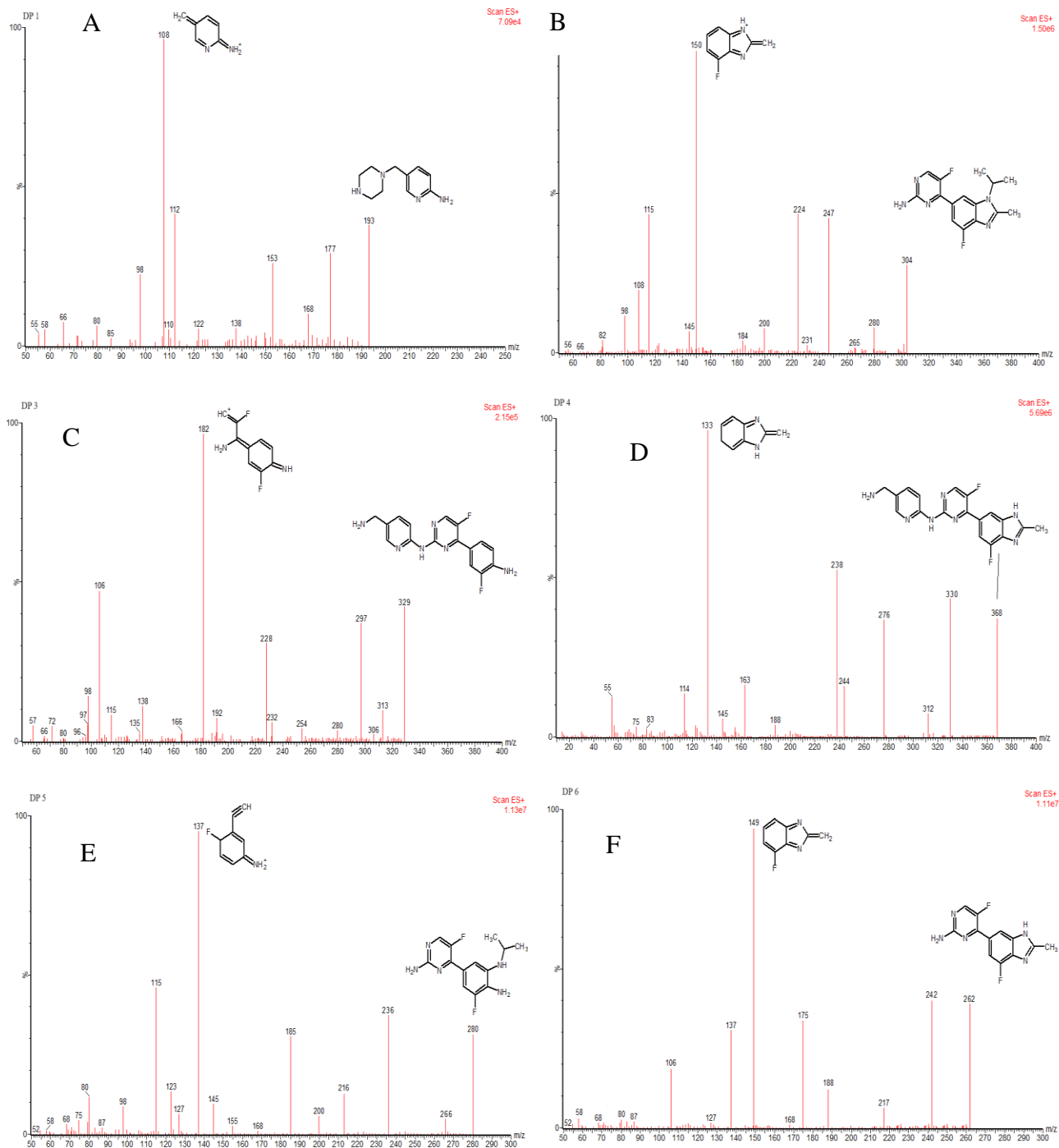
DP Mass spectra observed at t_R of 0.9 min for DP 1 (A), 1.5 min for DP 2 (B), 3.1 min for DP 3 (C), 7.5 min for DP 4 (D), 9.95 min for DP 5 (E) and 10.12 min for DP 6 (F)

Figure 5: Mass spectra of DPs
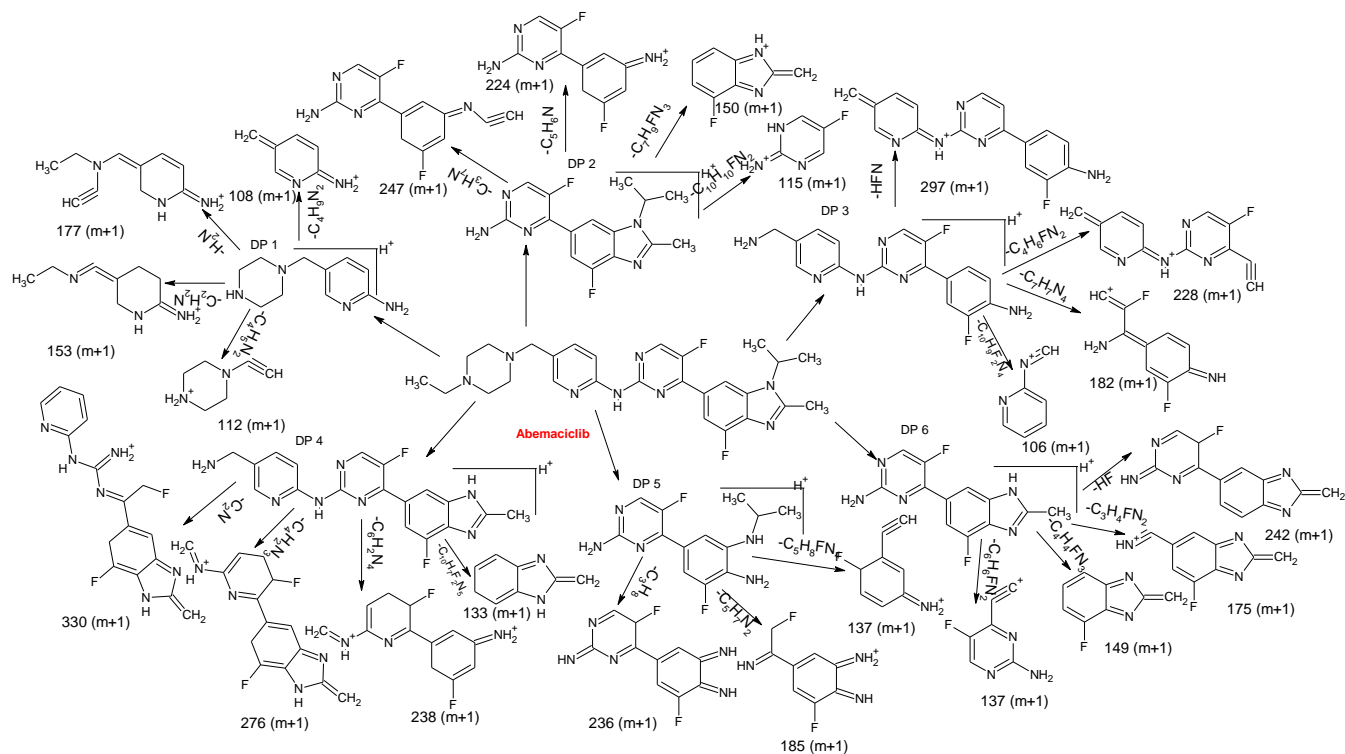
Figure 6: DPs of abemaciclib identified during forced degradation study

The method was applied for the detection and quantification of process-related impurities in pharmaceutical formulations. In this study, we selected the Verzenio® brand formulation of abemaciclib. Both impurities-spiked and unspiked formulation solutions of abemaciclib were subjected to analysis using the proposed method. The chromatogram of the impurities-spiked formulation solution (Figure 7A) clearly revealed the presence of impurities in addition to the standard abemaciclib peak. Conversely, the chromatogram of the unspiked formulation solution (Figure 7B) exhibited no peaks corresponding to impurities. This observation confirmed that the studied impurities were either absent in the formulation or present at concentrations below the detection limit of the method. Furthermore, there was no detection of any unknown impurities or formulation excipients in either the impurities-spiked or unspiked formulation analyses. As a result, this method was deemed suitable for the routine analysis of abemaciclib and its process-related impurities in the given formulation.
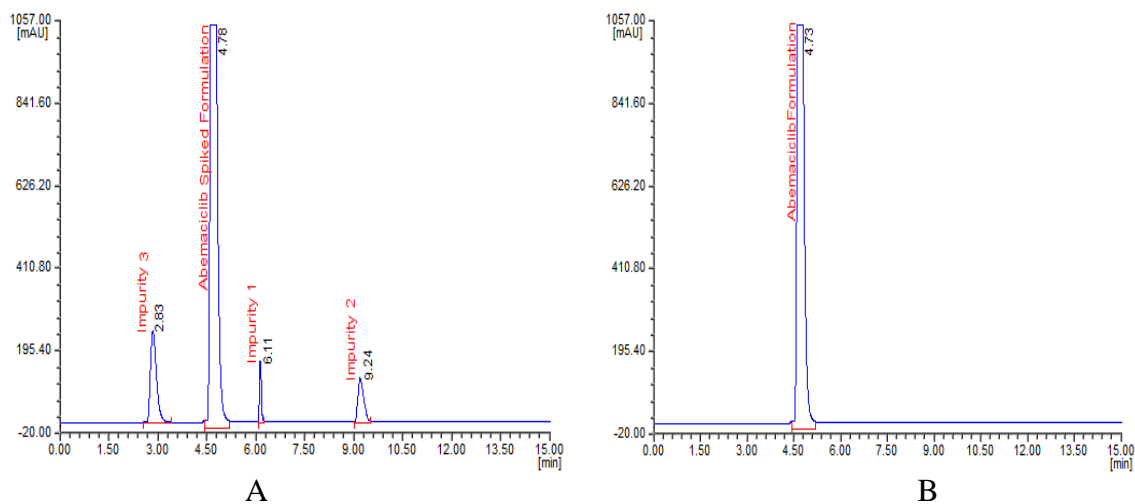
A) Verzenio® formulation solution spiked with 0.1 % of each impurity; B) Verzenio®
formulation solution spiked with no impurities

Figure 8: formulation analysis chromatograms in the proposed method

Conclusion:

The proposed method for analyzing abemaciclib and its related impurities in both bulk and pharmaceutical dosage forms has demonstrated exceptional specificity, precision, accuracy, speed, and cost-effectiveness. The method produce calibration curve linear within the range of 30 – 210 μg/mL for abemaciclib and 0.3 – 0.21 μg/mL for impurities. The drug was subjected to various degradation conditions, including acidic, basic, peroxide, thermal, and UV light stress. Chromatograms obtained under different stress conditions revealed the emergence of DPs, the number of which depended on the specific stress condition applied. Notably, the drug exhibited substantial degradation under acidic and basic conditions, while minimal degradation occurred under thermal stress, with no detectable degradation compounds observed. The structures of these DPs were elucidated using LC-MS/MS analysis, which proved invaluable in understanding the degradation pathway and assessing the overall stability of abemaciclib. The developed isocratic HPLC method was subjected to rigorous validation and demonstrated specificity, linearity, precision, and accuracy. This method serves as a valuable tool for the identification and quantification of both process-related impurities and degradation products of abemaciclib.

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