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# *CREATION AND VERIFICATION OF AN RP-UHPLC TECHNIQUE FOR ABEMACICLIB AND ASSOCIATED IMPURITIES IN FORMULATIONS AND APIS USING BOX-BEHNKEN DESIGN*

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***ABSTRACT***

**Background:** Strict impurity profiling is necessary to ensure the safety, effectiveness, and regulatory compliance of Abemaciclib (AbB), a selective CDK4/6 inhibitor frequently used in breast cancer treatment. However, a lack of thorough impurity separation, lengthy run times, and low sensitivity are common problems with current analytical techniques. **Methodology:** A novel reverse-phase ultra-high-performance liquid chromatography (RP-UHPLC) method was developed using a Quality by Design (QbD) framework. Critical method parameters, including mobile-phase pH, flow rate, and column temperature, were optimized using the Box-Behnken Design (BBD). Chromatographic separation was achieved on a C18 column using an isocratic mobile phase consisting of acetonitrile (40:60, v/v) and ammonium formate buffer (pH 2.7). The technique was validated in accordance with the ICH Q2(R1) requirements. **Results and Discussion:** The optimized method demonstrated exceptional linearity over the range of 0.02–150 µg/mL ($R^2 > 0.999$) with excellent precision (RSD < 2% for AbB and < 10% for related impurities) and showed acceptable accuracy, with mean recoveries ranging from 92.76% to 102.88% across AbB and its related impurities. The detection and quantification limits for AbB were 0.01 µg/mL and 0.02 µg/mL, respectively. Forced degradation studies that effectively distinguished degradation products in oxidative, acidic, and alkaline settings confirmed the method's stability-indicating nature. The 8-minute runtime allowed for a quick examination. The integration of BBD-based QbD significantly enhanced the method's robustness, resolution, and sensitivity compared with conventional chromatographic approaches, thereby enabling simultaneous quantification of AbB and its related impurities. **Conclusion:** For routine quality control, stability investigations, and regulatory-compliant impurity profiling of Abemaciclib, the developed RP-UHPLC method is quick, sensitive, reliable, and appropriate.

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## *INTRODUCTION*

Abemaciclib (AbB), a potent inhibitor of cyclin-dependent kinase 4 and 6 (CDK4/6), has garnered significant attention in oncology, particularly for the treatment of advanced or metastatic HER2-negative and hormone receptor-positive (HR+) breast cancer [1-3]. In 2017, the FDA authorized AbB for use as a monotherapy in patients with advanced breast cancer that had progressed after endocrine therapy, after comprehensive clinical trials demonstrated its safety & effectiveness. For patients whose disease had progressed after endocrine therapy & previous radiation therapy, it was also recommended in conjunction with fulvestrant [4,5].

Degradants, unreacted starting materials, process-related impurities, and byproducts of side reactions are among the unwanted chemicals that may arise during the multistep transformations involved in AbB synthesis. Toxicity, safety, and regulatory compliance are major issues when related impurities and degradation products are present in the drug product and the active pharmaceutical ingredient (API) [6]. Regulatory agencies such as the U.S. Food and Drug Administration (FDA) and the International Council for Harmonization (ICH) demand stringent impurity profiling to ensure the safety and efficacy of medications. Thus, the development of highly sensitive, specific, and fast analytical techniques for impurity quantification is crucial [7].

Although reliable impurity profiling is essential, current liquid chromatography (LC) techniques have several drawbacks. Due to the restricted sensitivity and higher limits of detection (LOD) and quantification (LOQ) of previously reported HPLC and LC-MS/MS methods for AbB quantification, trace-level impurity analysis is challenging [8,9]. Furthermore, traditional methods require prolonged run times, making them inefficient for high-throughput pharmaceutical analysis [10]. Another critical limitation is the lack of simultaneous quantification, as most approaches either quantify AbB alone or focus on specific impurities, rather than on comprehensive impurity profiling in a single run [11,12]. Additionally, many existing methods employ the one-factor-at-a-time (OFAT) optimization paradigm, which is ineffective for optimizing multiple variables simultaneously [13]. Moreover, few studies have thoroughly investigated the forced degradation behavior of AbB, thereby limiting the ability of standard approaches to assess stability [10].

To overcome these obstacles, a novel reverse-phase ultra-high-performance liquid chromatography (RP-UHPLC) approach is developed and optimized in this study for the simultaneous measurement of AbB and its process-related impurities (IMP-I, II, III, and IV) in pharmaceutical formulations and API. Figure 1 illustrates the molecular structures of these impurities, which primarily arise from the Suzuki coupling reaction and reductive amination processes involved in AbB synthesis [14]. In contrast to traditional approaches, this study incorporates Quality by Design (QbD) concepts to improve method performance, guaranteeing the development of reliable, reproducible, and regulatory-compliant analytical procedures. Regulatory agencies, including the FDA and ICH, strongly advocate for QbD-based method development to improve analytical reliability [15].

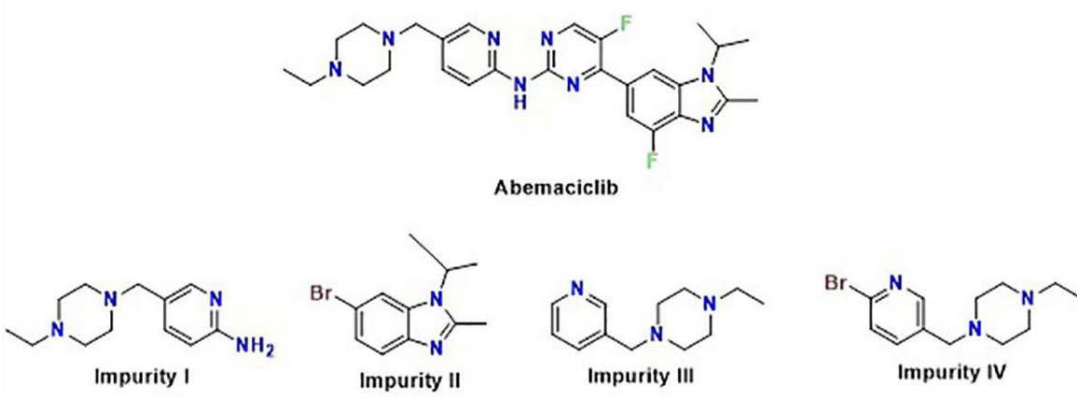


**Figure 1: Chemical structures of Abemaciclib (AbB) and its four process-related impurities, IMP-I, IMP-II, IMP-III, and IMP-IV. These impurities primarily arise during the Suzuki coupling & reductive amination steps involved in AbB synthesis.**

This work uses the Box-Behnken Design (BBD), a popular statistical technique based on response surface methodology (RSM), to optimize key method parameters (CMPs), including pH, column temperature, and flow rate. Compared to alternative design of experiments (DoE) methodologies, BBD offers several advantages. It minimizes the number of experimental runs

required, optimizing resource efficiency while maintaining model fidelity [16]. Additionally, BBD effectively captures interactions between CMPs, ensuring method robustness and avoiding the pitfalls of the OFAT approach. Furthermore, it enhances chromatographic resolution, precision, and sensitivity, which are critical for impurity profiling in pharmaceutical analysis. Given its strong regulatory alignment, DoE-based method development, particularly using BBD, is highly recommended by agencies such as the FDA and ICH to ensure method reproducibility and robustness [17].

This work successfully overcomes the drawbacks of current chromatographic techniques by establishing a highly sensitive, reliable, and time-efficient UHPLC (Ultra-High Performance Liquid Chromatography) approach for impurity profiling through the integration of BBD-based optimization.

## *MATERIALS AND METHODS*

### Reagents and Chemicals

The operating standard for AbB and impurities (IMP-I, II, III, and IV) was purchased from Rx Innovations in Hyderabad, India. We purchased 50mg AbB tablets from a nearby drugstore. The acetonitrile (HPLC grade, ≥99.9%), ammonium formate (suited for HPLC, ≥99%), and formic acid (EMSURE® ACS, Reag, Ph. Eur-98-100%) were supplied by Sigma-Aldrich Chemicals Private Limited in Bangalore, India. Milli-Q water from the Millipore Milli-Q water purification system was used. All other reagents used in the study were of analytical grade.

### Chromatographic conditions and equipment

In this investigation, a high-sensitivity UHPLC system from the Shimadzu Nexera X3 Series, including a PDA detector, binary liquid management, and an automated sample manager, was utilized. Reverse-phase isocratic elution using a Kinetex® LC C18 Column (2.1 mm x 100 mm, 1.7 µm) was used to accomplish chromatographic separation. The optimized mobile phase consisted of 10 mM ammonium formate buffer (pH 2.7, adjusted with formic acid) and acetonitrile in a 40:60 v/v ratio. The injection volume was 4µl, and the detection wavelength was 210 nm. Lab Solutions software was used to process the chromatographic data.

The reputable Micro Systems RB-1201 Raptec was used to weigh the samples. The pH was measured using the Elico India LI615 pH meter. The ultrasonicator (CV334, Sonico Vibra, Cell Sonics, USA), the computerized shaking bath with water (Model-1113, Gesellschaft für Labortechnik GmbH, Germany), and the Spinix Vortex mixer (Spinix Corporation, USA) were all utilized. Class-A glassware bulb pipettes (BLAUBRAND®) and Mettler Toledo single-channel micropipettes made of polypropylene were utilized. Key method parameters were optimized using Design-Expert® software (version 13.0.5.0, Stat-Ease, Inc., Minneapolis).

### Preparation of Sample Solution

After the Ramiven pills were ground into a fine powder, 100 mg of pure Abemaciclib was carefully weighed and added to a 100 mL volumetric flask. A 60:30 acetonitrile: water diluent was used to ultrasonically dissolve the powder in 60 mL. The volume was then adjusted to 100 mL (1 mg/mL; sample stock solution). The sample solution was filtered through a 0.45 µm membrane filter. An aliquot (10 mL) of the filtered solution was diluted to 100 mL in another volumetric flask, yielding a final concentration of 100 µg/mL.

### Standard AbB Solution (0.5% relative to sample concentration)

After carefully weighing 25 mg of AbB, it was transferred to a 100 mL volumetric flask and ultrasonically dissolved in the appropriate diluent. To remove any remaining particles, the finished solution was passed through a 0.45-micron membrane filter. To obtain a final volume of 100 mL, 2 mL of the filtered solution was diluted with the same diluent in a different volumetric flask. A concentration of 0.5 µg/mL was obtained by further diluting 10 mL of the diluted solution with the diluent.

### Related substances stock solution

About 10 mg of each related impurity (IMP-I, IMP-II, IMP-III, and IMP-IV) was weighed precisely. They were put into a 100 mL volumetric flask. The volume was raised to 100 mL after the related impurities were sonicated with 70 mL of the appropriate diluent. To make a stock solution with 1 µg/mL impurities, 1 mL of the generated solution was mixed with the solvent in another 100 mL volumetric flask.

### Sample Solution Spiked

A spiked sample solution was prepared by transferring 10 mL of AbB stock solution (1 mg/mL) and 10 mL of mixed related-impurity stock solution (1 µg/mL) into a 100 mL volumetric flask. The volume was made up to the mark with diluent to obtain a final solution containing 100 µg/mL of AbB and 0.1 µg/mL of each related impurity.

**Mobile phase**

The optimized mobile phase consisted of 10 mM ammonium formate buffer adjusted to pH 2.7 with formic acid, and acetonitrile in a 40:60 (v/v) ratio. The buffer was filtered through a 0.22 µm membrane filter and degassed before use. The same mobile phase composition was used for method validation, forced degradation analysis, assay, and related-substance determination.

**UHPLC method development**

To measure trace-level potential associated substances in a sample of AbB medicine, the UHPLC technique was developed. The chromatographic method was developed and refined in compliance with the QbD technique using a suitable response surface design. The developed method's compliance with ICH standards was then confirmed. Isocratic elution was selected based on preliminary chromatographic screening and the observed retention behavior of AbB and its four process-related impurities.

Although gradient elution is commonly useful for analytes with widely different hydrophobicity or broad retention behavior, AbB and the selected impurities eluted within a relatively narrow chromatographic window under acidic reversed-phase conditions; the optimized ammonium formate buffer-acetonitrile mobile phase maintained controlled protonation of AbB and amine-containing impurities, reduced variable secondary interactions, and improved peak symmetry and selectivity on the C18 stationary phase. In addition, the use of a high-efficiency Kinetex® C18 UHPLC column with 1.7 µm particle size improved column efficiency and peak capacity, enabling baseline separation without gradient programming.

Therefore, isocratic elution was selected because it provided sufficient selectivity and resolution while avoiding gradient re-equilibration time, improving reproducibility, reducing solvent consumption & simplifying routine quality-control applications. First, key parameters were identified through screening experiments. The next step was to apply the Box-Behnken design to optimize the critical components influencing the analytical results. The impacts of each variable were evaluated separately and in combination using statistical techniques and experimental design. The pH of the mobile phase was adjusted with 0.05% formic acid. To eliminate any possible carryover, a strong washing solution consisting of equal parts methanol, acetonitrile, isopropanol, and water was used both before and after each injection.

**Screening studies**

To select a column, phase of mobility, and flow rate, initial screening studies were carried out. The trials were carried out using the OFAT approach. The initial phase involved screening five different columns: ACQUITY UHPLC® BEH Phenyl (2.1 mm x 100 mm, 1.7 µm), Hypersil BDS C18 (2.1 mm x 100 mm, 1.7 µm), LUNA® C18 (2.1 mm x 100 mm, 1.7 µm), and Kinetex® LC C18 (2.1 mm x 100 mm, 1.7 µm). Three different mobile phases with different pH levels (1. Acetonitrile and water with 10 mM ammonia formate (50:50 v/v) and 0.1% formic acid at pH = 6.8. A pH 2.5 solution consisting of a water phase (10 mL ammonia formate with 0.05% formic acid) and an organic phase (40:60 v/v). 3. Screening was conducted using a pH of 9.2 (a mixture of 10 mM acetonitrile and ammonia bicarbonate). The effect of flow rate on resolution, sensitivity, and retention times was examined using a Kinetex® LC C18 Column (2.1 mm × 100 mm, 1.7 µm) and an aqueous phase containing 10 mM ammonia formate in an organic phase of acetonitrile (40:60 v/v) at pH - 2.7. The flow rate ranged from 0.2 to 0.8 mL/min. The column temperatures were maintained at 30 °C for each experiment. The 100% specification level for all screening studies was a diluted solution of AbB standards (100 µg/mL) and a stock solution of AbB-related compounds (100 µg/mL of AbB and 0.1 µg/mL of related substances).

The selection of mobile phase pH, flow rate, and column temperature as independent variables was based on the physicochemical properties of AbB and the chromatographic behavior observed during preliminary screening. Abemaciclib is a weakly basic, nitrogen-containing molecule with a reported pKa of approximately 5.1. Therefore, mobile phase pH was considered a critical variable because it directly controls the protonation state of Abemaciclib and its related impurities, thereby influencing retention, peak symmetry, selectivity, and resolution on the reversed-phase C18 column. At acidic pH values below the pKa, particularly within the screened range of pH 2.5-3.5, Abemaciclib remains predominantly protonated, which provided more reproducible retention and improved peak shape. In contrast, neutral and alkaline screening conditions may reduce or alter ionization, increase variable secondary interactions, and adversely affect the resolution of structurally related impurity peaks.

Flow rate was selected as a second independent variable because AbB and its process-related impurities have structurally related heteroaromatic and amine-containing features and elute within a relatively narrow retention window. Therefore, changes in flow rate directly affect analyte residence time in the column, peak width, column efficiency, and the resolution of critical pairs tested. Column temperature was selected because AbB possesses hydrophobic and aromatic structural regions that interact with the C18 stationary phase; temperature changes can alter mobile phase viscosity, analyte diffusivity, mass-transfer kinetics, and partitioning behavior, thereby influencing retention and selectivity. Based on these chemical and chromatographic considerations, mobile phase pH, flow rate, and column temperature were selected as critical method parameters for Box-Behnken optimization.

**QbD approach**

Using the Quality by Design (QbD) methodology, the current work develops a UHPLC method for the precise determination of AbB and related substances in the therapeutic product under investigation. The study uses QbD to establish an accurate UHPLC technique for measuring AbB and related substances in the medicinal product under examination. The Analytical Target Profile (ATP) sets the performance criteria by product requirements [16].

The QbD approach considers factors such as resolution, theoretical plates, tailing factor, and retention time to identify crucial method variables. Risk assessment is crucial for connecting the design space and control strategy, as well as for assessing the impact of various factors on analytical responses [17]. If the drug peak or its degradation peak co-elutes, it might affect the method's selectivity. The mobile-phase pH, structure, column temperature, and flow rate are examples of critical method parameters that interact with critical technique characteristics [18].

***Optimization of CMP - Design of experiments (DOE)***

The results of screening studies were used to identify the CMPs that impact the CMAs. A full factorial, response surface Box-Behnken Design (BBD) was used to assess the effects of CMPs and their reciprocal impacts on the selected CMAs. The method elements (mobile-stage pH, column temperatures, and flow rate) that affect the technique's precision, accuracy, robustness, and specificity were identified through the risk assessment. Drug retention times (R1), resolving between drug peaks and IMP-I (R2), resolving between drug peaks and IMP-II (R3), and resolving between IMP-III and IMP-IV (R4) were all deemed pertinent method input variables for assessing their impact on output parameters. It was decided to set the column temperature (B) between 25 and 35 °C, the pH (A) range between 2.5 and 3.5, and the circulation rate (C) between 0.4 and 0.8 mL/min. Using Design-Expert® software, 17 tests were selected via BBD and randomized. The testing variable levels for each authorized trial are listed in Table 1. A standard suspension of AbB-IMP (100 µg/mL-AbB and 0.1 µg/mL-IMP) was used for all design investigations.

**Verification of the approach**

The ICH-Q2(R1) standards were followed in the validation of the QbD-based UHPLC procedure. Linearity, specificity, accuracy, precision, solution stability, resilience, system suitability, limit of detection (LOD), and limit of quantification (LOQ) were all evaluated**.** LOD and LOQ were estimated experimentally using the signal-to-noise approach.

Serially diluted standard and impurity-spiked solutions were injected, and the signal-to-noise ratios were calculated by comparing the analyte peak response with baseline noise near the corresponding retention time. The LOD was assigned to the concentration yielding an S/N ratio of approximately 3:1, and the LOQ to the concentration yielding an S/N ratio of approximately 10:1, with acceptable replicate precision.

**Analysis of statistics**

Response surface methodology (RSM) was used to investigate the impact of CMPs on CMAs, and ANOVA was used for statistical analysis. Visualization was done using contours, response surfaces, and perturbation plots. Utilizing a polynomial prediction model, the quadratic influence of CMPs on CMAs was explained. Statistical analysis was used to determine the Methodology Operable Design Range (MODR). It indicates the region that, when ATP criteria are met, falls inside the CMP variable boundaries. The design space was constructed, and MODR was visualized using contour plots, which placed limitations on CMPs. Confirmation experiments validated the design-recommended ideal procedure circumstances, ensuring the desired analytical outcomes. The UHPLC method verification was used to assess the recommended model predictability.

## *RESULT*

### Method development and Design of experiments

Based on preliminary trial data, the initial chromatography conditions for separating AbB and its known related molecules were selected. AbB pKa is 5.1. Among the numerous mobile phases investigated, the one with a pH between 2.5 and 3.5 was selected as the best choice due to the pKa values of AbB and its impurities. UV spectra of AbB and related compounds in diluent solution were recorded. The greatest absorbance of AbB and similar compounds was observed between 225 and 245 nm; however, the final detection wavelength of 210 nm was selected to ensure sufficient sensitivity for the lower-absorbing process-related impurities, which showed adequate response at this wavelength, and to achieve uniformity of detection across all analytes in the impurity profiling method. The Kinetex® LC C18 Column (2.1 mm x 100 mm, 1.7 µm) offered the best peak shape and system suitability parameters for AbB detection among all the columns examined. A pH 2.5 mixture of an organic phase (40:60 v/v) and an aqueous phase (10 mM ammonium formate) showed high system compatibility among the various mobile phases. Flow rates between 0.4 and 0.8 mL/min satisfy system compatibility criteria.

The findings of the early experiments were used to identify the important technique aspects, including the drug retention time (R1), the degree of separation between the IMP-I and AbB peaks (R2), the resolution between the AbB and IMP-II peaks (R3), and the resolution between the IMP-III and IMP-IV peaks (R4). Mobile-phase pH (A), column temperature (B), and flow rate (C) were identified as CMPs affecting CMAs. Using the BBD (three factors, three levels), the proposed UHPLC CMPs were optimized. In each experiment, 40 µg/mL of AbB and 0.06 µg/mL of each IMP were injected into a spiked sample of related compounds. Table 1 displays the results of each DOE trial.

**Table 1: Box-Behnken design matrix with experimental runs (1–17) showing independent variables (mobile phase pH, flow rate in mL/min, column temperature in °C) and dependent responses (retention time of AbB in minutes, resolution R2 between IMP-I and AbB, resolution R3 between IMP-II and AbB, resolution R4 between IMP-III and IMP-IV).**

| Run | Independent variables | | | Dependent variables | | | |
|---|---|---|---|---|---|---|---|
| | pH of mobile phase | Flow rate | Temp. (0C) | Retention time of AbB | R2 (resolution between IMP-I and AbB | R3 (resolution between IMP-II and AbB) | R4 (resolution between IMP-III and IMP-IV |
| **1** | 3.5 | 0.4 | 30 | 4.5±0.12 | 1.4±0.05 | 1.54±0.06 | 2.02±0.08 |
| **2** | 3 | 0.6 | 30 | 3.1±0.11 | 1.63±0.07 | 1.78±0.08 | 2.31±0.06 |
| **3** | 3 | 0.8 | 25 | 2.5±0.09 | 1.5±0.02 | 1.65±0.09 | 2.15±0.06 |
| **4** | 3 | 0.4 | 25 | 3.1±0.11 | 1.9±0.05 | 2.09±0.03 | 2.72±0.08 |
| **5** | 3.5 | 0.8 | 30 | 3.9±0.10 | 1.15±0.06 | 1.41±0.01 | 1.8±0.03 |
| **6** | 3.5 | 0.6 | 25 | 4.1±0.13 | 1.4±0.07 | 1.54±0.02 | 2.02±0.02 |
| **7** | 2.5 | 0.4 | 30 | 2.8±0.14 | 3.1±0.06 | 3.29±0.08 | 4.28±0.06 |
| **8** | 3 | 0.8 | 35 | 2.6±0.16 | 1.8±0.04 | 1.98±0.03 | 2.55±0.03 |
| **9** | 3 | 0.6 | 30 | 3.05±0.11 | 1.56±0.03 | 1.69±0.02 | 2.22±0.03 |
| **10** | 3.5 | 0.6 | 35 | 4.3±0.14 | 1.2±0.02 | 1.32±0.07 | 1.72±0.04 |
| **11** | 2.5 | 0.6 | 35 | 2.7±0.15 | 2.94±0.06 | 3.06±0.06 | 3.98±0.13 |
| **12** | 2.5 | 0.8 | 30 | 2.4±0.11 | 2.51±0.07 | 2.73±0.09 | 3.55±0.11 |
| **13** | 3 | 0.6 | 30 | 3.09±0.12 | 1.7±0.07 | 1.87±0.02 | 2.33±0.08 |
| **14** | 3 | 0.6 | 30 | 3.16±0.12 | 1.78±0.02 | 1.92±0.08 | 2.49±0.08 |
| **15** | 3 | 0.6 | 30 | 3.12±0.12 | 1.84±0.03 | 1.96±0.05 | 2.54±0.09 |
| **16** | 3 | 0.4 | 35 | 3.2±0.13 | 2.1±0.05 | 2.31±0.09 | 3.0±0.11 |
| **17** | 2.5 | 0.6 | 25 | 2.5±0.12 | 2.26±0.05 | 2.39±0.04 | 3.11±0.12 |

***Note. The values are shown as mean ± standard deviation (n = 3). AbB stands for abemaciclib, IMP for impurity, and R2, R3, and R4 for resolution values determined according to USP recommendations (2 × (tR2 − tR1) / (w1 + w2), where tR is retention time and w is peak width at baseline).***

The adequacy of the fitted quadratic models was evaluated by ANOVA for all four chromatographic responses. The complete ANOVA outputs for R1, R2, R3, and R4 are presented in Tables 2a-2d, respectively. These tables include the model sum of squares, degrees of freedom, mean square, F-value, p-value, lack-of-fit analysis, and model-fit statistics, including $R^2$, adjusted $R^2$, predicted $R^2$, and adequate precision. The model was considered statistically significant when $p < 0.05$, while a non-significant lack-of-fit value was considered evidence of adequate model fit.

**Table 2a: ANOVA for the quadratic model of R1 (retention time of abemaciclib).**

| Source | SS | df | MS | F-value | p-value | Significance |
|---|---|---|---|---|---|---|
| Model | 6.6315 | 9 | 0.7368 | 312.22 | <0.0001 | *** |
| A (pH) | 5.1200 | 1 | 5.1200 | 2169.49 | <0.0001 | *** |
| B (Temperature) | 0.0450 | 1 | 0.0450 | 19.07 | 0.0033 | ** |
| C (Flow Rate) | 0.6050 | 1 | 0.6050 | 256.36 | <0.0001 | *** |
| AB | <0.0001 | 1 | <0.0001 | <0.01 | 0.9999 | ns |
| AC | 0.0100 | 1 | 0.0100 | 4.24 | 0.0785 | ns |
| BC | <0.0001 | 1 | <0.0001 | <0.01 | 0.9999 | ns |
| $A^2$ | 0.7534 | 1 | 0.7534 | 319.23 | <0.0001 | *** |
| $B^2$ | 0.0679 | 1 | 0.0679 | 28.78 | 0.0010 | ** |
| $C^2$ | 0.0679 | 1 | 0.0679 | 28.78 | 0.0010 | ** |
| Residual | 0.0165 | 7 | 0.0024 | - | - | |
| Lack of Fit | 0.0100 | 3 | 0.0033 | 2.04 | 0.2502 | ns (good) |
| Pure Error | 0.0065 | 4 | 0.0016 | - | - | |
| Total | 6.6480 | 16 | - | - | - | |

***Note. Model F = 312.22 (p < 0.0001, significant); R2 = 0.9975; Adj-R2 = 0.9943; Pred-R2 = 0.9744; S/N = 57.704; LOF p = 0.2502 (not significant). ** p < 0.01; * p < 0.05; ns = not significant. SS = sum of squares; df = degrees of freedom; MS = mean square.***

**Table 2b: ANOVA for the quadratic model of R2 (resolution between IMP-I and abemaciclib).**

| Source | SS | df | MS | F-value | p-value | Significance |
|---|---|---|---|---|---|---|
| Model | 4.9363 | 9 | 0.5485 | 48.34 | <0.0001 | *** |
| A (pH) | 4.0044 | 1 | 4.0044 | 352.90 | <0.0001 | *** |
| B (Temperature) | 0.1201 | 1 | 0.1201 | 10.58 | 0.0140 | * |
| C (Flow Rate) | 0.2965 | 1 | 0.2965 | 26.13 | 0.0014 | ** |
| AB | 0.1936 | 1 | 0.1936 | 17.06 | 0.0044 | ** |
| AC | 0.0289 | 1 | 0.0289 | 2.55 | 0.1545 | ns |
| BC | 0.0025 | 1 | 0.0025 | 0.22 | 0.6531 | ns |
| $A^2$ | 0.2257 | 1 | 0.2257 | 19.89 | 0.0029 | ** |
| $B^2$ | 0.0011 | 1 | 0.0011 | 0.10 | 0.7599 | ns |
| $C^2$ | 0.0478 | 1 | 0.0478 | 4.21 | 0.0794 | ns |
| Residual | 0.0794 | 7 | 0.0113 | - | - | |
| Lack of Fit | 0.0290 | 3 | 0.0097 | 0.76 | 0.5702 | ns (good) |
| Pure Error | 0.0505 | 4 | 0.0126 | - | - | |
| Total | 5.0158 | 16 | - | - | - | |

***Note. Model F = 48.34 (p < 0.0001, significant); R2 = 0.9842; Adj-R2 = 0.9638; Pred-R2 = 0.8919; S/N = 23.011; LOF p = 0.5702 (not significant). ** p < 0.01; * p < 0.05; ns = not significant. SS = sum of squares; df = degrees of freedom; MS = mean square.***

**Table 2c: ANOVA for the quadratic model of R3 (resolution between IMP-II and abemaciclib).**

| Source | SS | df | MS | F-value | p-value | Significance |
|---|---|---|---|---|---|---|
| Model | 5.0078 | 9 | 0.5564 | 49.57 | <0.0001 | *** |
| A (pH) | 4.0045 | 1 | 4.0045 | 356.77 | <0.0001 | *** |
| B (Temperature) | 0.1250 | 1 | 0.1250 | 11.14 | 0.0125 | * |
| C (Flow Rate) | 0.2665 | 1 | 0.2665 | 23.74 | 0.0018 | ** |
| AB | 0.1980 | 1 | 0.1980 | 17.64 | 0.0040 | ** |
| AC | 0.0462 | 1 | 0.0462 | 4.12 | 0.0820 | ns |
| BC | 0.0030 | 1 | 0.0030 | 0.27 | 0.6197 | ns |
| $A^2$ | 0.2310 | 1 | 0.2310 | 20.58 | 0.0027 | ** |
| $B^2$ | <0.0001 | 1 | <0.0001 | <0.01 | 0.9888 | ns |
| $C^2$ | 0.1136 | 1 | 0.1136 | 10.12 | 0.0155 | * |
| Residual | 0.0786 | 7 | 0.0112 | — | — | |
| Lack of Fit | 0.0308 | 3 | 0.0103 | 0.86 | 0.5298 | ns (good) |
| Pure Error | 0.0477 | 4 | 0.0119 | — | — | |
| Total | 5.0864 | 16 | — | — | — | |

***Note. Model F = 49.57 (p < 0.0001, significant); R2 = 0.9846; Adj-R2 = 0.9647; Pred-R2 = 0.8883; S/N = 24.213; LOF p = 0.5298 (not significant). ** p < 0.01; * p < 0.05; ns = not significant. SS = sum of squares; df = degrees of freedom; MS = mean square.***

**Table 2d: ANOVA for the quadratic model of R4 (resolution between IMP-III and IMP-IV).**

| Source | SS | df | MS | F-value | p-value | Significance |
|---|---|---|---|---|---|---|
| Model | 8.5254 | 9 | 0.9473 | 52.11 | <0.0001 | *** |
| A (pH) | 6.7712 | 1 | 6.7712 | 372.47 | <0.0001 | *** |
| B (Temperature) | 0.1953 | 1 | 0.1953 | 10.74 | 0.0135 | * |
| C (Flow Rate) | 0.4851 | 1 | 0.4851 | 26.68 | 0.0013 | ** |
| AB | 0.3422 | 1 | 0.3422 | 18.82 | 0.0034 | ** |
| AC | 0.0650 | 1 | 0.0650 | 3.58 | 0.1005 | ns |
| BC | 0.0036 | 1 | 0.0036 | 0.20 | 0.6698 | ns |
| $A^2$ | 0.4271 | 1 | 0.4271 | 23.50 | 0.0019 | ** |
| $B^2$ | 0.0005 | 1 | 0.0005 | 0.03 | 0.8718 | ns |
| $C^2$ | 0.1964 | 1 | 0.1964 | 10.81 | 0.0134 | * |
| Residual | 0.1273 | 7 | 0.0182 | — | — | |
| Lack of Fit | 0.0566 | 3 | 0.0189 | 1.07 | 0.4566 | ns (good) |
| Pure Error | 0.0707 | 4 | 0.0177 | — | — | |
| Total | 8.6526 | 16 | — | — | — | |

***Note. Model F = 52.11 (p < 0.0001, significant); R2 = 0.9853; Adj-R2 = 0.9664; Pred-R2 = 0.8826; S/N = 25.177; LOF p = 0.4566 (not significant). ** p < 0.01; * p < 0.05; ns = not significant. SS = sum of squares; df = degrees of freedom; MS = mean square.***

As shown in Table 2a, the quadratic model for R1, representing the retention time of AbB, was statistically significant, with a model F-value of 312.22 and $p < 0.0001$. The model showed excellent goodness of fit, with $R^2 = 0.9975$, adjusted $R^2 = 0.9943$, predicted $R^2 = 0.9744$, and adequate precision of 57.704. The lack-of-fit test was non-significant, confirming that the model adequately described the experimental design space for AbB retention time.

Among the investigated critical method parameters, the mobile-phase pH (A) had the most pronounced effect on R1, indicating that the ionization state of AbB strongly influenced its retention behavior. Based on the response surface plots (Figure 2), perturbation plots, and the regression equation (Equation 1), mobile-phase pH increased AbB retention time, whereas increasing flow rate reduced retention time by shortening the analyte's residence time in the column.

$$R1=3.104 + 0.8 * A + -0.275 * B + 0.075 * C + -0.05 * AB + 1.5156e\text{-}18 * AC + -1.77855e\text{-}17 * BC + 0.423 * A^2 + -0.127 * B^2 + -0.127 * C^2 \quad \text{Equation 1}$$

As shown in Table 2b, the quadratic model for R2, representing the resolution between IMP-I and AbB, was statistically significant, with a model F-value of 48.34 and $p < 0.0001$. The model demonstrated satisfactory goodness of fit, with $R^2 = 0.9842$, adjusted $R^2 = 0.9638$, predicted $R^2 = 0.8919$, and adequate precision of 23.011. The non-significant lack-of-fit value indicated that the quadratic model was suitable for predicting this critical resolution response. Among the investigated variables, mobile-phase pH (A) had the strongest influence on R2, confirming that pH-dependent ionization and analyte–stationary-phase interactions played a major role in controlling the separation between IMP-I and AbB. The analysis of perturbation plots, response surface plots (Figure 2), and the regression equation (Equation 2) indicated that mobile-phase pH (A) and flow rate (C) negatively affected R2, whereas column temperature (B) had a positive effect within the studied design range. The significant AC interaction further indicated that the combined influence of mobile-phase pH and flow rate was important in controlling the resolution between IMP-I and AbB.

$$R2=1.732 + -0.7075 * A + -0.1925 * B + 0.1225 * C + 0.085 * AB + -0.22 * AC + 0.025 * BC + 0.2165 * A^2 + 0.0915 * B^2 + 0.0015 * C^2 \quad \text{Equation 2}$$

As shown in Table 2c, the quadratic model for R3, representing the resolution between IMP-II and AbB, was statistically significant, with a model F-value of 49.57 and $p < 0.0001$. The model showed satisfactory goodness of fit, with $R^2 = 0.9846$, adjusted $R^2 = 0.9647$, predicted $R^2 = 0.8883$, and adequate precision of 24.213. The non-significant lack-of-fit test supported the model's adequacy in predicting the IMP-II/AbB resolution response. Among the investigated variables, mobile-phase pH (A) had the strongest influence on R3, confirming that pH-dependent ionization and analyte–stationary-phase interactions played a major role in controlling the separation between IMP-II and AbB. The response surface plots, perturbation plots (Figure 2), and regression equation (Equation 3) further indicated that the combined influence of mobile phase

pH and flow rate/temperature was important for this response, as reflected by the significant interaction term in the ANOVA model.

$$R3=1.872 + -0.7075 * A + -0.1825 * B + 0.125 * C + 0.1075 * AB + -0.2225 * AC + 0.0275 * BC + 0.22025 * A^2 + 0.15025 * B^2 + -0.01475 * C^2 \text{……..Equation 3}$$

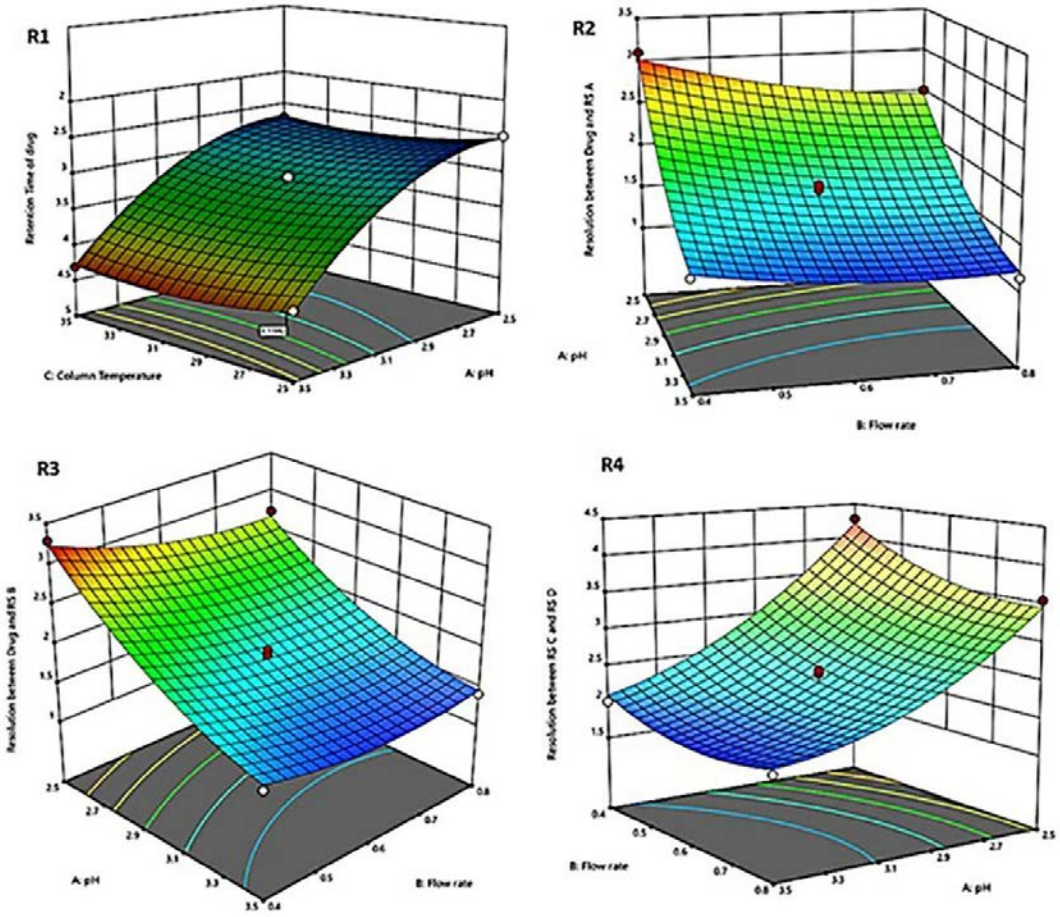


**Figure 2: 3D Response surface plots for method responses R1, R2, R3, and R4. Response surface graphs in three dimensions that show how important technique parameters interact with chromatographic responses. (A) Effects of column temperature (B) and mobile phase pH (A) on AbB retention time (R1). (B) Effects of mobile phase pH (A) and flow rate (C) on the resolution between IMP-I and AbB (R2). (C) The effect of flow rate and pH on the resolution between AbB and IMP-II (R3). (D) Effects of mobile phase pH (A) and flow rate (C) on the resolution between IMP-III and IMP-IV (R4). For panels A and B, the column temperature for panels C and D was set to 30 °C, the flow rate to 0.6 mL/min, and the pH for panel D to 3.0 (as shown).**

As shown in Table 2d, the quadratic model for R4, representing the resolution between IMP-III and IMP-IV, was statistically significant, with a model F-value of 52.11 and $p < 0.0001$. The model showed satisfactory goodness of fit, with $R^2 = 0.9853$, adjusted $R^2 = 0.9664$, predicted $R^2 = 0.8826$, and adequate precision of 25.177. The non-significant lack-of-fit value confirmed that the quadratic model adequately described the response behavior within the studied design space. Among the investigated variables, mobile-phase pH (A) had the strongest influence on R4, indicating that pH-dependent ionization and selectivity were critical for resolving IMP-III and IMP-IV. The response surface plots, perturbation plots (Figure 2), and regression equation (Equation 4) further supported the influence of the selected critical method parameters and their interaction effects on this resolution response.

$$R4=2.414 + -0.92 * A + -0.24625 * B + 0.15625 * C + 0.1275 * AB + -0.2925 * AC + 0.03 * BC + 0.3005 * A^2 + 0.198 * B^2 + -0.007 * C^2 \text{…….Equation 4}$$

Figure 3 demonstrates the overlay plot used for design optimization and MODR identification.

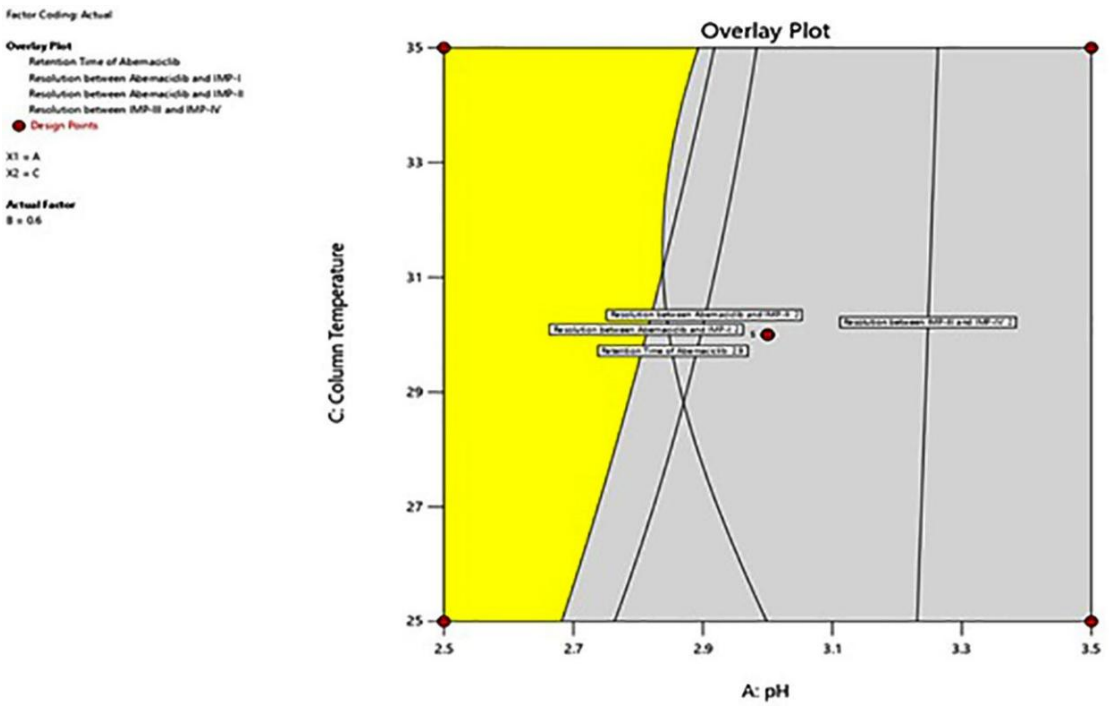


**Figure 3: Overlay plot showing the method operable design region (MODR) for the optimized RP-UHPLC method. The yellow-shaded region represents the design space in which the ATP criteria were satisfied, including acceptable AbB retention time and baseline resolution of the critical peak pairs R2, R3, and R4. The red point indicates the selected optimized condition: mobile phase pH 2.7, flow rate 0.6 mL/min, and column temperature 30 °C. The overall desirability value was 0.698, indicating a balanced compromise among resolution, retention time, and robustness.**

The MODR was established from the overlay plot by applying the ATP acceptance criteria to all model-predicted responses. The acceptance criteria included a short, reproducible AbB retention time and baseline resolution for the critical peak pairs: IMP-I/AbB, IMP-II/AbB, and IMP-III/IMP-IV. Based on the model prediction and overlay plot constraints, the acceptable working region was identified as mobile-phase pH 2.55-2.70, flow rate 0.50-0.65 mL/min, and column temperature 28-32 °C. Within this region, the predicted AbB retention time remained within the desired short-run window, and all critical resolution values met the baseline-separation criterion of Rs > 2.0. The final optimized condition of mobile phase pH 2.7, flow rate 0.6 mL/min, and column temperature 30 °C was selected because it lies within the MODR and provides a balanced compromise between resolution, retention time, robustness, and routine applicability.

The overall desirability value of 0.698 indicates that the optimized condition represents a balanced compromise among multiple chromatographic responses rather than maximizing a single response. In this method, the resolution responses R2, R3, and R4 were given primary importance because the method was intended for stability-indicating impurity profiling, where inadequate separation of AbB from its related impurities or degradation products would compromise specificity and quantitative reliability. Minimization of AbB retention time was considered important for rapid analysis; however, it was not prioritized at the expense of baseline resolution. Although higher predicted resolution was observed in the lower pH region, the final optimized condition was selected because it maintained all critical peak-pair resolutions above Rs >2.0 while preserving a short run time and practical routine operating conditions. Therefore, the desirability value of 0.698 reflects an intentional trade-off between resolution, retention time, and method robustness.

**Validation**

Validation entails several tests to provide a documented demonstration, with a high level of assurance, that an analytical method is appropriate for its intended use. The developed method was validated in compliance with ICH guidelines. Six replicate injections of a standard AbB solution at a concentration of 100 µg/mL (100% specification level) were performed to assess system suitability. The tailing factor, peak area,

theoretical plate count, and percent relative standard deviation (%RSD) were identified as important characteristics. The calculated mean retention time of the drug was 2.89 minutes, with a %RSD of 0.70 for the six replicate doses. All assessed parameters lay within the allowable limits of system suitability. In particular, a tailing factor of less than 1.1 and a theoretical plate count greater than 6000 are system suitability characteristics for the traditional method.

The ability to accurately evaluate the target analyte reaction in analytical methodology even in the presence of other comparable chemicals that are anticipated to be present is known as specificity. Standard and blank samples, two distinct AbB test samples, related chemicals, related substances, spiked samples, and forced degradation samples were injected to assess the procedure's specificity. The specificity results indicate that AbB does not interfere with any known related impurities. To verify the specificity and stability-indicating capability of the established UHPLC method, chromatograms were obtained from forced-degradation samples. To verify the specificity and stability-indicating capability of the developed UHPLC method, chromatograms were obtained for blank, standard, impurity-spiked, and forced-degradation samples. The optimized UHPLC chromatogram shown in Figure 4 confirms baseline separation of AbB from IMP-I, IMP-II, IMP-III, and IMP-IV under the final chromatographic conditions. Under the optimized isocratic condition, all analytes were resolved within the analytical run, with IMP-IV, AbB, IMP-I, IMP-II, and IMP-III eluting at approximately 2.212, 2.896, 3.346, 3.951, and 4.256 min, respectively. The narrow elution window, controlled acidic mobile phase, and high-efficiency 1.7 µm C18 column collectively enabled baseline separation of all critical peak pairs while retaining an 8-minute total run time. The representative optimized chromatogram is shown in Figure 4.

Forced degradation behavior was evaluated separately under oxidative, acidic, alkaline, neutral, photolytic, UV, and thermal stress conditions, and the degradation results, unknown degradation products, mass balance, and peak purity data are summarized in Tables 3–5. The goal of these studies was to verify the method's ability to identify and characterize degradation products, thereby enabling evaluation of its viability for stability testing. Both the test solution of the medication product (100 µg/mL) and the reference solution of the active pharmaceutical ingredient (100 µg/mL) were subjected to oxidation, including temperature changes, light exposure, hydrolysis (acidic, alkaline, and balanced), and other stressors. All stressed samples were filtered using a 0.22 µm syringe filter before the analysis. The filtered, pressurized samples were then loaded into the UHPLC apparatus.

Under heat stress, visible light, UV radiation, and water hydrolysis, no discernible degradation was observed. Nonetheless, significant deterioration was noted in the presence of oxidation, alkaline hydrolysis, and acid hydrolysis. Surprisingly, each degradation peak was clearly distinguished from the peaks of the medicinal compound. The specificity of the established UHPLC technique for the analysis of chemicals, as demonstrated by the results collected from the degradation studies, demonstrated AbB. The assay for every stressed sample was computed as part of the mass balance analysis. Over 99% of the assay findings were obtained. For eight hours at room temperature, a standard solution of the pharmaceutical substance (100 µg/mL) and the drug sample solutions were treated with 10% hydrogen peroxide as part of the oxidative degradation investigation. In drug substances and pharmaceutical solutions, two unknown degradation products (DP-I and DP-II) were identified by UV/PDA spectral comparison and relative retention times; no MS characterization was performed, and structural identities were therefore not assigned. To achieve hydrolytic disintegration of the drug substance (100 µg/mL) and to test the solution containing the drug product (100 µg/mL), the drug specimen was dissolved in 1N HCl at 80 °C for eight hours. The UHPLC method was used to analyze the stressed samples after extraction with 1 M NaOH.

Two unknown degradation products (DP-III and DP-IV), accounting for approximately 3.21% total degradation, were generated in both solutions. To perform an alkaline hydrolytic breakdown of the drug substance (100 µg/mL) and to test the solution containing the drug product (100 µg/mL), the drug substance was treated with 1 M NaOH at 80 °C for eight hours. Using 1 N HCl, the stressed samples were removed and subsequently analyzed by UHPLC. One major unknown degradant (DP-V) was produced. The relative retention times (RRT) of all five degradation products (DP-I through DP-V), along with their UV/PDA spectral maxima and observed percentage degradation under each stress condition, are summarised in Table 3. The results demonstrate that the drug is durable when treated with water under neutral conditions.

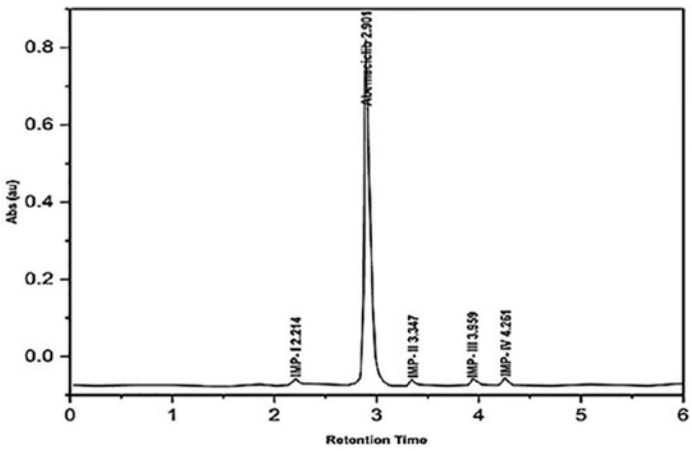


**Figure 4: An illustration of a final validation experiment's optimized UHPLC chromatogram (mobile phase: ammonium formate buffer pH 2.7: acetonitrile = 40:60 v/v; flow rate 0.6 mL/min; column temperature 30 °C; detection wavelength 210 nm; injection volume 4 µL). The peaks are IMP-IV (2.212 min), AbB (2.896 min), IMP-I (3.346 min), IMP-II (3.951 min), and IMP-III (4.256 min). Baseline separation is shown by all resolutions being greater than 2.0.**

**Table 3: Unknown degradation products identified under forced degradation conditions, with relative retention time (RRT), approximate retention time (RT, min), UV spectral maxima, and estimated percentage degradation.**

| Degradant | Stress Condition | RT (min) | RRT | Degradation (%) | UV max (nm) |
|---|---|---|---|---|---|
| DP-I | Oxidative (H2O2, 10%, 8 h RT) | 1.82 | 0.63 | ~1.9 | 220, 248 |
| DP-II | Oxidative (H2O2, 10%, 8 h RT) | 4.15 | 1.43 | ~1.5 | 215, 262 |
| DP-III | Acid (1N HCl, 80 C, 8 h) | 1.48 | 0.51 | ~1.7 | 218, 245 |
| DP-IV | Acid (1N HCl, 80 C, 8 h) | 4.87 | 1.68 | ~1.6 | 222, 258 |
| DP-V | Alkaline (1M NaOH, 80 C, 8 h) | 5.34 | 1.84 | ~4.1 | 225, 271 |

***Note. RRT = RT(degradant)/RT(AbB); RT(AbB) = 2.896 min. Structural identities not assigned (no MS characterization performed). Degradation (%) estimated from peak-area ratio to total area.***

The standard AbB medicinal solution was subjected to 1.2 million lux-hours of visible light and 200 W-h/m2 of UV radiation for seven days. When the drug sample was exposed to photolytic degradation, no discernible deterioration was seen. The drug dried form (20 mg) was heated to 80 °C for 24 hours. There was no detectable heat degradation in AbB. Peak purity was evaluated for the AbB main peak in all stressed samples using the PDA detector: the purity angle (spectral angle across the peak width) was below the purity threshold for every stressed sample, confirming spectral homogeneity of the AbB peak and ruling out co-elution with any degradant (Table 4). A summary of the degradation products with their relative retention times (RRT), approximate retention times, UV spectral maxima, and % degradation under each stress condition is provided in Table 3. All forced degradation results are tabulated in Table 5.

**Table 4: Peak purity assessment of the abemaciclib main peak under forced degradation conditions by PDA spectral correlation (purity angle vs. threshold).**

| Stress Condition | Purity Angle (°) | Purity Threshold (°) | Pass/Fail | Assay (%) | Degradation (%) | Inference |
|---|---|---|---|---|---|---|
| Control (untreated) | 0.12 | 0.85 | Pass | 99.58 | — | Peak homogeneous |
| Oxidation ($H_2O_2$) | 0.38 | 0.89 | Pass | 94.78 | 3.43 | AbB pure; DPs resolved. |
| Acid hydrolysis (1N HCl) | 0.29 | 0.91 | Pass | 96.76 | 3.28 | AbB pure; DPs resolved |
| Neutral hydrolysis ($H_2O$) | 0.09 | 0.84 | Pass | 99.13 | ND | No co-elution |
| Alkaline hydrolysis (1N NaOH) | 0.44 | 0.93 | Pass | 90.12 | 4.13 | AbB pure; DPs resolved |
| Photolytic (VIS + UV) | 0.11 | 0.86 | Pass | 98.89 | ND | No co-elution |
| Thermal (80 °C, 24 h) | 0.10 | 0.85 | Pass | 99.02 | ND | No co-elution |

***Note. A purity angle < purity threshold in all cases confirms the spectral homogeneity of the AbB peak and rules out co-elution with degradants.***

**Table 5: Abemaciclib drug substance and drug product forced degradation study results under various stress settings.**

| S.No | Category | Degradation | Assay (%) | Degradation (%) | Mass balance (%) |
|---|---|---|---|---|---|
| **1** | Drug substance | Control (untreated) | 99.58±6.24 | - | |
| | Drug product | | 99.34±8.24 | - | |
| **2** | Drug substance | Oxidation | 94.78±5.88 | 3.43±0.41 | 99.62±8.51 |
| | Drug product | | 95.21±5.76 | 3.32±0.39 | 100.19±8.45 |
| **3** | Drug substance | Acid hydrolysis | 96.76±6.17 | 3.28±0.37 | 100.15±7.11 |
| | Drug product | | 97.08±6.68 | 3.14±0.41 | 100.52±7.71 |
| **4** | Drug substance | Neutral (water) | 99.13±7.81 | No significant degradation | 99.54±6.78 |
| | Drug product | | 98.94±6.52 | | 99.59±8.28 |
| **5** | Drug substance | Alkaline hydrolysis | 90.12±5.67 | 4.13±0.65 | 99.66±6.93 |
| | Drug product | | 90.96±6.45 | 4.34±0.81 | 99.95±7.18 |
| **6** | Drug substance | Photolytic degradation | 98.89±7.91 | No significant degradation | 99.30±5.44 |
| | Drug product | | 99.07±8.21 | | 99.72±7.29 |
| **7** | Drug substance | UV degradation | 99.12±8.24 | No significant degradation | 99.53±7.85 |
| | Drug product | | 98.76±8.33 | | 99.41±9.21 |
| **8** | Drug substance | Thermal degradation | 99.02±6.24 | No significant degradation | 99.43±8.24 |
| | Drug product | | 99.15±8.63 | | 99.80±8.71 |

***Note. Mass balance (%), assay (%), and degradation (%) are shown as mean ± standard deviation (n = 3). DP stands for degradation product; AbB stands for abemaciclib. Stress conditions include: oxidation (10% $H2O_2$, 8 hours, room temperature); acid hydrolysis (1N HCl, 80 °C, 8 hours); neutral hydrolysis (water, 80 °C, 8 hours); alkaline hydrolysis (1N NaOH, 80 °C, 8 hours); photolytic degradation (1.2 million lux hours visible light + 200 W·h/m² UV); and thermal deterioration (80 °C, 24 hours). "No significant degradation" denotes degradation products that are less than the limit of quantification (0.02 µg/mL).***

The proposed UHPLC technique, encompassing AbB and related impurities, was tested for linearity using 10 different doses of the related substances spiked into the AbB standard solution. Related chemicals had concentrations between 0.04 and 0.2 µg/mL, whereas AbB concentrations ranged from 0.2 to 150 µg/mL. The results of the linearity study are shown in Table 6. The optimized UHPLC method demonstrated an acceptable correlation between the analyte and IMP concentrations and the UHPLC-UV detector response, as indicated by the linearity test results (Table 6). For AbB, IMP-I, IMP-II, IMP-III, and IMP-IV, the observed correlation coefficient values are 0.9998, 0.9998, 0.9967, and 0.9994, respectively. The relative response factor (RRF) values for the relevant chemicals were calculated from the slopes of the calibration curves. LOD and LOQ were determined using the signal-to-noise approach. The LOD was defined as the lowest concentration producing an S/N ratio of approximately 3:1, and the LOQ was defined as the lowest concentration producing an S/N ratio of approximately 10:1 with acceptable replicate precision. The LOD and LOQ for AbB were 0.01 µg/mL and 0.02 µg/mL, respectively. The LOQ level was verified by replicate injections to confirm acceptable response and precision. For related impurities, the LOQ was confirmed at the lowest validated impurity level that produced an S/N ratio of approximately 10:1 with acceptable precision. Six samples of the standard drug solution (100% specification level) and six samples of the test drug product (100% specification level) were injected to evaluate the assay method's accuracy. The AbB test samples assay percentage RSD fell within the permitted range of less than 2%. Six replicate samples of distinct related compounds were injected to evaluate the relative substance method accuracy. The RRF value was used to determine the percentage for each IMP assay (Table 6). The impurity assay percentage RSD falls within the permissible range of less than 10%. For three consecutive days, the same concentration range was used to calculate the intra-day and inter-day precision. The acceptable range is covered by the intermediate precision percentage RSD results.

The accuracy of the developed RP-UHPLC method was evaluated using recovery studies at three validation levels. For AbB, recovery was assessed at 50%, 100%, and 150% levels, whereas the related impurities were assessed at LOQ, 100%, and 150% of the specification level. Each level was analyzed in triplicate, and impurity recoveries were calculated using the respective relative response factor (RRF) values. The mean recovery ranged from 99.36% to 102.88% for AbB, 96.57% to 101.41% for IMP-I, 95.58% to 100.53% for IMP-II, 92.76% to 99.66% for IMP-III, and 94.55% to 101.17% for IMP-IV. Across all analytes and validation levels, the overall mean recovery ranged from 92.76% to 102.88%, with individual replicate

recoveries ranging from 92.27% to 103.24%. The %RSD values were low and within the predefined acceptance limits, confirming the method's accuracy for AbB and its related impurities. The per-analyte and per-level recovery results are presented in Table 7.

**Table 6: There is linearity data for abemaciclib (AbB) and its related impurities, IMP-I, IMP-II, IMP-III, and IMP-IV.**

| S.No. | Linearity level of AbB | Peak area | Linearity level of Impurities | Peak area | | | |
|---|---|---|---|---|---|---|---|
| | (ppm) | | (ppm) | I | II | III | IV |
| **1** | 0.02 (LOQ) | 2185.34 | | | | | |
| **2** | 0.05 | 4253.76 | | | | | |
| **3** | 0.5 | 20672.34 | | | | | |
| **4** | 1 | 41789.57 | | | | | |
| **5** | 2 | 209874.65 | | | | | |
| **6** | 5 | 421758.78 | | | | | |
| **7** | 10 | 1065432.87 | 0.02(LOQ) | 1865.43 | 1402.24 | 1924.73 | 1567.87 |
| **8** | 50 | 2167543.43 | 0.03 | 3612.32 | 2812.65 | 3886.46 | 3178.82 |
| **9** | 70 | 4231256.76 | 0.06 | 7082.54 | 5576.87 | 7686.54 | 6234.54 |
| **10** | 100 | 6235873.32 | 0.09 | 10363.42 | 8034.78 | 11212.54 | 8998.89 |
| **11** | 150 | 8376542.17 | 0.12 | 13768.73 | 10234.89 | 14786.93 | 11876.65 |
| **RRF** | | | | 1.08 | 0.80 | 1.16 | 0.93 |
| **$R^2$** | | 0.999 | | 0.9998 | 0.9967 | 0.9994 | 0.9992 |

***Note. AbB 0.02–150 µg/mL and impurities 0.02–0.12 µg/mL (except the LOQ threshold at 0.02 µg/mL) are the concentration ranges. Mean values (n = 3) represent peak areas. The relative response factor, or RRF, is calculated by dividing the slope of the impurity calibration curve by the slope of the AbB calibration curve. LOQ stands for limit of quantification (signal-to-noise ratio > 10). R2 is the coefficient of determination.***

Solution stability was evaluated using the AbB standard stock solution, a diluted standard solution, a test sample solution, and an impurity-spiked sample solution. The solutions were stored in tightly capped volumetric flasks at room temperature & analysed at 6hr intervals. AbB assay and related-substance responses remained consistent throughout the study period, with %RSD values within the predefined acceptance limits. Robustness was evaluated by making deliberate minor variations in critical chromatographic parameters, including mobile-phase composition, detection wavelength & flow rate. These changes did not produce any meaningful effect on retention time, peak shape, assay response, or resolution between AbB and its related impurities, confirming the robustness of the developed method.

**Table 7: Accuracy (percent recovery +/- %RSD) for abemaciclib and related impurities at LOQ, 100%, and 150% spiking levels (*n* = 3).**

| Analyte | Level (%) | Spiked (µg/mL) | Recovery Replicate 1 (%) | Recovery Replicate 2 (%) | Recovery Replicate 3 (%) | Mean Recovery (%) | SD | %RSD |
|---|---|---|---|---|---|---|---|---|
| AbB | 50 | 50 | 99.12 | 98.75 | 100.21 | 99.36 | 0.74 | 0.74 |
| AbB | 100 | 100 | 101.23 | 100.84 | 101.58 | 101.22 | 0.37 | 0.37 |
| AbB | 150 | 150 | 102.48 | 103.24 | 102.91 | 102.88 | 0.38 | 0.37 |
| IMP-I | LOQ | 0.02 | 96.58 | 97.24 | 95.89 | 96.57 | 0.68 | 0.70 |
| IMP-I | 100 | 0.10 | 98.42 | 99.15 | 97.88 | 98.48 | 0.64 | 0.65 |
| IMP-I | 150 | 0.15 | 101.25 | 100.87 | 102.11 | 101.41 | 0.63 | 0.62 |
| IMP-II | LOQ | 0.02 | 95.74 | 94.89 | 96.12 | 95.58 | 0.62 | 0.65 |
| IMP-II | 100 | 0.10 | 97.65 | 98.21 | 97.44 | 97.77 | 0.40 | 0.41 |
| IMP-II | 150 | 0.15 | 100.48 | 101.23 | 99.87 | 100.53 | 0.68 | 0.68 |
| IMP-III | LOQ | 0.02 | 92.27 | 93.14 | 92.88 | 92.76 | 0.44 | 0.47 |
| IMP-III | 100 | 0.10 | 96.32 | 95.87 | 97.21 | 96.47 | 0.69 | 0.71 |
| IMP-III | 150 | 0.15 | 99.78 | 100.24 | 98.97 | 99.66 | 0.65 | 0.65 |
| IMP-IV | LOQ | 0.02 | 94.56 | 95.23 | 93.87 | 94.55 | 0.68 | 0.72 |
| IMP-IV | 100 | 0.10 | 97.89 | 98.34 | 97.56 | 97.93 | 0.40 | 0.41 |
| IMP-IV | 150 | 0.15 | 100.75 | 101.54 | 101.21 | 101.17 | 0.40 | 0.39 |

***Note. Recovery values are expressed as individual replicate recoveries and mean recovery ± SD from triplicate determinations. The overall mean recovery ranged from 92.76% to 102.88% across AbB and its related impurities. The lower mean recovery observed for IMP-III at the LOQ level (92.76%) and IMP-IV at the LOQ level (94.55%) remained within the accepted range for trace-level impurity recovery. %RSD values were <2% for AbB and <10% for all related impurities.***

***DISCUSSION***

Drug impurity profiling is a crucial component of medicinal quality assurance that ensures the effectiveness and safety of pharmaceutical products. According to Dhangar et al. (2017), the main goal of this work was to create an exceptionally sensitive, specific, precise, and robust RP-UHPLC approach for the simultaneous detection of AbB, its process-associated impurities, and product degradation at trace levels. This work employs state-of-the-art experimental design approaches to optimize chromatographic performance, thereby improving resolution and reproducibility compared to standard methods [19].

Chromatographic conditions were designed with AbB's properties in mind. Statistical design of experiments (DoE) optimized peak shape, retention time, and resolution between key impurity peaks to improve precision. To separate AbB and its impurities, the mobile-phase pH (A), column temperature (B), and flow rate (C) were optimized using the BBD [20]. The robustness of the method is illustrated by the overlay plot in Figure 3, which identifies an MODR that satisfies the ATP criteria. The desirability value of 0.698 indicates that the optimized condition was selected as a balanced compromise, with critical resolution responses prioritized over simple retention-time minimization to preserve the stability-indicating and impurity-profiling capability of the method.

Confirmation studies, in which sample solutions were injected under ideal conditions and yielded chromatographic findings consistent with statistical predictions, were used to evaluate the validity of the optimization procedure recently reported by Gandu and co-workers (2025) [21]. AbB and its related compounds were effectively measured using the devised approach, which produced distinct, well-separated peaks. With all peaks resolving greater than 2, specificity and reliability were guaranteed [22]. The mean retention time for AbB was 2.896 min, whereas those for the associated impurities were 2.212, 3.346, 3.951, and 4.256 min.

The new UHPLC method has implications for pharmaceutical research and industry beyond API analysis, particularly in QC labs, stability studies, and clinical batch testing. It serves as a stability-indicating tool for tracking impurities and degradation, as required by ICH guidelines for rigorous stability assessment [10]. This technique makes it easier to meet regulatory standards for medication stability and shelf-life by enabling precise measurement of AbB and its related impurities [7]. In pharmaceutical manufacturing, clinical batch testing requires highly sensitive analytical techniques to detect impurities at the trace level. Because of its low LOQ (0.02 ± 0.00 µg/mL) and LOD (0.01 ± 0.00 µg/mL), the developed technique ensures compliance with stringent regulatory criteria by precisely detecting impurities that may arise during synthesis and storage [8]. Additionally, its 8-min run time improves laboratory productivity, making it a valuable tool for commercial production and high-throughput batch release testing [9,14]. To ensure product consistency across multiple manufacturing cycles, regulatory bodies emphasize that thorough impurity profiling is a crucial component of routine batch testing [15].

Because of its robustness and reproducibility, the approach is also appropriate for routine QC analysis of AbB in bulk medications and formulated goods. Unlike traditional techniques that require multiple analytical runs for different impurities, this method measures multiple impurities simultaneously and with high resolution, saving time and resources [11,12]. It aligns with trends for rapid, sustainable analytics, offering a practical, economical option for pharmaceutical QC workflows [15]. This study significantly advances the field of pharmaceutical analytical chemistry by filling important gaps in the literature. Most notably, it is the first time that Box-Behnken Design (BBD) has been used to optimize a UHPLC technique specifically for AbB impurity profiling. By carefully adjusting column temperature, flow rate, and mobile phase composition, BBD improves chromatographic efficiency and resolution while enhancing procedural accuracy and robustness [16,17].

Building on this, the method developed in this work enables the simultaneous assessment of process-relevant impurities and degradation products in a single analytical run. This advancement reduces analysis time and improves sensitivity compared to prior HPLC and LC-MS/MS methods, which often address either the drug substance alone or only a subset of related impurities [9]. This method outperforms current methods in sensitivity by obtaining much lower LOQ (0.02 ± 0.00 µg/mL) and LOD (0.01 ± 0.00 µg/mL) values, enabling trace-level impurity detection [9,14].

Additionally, the study confirms the method's regulatory appropriateness by validating its specificity, precision, accuracy, and robustness in accordance with ICH Q2(R1) requirements [17]. This method's improved sensitivity, high resolution, and

methodical improvements make it a useful tool for pharmaceutical impurity profiling, applicable in both commercial and research settings. A side-by-side comparison with previously reported AbB analytical methods is provided in Table 8. Compared with earlier RP-HPLC-UV methods developed for assay or related-substance analysis, the present RP-UHPLC method provides lower LOD/LOQ values, a shorter 8-min isocratic run time, simultaneous profiling of AbB and four process-related impurities, and QbD/BBD-based optimization. Although some LC-MS/MS bioanalytical methods report lower plasma LOD/LOQ values, these methods are designed for biological matrices and pharmacokinetic applications. They are not directly comparable with pharmaceutical API/formulation impurity profiling methods. Therefore, the major novelty of the present method lies in its stability-indicating, QbD-optimized, rapid RP-UHPLC platform for AbB impurity profiling.

**Table 8: Comparison of the present RP-UHPLC method with previously reported methods for Abemaciclib analysis.**

| Method/ Detector | Application / Matrix | Column | Mobile phase / Elution | LOD µg/mL | LOQ µg/mL | Analytes covered | Run time | Validation / Optimization | Key distinction | Ref. |
|---|---|---|---|---|---|---|---|---|---|---|
| RP-HPLC-UV | Bulk drug substance & pharmaceutical dosage forms | Ascentis C18, 250 × 4.6 mm, 5 µm | 0.5% TFA: Methanol: Acetonitrile (35:45:20, v/v/v), isocratic | 0.60 | 1.80 | Abemaciclib | 3.72 min | ICH Q2(R1) validation | Simple assay method for routine QC | 23 |
| RP-HPLC-UV | Tablet dosage form | Inertsil ODS C18, 250 × 4.6 mm, 5 µm | 25 mM dipotassium hydrogen phosphate + 0.1% TEA (pH 2.5): Acetonitrile (60:40, v/v), isocratic | **1.14 µg/mL** | **3.47 µg/mL** | Abemaciclib | **5.60 min** | ICH Q2(R1) | QC method for tablet assay | 24 |
| UHPLC-MS/MS | Human & mouse plasma | Kinetex C18, 150 × 2.1 mm, 2.6 µm | 10 mM ammonium bicarbonate (A) / methanol-water (9:1) with gradient | 0.2 ng/mL* | 0.2 ng/mL (LLOQ) | Abemaciclib + M2 + M20 + M18 | ~8 min | FDA/EMA bioanalytical validation | Simultaneous determination of parent drug and active metabolites | 25 |
| RP-UHPLC-PDA/UV | API and pharmaceutical formulation; stability-indicating impurity method | Kinetex® LC C18, 100 × 2.1 mm, 1.7 µm | 10 mM ammonium formate buffer, pH 2.7: ACN, 40:60 v/v; isocratic | 0.01 | 0.02 | AbB + IMP-I, IMP-II, IMP-III, IMP-IV; unknown DPs resolved during forced degradation | 8 min | ICH Q2(R1); QbD/BBD | Simultaneous impurity profiling, stability-indicating capability, short isocratic run, and defined MODR | Present study |

***Note. LOD/LOQ values for plasma-based LC-MS/MS methods are not directly comparable with pharmaceutical API/formulation impurity methods because they are developed for bioanalytical matrices and validated under ICH M10 rather than ICH Q2(R1). ACN = acetonitrile; AbB = Abemaciclib; IMP = process-related impurity; DP = degradation product; QbD = Quality by Design; BBD = Box-Behnken Design; MODR = method operable design region.***

## *CONCLUSION*

This study successfully developed and validated a stability-indicating RP-UHPLC method for the simultaneous determination of AbB and four process-related impurities, IMP-I, IMP-II, IMP-III, and IMP-IV, in the API and pharmaceutical formulation. The application of a Box-Behnken Design within a QbD framework enabled systematic optimization of critical method parameters, namely mobile-phase pH, flow rate, and

column temperature. It established the optimized chromatographic conditions of mobile phase pH 2.7, flow rate 0.6 mL/min, and column temperature 30 °C. This QbD-guided optimization produced measurable analytical benefits, including baseline separation of all critical impurity pairs with resolution values greater than 2.0, a short total run time of 8 min, and reproducible elution of IMP-IV, AbB, IMP-I, IMP-II, and IMP-III at approximately 2.212, 2.896, 3.346, 3.951, and 4.256 min, respectively.

The optimized method demonstrated high sensitivity, with AbB LOD and LOQ values of 0.01 µg/mL and 0.02 µg/mL, respectively. The method also demonstrated acceptable validation performance in accordance with ICH Q2(R1), including good linearity, precision within accepted limits, and recovery between 92.27% and 103.24%. Forced degradation studies under acidic, alkaline, and oxidative conditions confirmed the stability-indicating capability of the method, as degradation peaks were adequately separated from the AbB peak and mass balance remained above 99% across the evaluated stress conditions. Therefore, the QbD-based RP-UHPLC method provides a rapid, sensitive, reproducible, and stability-indicating platform for routine quality-control analysis, stability testing, and impurity profiling of AbB in pharmaceutical settings.

However, the study has certain limitations. The degradation products were assigned solely based on chromatographic retention behavior and PDA response; therefore, structural confirmation of unknown degradants would require hyphenated techniques such as UHPLC-MS/MS. In addition, the method was validated using a single UHPLC system and column type; future inter-laboratory validation using different instrument platforms and column batches would further strengthen method transferability and regulatory confidence.

***FINANCIAL ASSISTANCE***
NIL

***CONFLICT OF INTEREST***
The authors declare no conflict of interest.

***AUTHOR CONTRIBUTION***
Kalpana Krishnaraju conceived and designed research, conducted experiments, and wrote the manuscript. Malarkodi Velraj analyzed data and reviewed the manuscript. All authors read and approved the manuscript.

***DECLARATION OF USE OF AI TOOLS***
The authors used Grammarly to assist with language editing and improving readability. All content, analysis, and conclusions were reviewed and verified by the authors. The authors take full responsibility for the originality, accuracy, and integrity of the work.

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