

# Analytical Quality by Design-Based RP-HPLC Method for the Quantification and Stability Assessment of Nilotinib in Bulk and Nanoformulations

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## ABSTRACT

**Background:** Nilotinib, a tyrosine kinase inhibitor of the second generation, is susceptible to stress-induced degradation that could undermine therapeutic effectiveness. **Materials and Methods:** The accelerated stability studies have been performed on both bulk Nilotinib as well as its nanoformulation in an acidic, basic, oxidative, thermal, and photolytic environment following ICH-recommended guidelines. The developed RP-HPLC method showed efficient separation of Nilotinib from its degraded products and thus is proven to be an appropriate stability-indicating analytical method for the analysis of Nilotinib and its nanoformulation. Supporting Fourier-Transform Infrared Spectroscopy (FTIR) analysis offered molecular-level proof of these changes. **Results:** Degradation processes included amide hydrolysis, dealkylation, oxidative N-oxide formation, thermal rearrangement, and photolytic radical-mediated cleavage. In all conditions of stress, the nanoformulation uniformly showed diminished degradation than the bulk drug, which emphasizes the shielding effect of the carrier matrix. **Conclusion:** In combination, HPLC and FTIR profiling created a complete degradation map of Nilotinib, highlighting the nanoformulation's improved stability and corroborating its viability for enhanced therapeutic dependability.

**Keywords:** Analytical Quality by Design (AQbD), Box-Behnken Design, Nilotinib, Pharmaceutical Analysis, RP-HPLC, Stability-Indicating Method.

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## INTRODUCTION

Nilotinib (NL) is a next generation tyrosine kinase inhibitor and has proven its therapeutic efficacy in the management of Chronic Myeloid Leukemia (CML). The biological activity of this drug is mainly due to the specific inhibition of BCR-ABL tyrosine kinase that is responsible for the development of CML. Despite its clinical significance, challenges related to its formulation and analytical assessment highlight the need for highly sensitive and reliable analytical methods to ensure consistent quality, therapeutic efficacy, and stability across various dosage forms.<sup>1</sup> Due to its accuracy, sensitivity, and flexibility, reverse phase High-Performance Liquid Chromatography (HPLC) has emerged as the most preferred method for pharmaceutical analysis and stability testing. In recent years, the adoption of the Analytical Quality by Design (AQbD) approach in method development has gained considerable momentum, offering a structured framework

for the systematic design and implementation of robust and reproducible analytical procedures.<sup>2</sup> In AQbD, importance is given to determining the Quality Attributes (CQAs) and Method Parameters (CMPs) that affect the efficiency of the method. In this approach, you can achieve method robustness, and it assures regulatory compliance by defining a design space where the method works reliably. Utilizing AQbD principles allows the optimization of chromatographic conditions for Nilotinib to achieve suitable resolution for quantification and stability studies. Formulation development through nanotechnology, like Nilotinib-loaded nanoparticles, can contribute immensely to better drug delivery and stability.<sup>3</sup> The stability of Nilotinib in formulations is influenced by numerous factors like pH, temperature, and exposure to oxidative or photolytic conditions, stressing the need for an effective stability-indicating analytical method for monitoring the degradation of Nilotinib.<sup>4</sup> The study elaborates on the design and validation of a stability-indicating RP-HPLC method for Nilotinib in bulk and nanoformulated states. Optimization was thoroughly conducted using Box-Behnken design, a statistical method that assesses the interaction among several factors to determine optimal conditions. The conditions for the chromatographic analysis were optimized by varying the composition of the mobile phase, flow rate, and column temperature to obtain good peak resolution, low



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tailing, and increased column efficiency. This method was further validated following the ICH Q2(R1) guidelines by evaluating the analytical performance of the method such as linearity, accuracy, precision, robustness, and sensitivity. Moreover, stability testing was conducted using different stress conditions like acidic, basic, oxidative, thermal, and photo degradation conditions to assess the stability profile of Nilotinib. The proposed analysis technique was found to provide effective separation of Nilotinib from its degradation products, thus proving its effectiveness as a stability-indicating test technique. Besides, the effective use of the technique for analysis of Nilotinib nanoparticles indicated its efficiency for routine quality testing and control, which could help meet applicable regulations. The inclusion of Analytical Quality by Design (A-QbD) principles in the RP-HPLC method development gave the quantitation a systematic approach that ensured reliable quantitation and detailed stability evaluation. Such a methodical approach tackles analytical issues inherent in nanoformulations as well as stress degradation profiling, highlighting the significance of analytical method development in research for pharmaceuticals. Finally, the research improves quality control procedures and aids better therapeutic effects for Nilotinib formulations.

## MATERIALS AND METHODS

### Chemicals and Reagents

The gift sample of Nilotinib (NL) was obtained from HRV Global Ltd., India. The other two reagents namely, phospholipon 90 G and HPLC grade methanol, were obtained from LIPOID® (Germany), and Sigma-Aldrich Corporation (Mumbai), respectively. While orthophosphoric acid and both sodium phosphates were obtained from Merck Specialties Pvt. Ltd., Mumbai. The remaining chemicals used in this study were of analytical grade.

### Instrumentation

The estimation of Nilotinib (NL) was done by a quantification method through the use of the Agilent 1100 series HPLC system that consists of the following components: Jasco UV-2075 Plus detector, autosampler, and Jasco LC-Net. The system also consisted of a G1310A isocratic pump for flow control and a C18 column (Agilent, 250 × 4.6 mm, 5 µM) for chromatographic separation. The ChemStation chromatography data software was applied for analysis in order to ensure accurate and reproducible results. Other instruments were a Essae-Teraoka analytical balance, a Systronics pH meter, and other laboratory equipment to ensure the reliability of the experimental procedures. All the data acquisition and processing of the chromatograms were done via ChemStation software (Version 3.2.1, Build 3.2.1.31). The method optimization was done using DoE and was implemented via the software Design-Expert (Version 13, Stat-Ease Inc., Minneapolis, USA) and was accomplished via Box-Behnken design. All

stability studies were conducted using a REMI Stability Chamber (Model No. 398/10/09-10) in a humidity-controlled environment. Ultrasonic dissolution of samples was done using PCI Mumbai 3.5 L 100H sonicator, which was followed by filtration through 0.45 µM membrane filter before HPLC. All the chemicals used in the experiment were weighed precisely with the help of Accuris™ precision analytical balance.

## Methods

### Chromatographic Condition

Nilotinib separation and quantitation were accomplished by means of an Agilent 1100 series HPLC apparatus equipped with a Jasco UV-2075 Plus detector while cross validation of results was achieved using an Agilent® 1200 series HPLC instrument. The optimized mobile phase used was a combination of methanol and 0.1% orthophosphoric acid in an 80:20 (v/v) proportion with pH being adjusted to 2.7. Chromatographic separation was accomplished at a flow rate of 0.7 mL/min, while the detection of Nilotinib took place at 265 nm. All samples were filtered using a 0.45 µM membrane prior to HPLC injection. Chromatographic data collection and analyses were done using ChemStation software (Version 3.2.1, Build 3.2.1.31).

### Preparation of stock and standard solutions

The preparation of the stock solution of Nilotinib (concentration 1000 µg/mL) involved weighing 100 mg of the compound and dissolving in 100 mL of methanol. The stock solution thus obtained was sonicated for 15 min to ensure complete dissolution of the compound. Appropriate dilutions of the stock solution were then made using the mobile phase and the concentration range for the working standard solutions was 5-25 µg/mL.<sup>5</sup>

### Preparation of Marketed NL Nanoparticles.

Nilotinib-loaded polymeric nanoparticles were prepared by the nanoprecipitation (solvent-displacement) technique using PLGA-PEG-FA as the carrier polymer. Briefly, Nilotinib (10 mg) and PLGA-PEG-FA (100 mg) were dissolved in 5 mL of acetone to form the organic phase, while the aqueous phase consisted of 20 mL of 0.5% w/v Poloxamer 188. The organic phase was added gradually to the aqueous phase with constant stirring using the magnetic stirrer operated at 700 rpm. The dispersion obtained was briefly sonicated in ice bath in order to reduce the size of particles formed. Afterwards, the dispersion was stirred for 3 hr at room temperature to achieve total evaporation of the organic solvent. The particles were recovered by centrifuging them at 15,000 × g for 20 min at 4°C. The isolated particles were thoroughly washed with deionized water twice to remove free drug and any stabilizer. Trehalose (5% w/v) was added as a cryoprotectant before freezing at -80°C, and the samples were lyophilized to obtain a dry nanoparticle powder. The prepared formulation was stored in a desiccator at 4°C until further characterization.<sup>6</sup>

## AQbD for Method Development

### Defining Analytical Target Profile and Critical Analytical Attributes

The process of designing and optimizing the analytical method started with determining the Analytical Target Profile (ATP), which is one of the main components of the Quality by Design (QbD) philosophy. It is a definition of desirable properties of the analytical method, and it helps to develop the method systemically. According to the expected performance of the method, Critical Analytical Attributes (CAAs) were defined to estimate the efficiency of the method. In the current work, three main CAAs were defined: theoretical plates number, peak tailing (asymmetry), and retention time. The theoretical plates number is considered a measure of chromatographic efficiency and performance, and peak tailing is used as a parameter of peak symmetry estimation. Retention time was defined to ensure correct elution of Nilotinib during chromatography.<sup>7</sup>

### Plackett-Burman design

Ishikawa's fishbone diagram (Figure S1) was generated as the first step in developing the HPLC method using QbD approach for estimating Nilotinib. On the basis of preliminary studies and literature data, the following process variables - flow rate, composition of mobile phase, buffer pH, temperature of the column, and injection volume - were selected for screening as they are expected to have significant influence on the performance of the HPLC method. To identify the factors having significant effect on the performance of the proposed HPLC method, Plackett-Burman design was used. In particular, the

flow rate was tested at 0.7 and 1.3 mL/min to determine its effect on retention time and resolution. Different ratios of methanol and 0.1% orthophosphoric acid were evaluated to define the optimal composition of the mobile phase also, pH was screened at conditions between 3 to 4. Effect of the column temperature at 25 and 30°C on peak symmetry and number of theoretical plates was estimated, whereas the impact of the injection volume at 5-20 µL on the peak area and analytical reproducibility was estimated. Two-level factorial design including 12 experimental runs with each of the selected factors tested at low and high level was used; these settings are summarized in Table S1. Retention time, peak symmetry, number of theoretical plates, and peak area were chosen as analytical responses for the analysis of the influence of the investigated factors.<sup>8</sup> As can be seen from the screening results, the flow rate and composition of the mobile phase had the strongest impact on peak symmetry and chromatographic resolution, whereas the buffer pH and column temperature had comparatively moderate impact. According to these results, the optimization of the chromatographic conditions was carried out based on the Box-Behnken Design (BBD).<sup>9</sup>

### Optimum Chromatographic Conditions by Box-Behnken Design

The important factors of the chromatographic process that were determined through the screening studies were further optimized by Box-Behnken design, which involves three factors. The design experiments were analyzed in terms of selected analytical responses like theoretical plate number, retention time, and tailing factor. The details of all the BBD experiments and factors are provided in Table 1. This disciplined methodology gave a

**Table 1: Box-Behnken Design Optimization for HPLC Method of Nilotinib in bulk form.**

| Run | Factor       |                             |                       | Response       |                    |                |
|-----|--------------|-----------------------------|-----------------------|----------------|--------------------|----------------|
|     | A: Flow rate | B: Mobile Phase Composition | C: Column Temperature | Retention Time | Theoretical Plates | Tailing Factor |
| 1   | 0.7          | 80                          | 30                    | 5.89±0.21      | 5021±23.45         | 1.06±0.06      |
| 2   | 0.7          | 90                          | 25                    | 5.67±0.16      | 5325±15.04         | 1.08±0.08      |
| 3   | 1            | 85                          | 30                    | 6.07±0.03      | 5352±25.62         | 1.1±0.10       |
| 4   | 1.3          | 85                          | 35                    | 6.4±0.09       | 5451±20.13         | 1.4±0.05       |
| 5   | 0.7          | 85                          | 25                    | 5.78±0.14      | 5300±18.99         | 1.12±0.09      |
| 6   | 1            | 90                          | 35                    | 6±0.08         | 5325±20.11         | 1.08±0.07      |
| 7   | 1.3          | 80                          | 30                    | 6.49±0.17      | 5322±17.84         | 1.08±0.08      |
| 8   | 1.3          | 90                          | 30                    | 6.31±0.13      | 5325±19.76         | 1.4±0.06       |
| 9   | 1            | 85                          | 30                    | 6.07±0.04      | 5187±12.01         | 1.04±0.04      |
| 10  | 1            | 90                          | 30                    | 6.01±0.10      | 5489±27.33         | 1.12±0.08      |
| 11  | 1.3          | 85                          | 25                    | 6±0.07         | 5250±22.09         | 1.09±0.09      |
| 12  | 1            | 90                          | 25                    | 6.16±0.18      | 5250±21.23         | 1.07±0.07      |
| 13  | 1            | 85                          | 30                    | 6.07±0.06      | 5320±18.97         | 1.04±0.03      |
| 14  | 1            | 80                          | 25                    | 6.72±0.20      | 5156±10.44         | 1.07±0.06      |
| 15  | 0.7          | 85                          | 25                    | 5.67±0.13      | 4998±12.35         | 1.06±0.05      |

clear perception of how the variations in the Critical Method Parameters (CMPs) affect chromatographic performance and hence allowed for accurate fine-tuning of the RP-HPLC method towards optimal outputs.<sup>10</sup>

### Statistical Analysis and Validation of the Optimization Model

Multiple Linear Regression (MLR) analysis of the data generated by the optimization experiment was performed via the software Design-Expert, Version 13.0 (Stat-Ease Inc., MN 55413, USA). Statistical analysis models were used to determine the impact of each of the selected chromatographic variables on the observed analytical responses. Terms which showed statistical significance at  $p < 0.05$  were taken into account when constructing the polynomials. Validity and appropriateness of the models were confirmed statistically through the evaluation of the following factors: coefficient of determination ( $R^2$ ), Predicted Residual Error Sum of Squares (PRESS), and lack-of-fit. Furthermore, a three-dimensional response surface was plotted to visualize the relationship between the studied factors and analytical responses. Insightful response surface plots of the generated data revealed the effect of independent variables chosen for analysis on critical performance parameters. Numerical optimization using the desirability function was then carried out to determine the conditions that met the pre-set criteria. The main goal of the numerical optimization was to find conditions that would minimize peak tailing and retention time but at the same time maximize the area and theoretical plates of the peaks. Furthermore, graphical optimization was also done in order to determine the design space and region with ideal conditions.<sup>11</sup>

### Analytical method validation

Optimized method was validated according to ICH Q2(R1) guidelines regarding some essential validation parameters such as linearity, accuracy, precision, specificity, robustness, LOD and LOQ. Linearity was studied by preparation of the set of Nilotinib standard solutions in the optimized mobile phase containing methanol and 0.1% orthophosphoric acid (80:20, v/v) within the concentration range 5-25 µg/mL. Chromatographic peak area was plotted versus Nilotinib concentration, and linear relationship was established by means of least squares regression analysis. The obtained data confirmed the applicability of the developed method for quantitation of Nilotinib within the investigated concentration range.<sup>12</sup> Accuracy of the method developed was evaluated using the standard addition approach. The stock solution of the standard Nilotinib was added with the amount of the compound to get different concentration levels of 80%, 100%, and 120% of the target concentration at concentrations of 24 µg/mL, 30 µg/mL, and 40 µg/mL, respectively. Accuracy of the method was calculated through percentage recovery, which was further supported by the values of mean recovery, Standard Deviation (SD), and Relative Standard Deviation (%RSD).<sup>13</sup>

Accuracy of the developed analytical procedure was checked out at three different concentrations of Nilotinib, that is, 30, 40, and 50 µg/mL. The intra-day accuracy of the technique was established by studying these particular concentrations on different days, whereas the inter-day accuracy was carried out by testing the procedure on a different day. The accuracy and reliability of the technique were analyzed by calculating the percentage relative standard deviation (%RSD) of the data.<sup>14</sup> Detection Limits (LODs) and Quantification Limits (LOQs) were obtained using the equation of the calibration graph and the standard deviation of the response. These values were estimated according to the formulae shown below (Equations 1 and 2) where  $S$  is the slope of the calibration graph and  $\sigma$  is the standard deviation of the response.

$$\text{LOD} = 3.6 \sigma / S \quad \text{Equation 1}$$

$$\text{LOQ} = 10 \sigma / S \quad \text{Equation 2}$$

To analyze the developed method's robustness, small intentional variations were added into the chromatographic conditions, such as modifying the acetonitrile proportion in the mobile phase by  $\pm 5\%$  v/v, changing the injection volume, varying the flow rate by  $\pm 8\%$ , and modifying the column oven temperature by  $\pm 5^\circ\text{C}$ . A working standard solution of 20 µg/mL Nilotinib was subjected to these changed conditions to check their influence on the performance of the method. Mean percentage recovery and %RSD values were obtained to check robustness. System suitability was additionally confirmed by conducting six repetitive injections of an identical standard solution, and Standard Deviation (SD) and %RSD for peak area, as well as retention time were measured.<sup>15</sup> Specificity was confirmed by subjecting a 20 µg/mL standard solution to conditions likely to cause degradation, followed by chromatographic analysis to check for potential interference of any degradation products with the critical analytical attributes of the reference standard.

### Application of the Validated HPLC Method for Nilotinib Nanoformulation

Subsequently, the developed HPLC method was utilized for the quantitative analysis of Nilotinib (NL) in an in-house nanoformulation of this drug. The application of this technique allowed estimating the drug content, as well as the stability of the nanocarrier formulation. Chromatographic analyses were performed on C18 Princeton column with a mobile phase containing methanol and 0.1% of orthophosphoric acid (80:20, v/v), adjusted to pH 2.7, and with a detector wavelength of 265 nm. With these optimized chromatographic conditions, Nilotinib was well separated from excipients used for the nanocarrier formation as well as any possible degradation products, thus allowing for reliable estimation of the drug content in the sample. Additionally, the proposed HPLC technique was utilized for evaluating the stability of the drug-loaded nanocarriers under

stress conditions, such as the thermal degradation, which allows to detect possible degradation products and analyze the stability of the active substance.<sup>16</sup>

### Forced Degradation Studies for Stability Indication

Forced degradation studies were carried out based on the ICH Q1A(R2) guidelines in order to determine the stability-indicating ability of the HPLC method developed. Both the pure Nilotinib and Nilotinib loaded nanofomulations were exposed to various stress conditions, such as acidic, alkaline, oxidative, thermal, and photo-induced stress. The purpose of these tests was to study the degradation behavior of Nilotinib under different stress conditions and to confirm the effectiveness of the proposed HPLC method in differentiating between the drug and its degradants. The aim was to check whether the method was capable of distinguishing Nilotinib from any resulting degradation product under these stress conditions. Acid hydrolysis was achieved using treatment of the samples with 0.1N HCl, followed by reflux for 2 hr at room temperature and neutralization with 0.1N NaOH. The same procedure was carried out for alkali hydrolysis using 0.1N NaOH for 2 hr and neutralized with 0.1N HCl. Oxidative degradation was triggered by exposing the samples to 3% hydrogen peroxide for 1 hr at room temperature. Thermal degradation was determined by keeping the samples in a hot air oven at 80°C for 72 hr. For photolytic degradation, the samples were subjected to UV light at 254 nm for 24 hr. Following each stress condition, the treated samples were assessed with the method developed HPLC for the detection and estimation of any degradation products.<sup>17</sup>

### FTIR Analysis of Degradation Products

The degradation products formed through the forced degradation studies were further analyzed using Fourier Transform Infrared Spectroscopy (FTIR) technique to study the functional groups in the possible impurities or degradation products. The solid residues obtained were ground with spectroscopic grade Potassium Bromide (KBr) to make transparent pellets under vacuum pressure. The FTIR spectrum was recorded in the wavelength region of 4000-400  $\text{cm}^{-1}$  with a resolution of 4  $\text{cm}^{-1}$ , recording 32 scans per sample. The spectra thus obtained were compared with the spectra of pure Nilotinib for any change in characteristic absorption peaks such as appearance, disappearance, and shift in peaks. The spectral changes were further interpreted to determine the structure modification in Nilotinib and hence obtain preliminary information on the degradation pathways of Nilotinib.

## RESULTS AND DISCUSSION

### Preliminary Chromatographic Method Development

Preliminary method development experiments were conducted in order to develop appropriate conditions for the quantitative estimation of Nilotinib (NL) in both the pure drug and its

nanofomulation. Various organic solvent systems and other chromatographic conditions were studied for obtaining satisfactory results in terms of separation and performance of the system. Based on the preliminary experiments, the mobile phase was selected which comprised of methanol and 0.1% orthophosphoric acid in the 80:20 (v/v) ratio and had a pH value of 2.7. The initial chromatographic experiments were performed using an Agilent C18 column having the flow rate of 0.7 mL/min and detection wavelength of 265 nm.

### Assessment of Tailing Factor as an Essential Analytical Attribute

Tailing was one of the CAA that is considered very critical for enhancing the efficiency of the separation process as well as enabling accurate quantification of the drugs under investigation. The model developed for tailing factor showed statistical significance ( $p$ -value=0.0121), and it indicated the presence of significant influence of the studied variables on peak symmetry. Of all the factors evaluated, flow rate (A), injection volume (E), and buffer pH (C) were found to have significant effect on the tailing factor while column temperature (D) and mobile phase composition (B) failed to show significant effect. The applicability of the developed model was confirmed by carrying out the diagnostic tests, including the normal probability plot and residuals versus run (Figure 1). The statistical data is presented in Table 2.<sup>18</sup>

### Column Efficiency Evaluation via Theoretical Plate Count

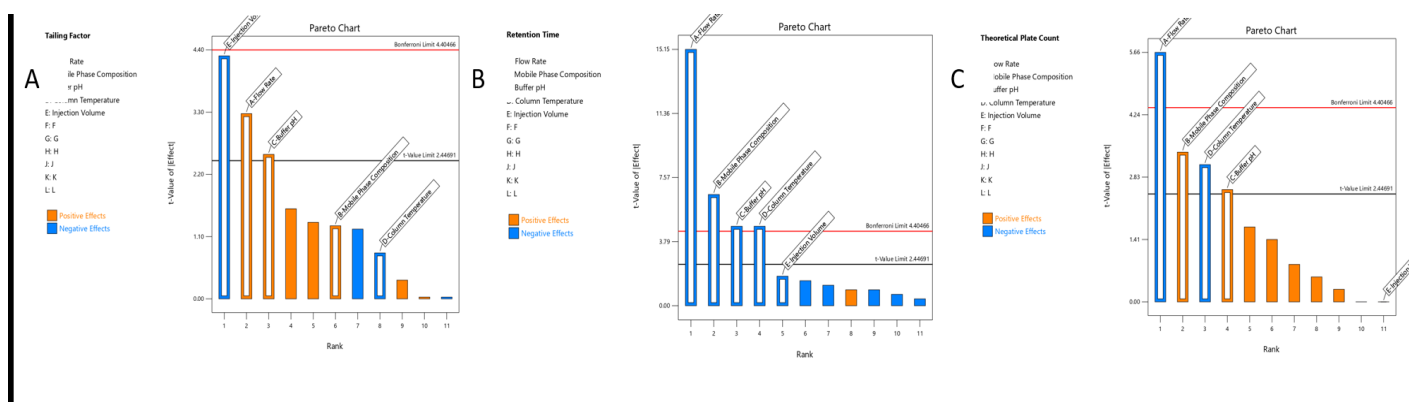
The theoretical plate count was chosen as a CAA in order to evaluate the efficiency of the separation process and the effectiveness of the developed chromatographic method. An increase in the value of the theoretical plate count means better column efficiency and leads to a more effective separation of the analyte from other interferences. In the case of the response of interest, the model was proven to be statistically significant, based on the value of  $p=0.0042$  (Table 2). The effect of the studied chromatographic variables on the response is confirmed. Of the considered variables, only the flow rate (A), mobile phase (B), buffer pH (C), and column temperature (D) influenced the value of the response, while the injection volume (E) had no effect on it (Figures 1A-1C). The quality of the developed model was additionally proven by diagnostic plots (Figure 2), showing proper residuals distribution and residuals-versus-run behavior.<sup>19</sup>

### Evaluation and Optimization of Retention Time

Retention time was regarded as one of the most important analytical attributes (CAA) used in the evaluation of the performance of the chromatography and the retention characteristics of Nilotinib. The significance of the developed factorial model for the retention time is highly significant ( $*p<0.0001$ ) showing that the selected chromatographic factors

**Table 2: Response Summary of ANOVA for data analysis of response variables.**

| Dependent Variable | Source                     | Sum of Squares | DF | Mean Square | f-Value | p-Value |             |
|--------------------|----------------------------|----------------|----|-------------|---------|---------|-------------|
| Retention Time     | Model                      | 8415.26        | 6  | 0.0048      | 95.22   | <0.0001 | significant |
|                    | A-Mobile Phase Composition | 1664.25        | 1  | 0.0012      | 0.9462  | 0.0026  |             |
|                    | B-Column Temperature       | 218.72         | 1  | 0.0031      | 6.90    | 0.0033  |             |
|                    | C-Flow rate                | 1123.20        | 1  | 0.0023      | 345.19  | <0.0001 |             |
| Tailing Factor     | Model                      | 1794.31        | 3  | 0.0015      | 38.14   | <0.0121 | significant |
|                    | A-Mobile Phase Composition | 90.17          | 1  | 0.0010      | 25.60   | 0.0004  |             |
|                    | B-Column Temperature       | 200.2          | 1  | 0.0003      | 7.90    | 0.0008  |             |
|                    | C-Flow rate                | 2561.17        | 1  | 0.0032      | 80.92   | <0.0001 |             |
| Theoretical Plate  | Model                      | 4.508E+05      | 9  | 50093.10    | 427.84  | <0.0042 | significant |
|                    | A-Mobile Phase Composition | 22578.13       | 1  | 22578.13    | 192.84  | <0.0023 |             |
|                    | B-Column Temperature       | 21012.50       | 1  | 21012.50    | 179.47  | <0.0041 |             |
|                    | C-Flow rate                | 1.907E+05      | 1  | 1.907E+05   | 1628.35 | <0.0011 |             |

**Figure 1:** Pareto chart showing the effect of factors on tailing factor (A) retention time(B), and theoretical plates (C).

have considerable effects on the response (Table 2). It can be seen from (Figure 1B) that flow rate (A), mobile phase composition (B), buffer pH (C) and column temperature (D) are some of the factors influencing retention time, while injection volume (E) is an insignificant factor.<sup>20</sup>

The adequacy of the developed model was further validated by some diagnostic plots such as the normal probability plot and the residual plot (Figure 2). In the normal probability plot, the residuals appear to be uniformly distributed about the reference line, thus validating the normality assumption. Moreover, the residuals are distributed equally around the X-axis in the residuals-versus-predicted response plot, thereby proving the lack of any systematic pattern among the residuals.

## Box-Behnken-Based Optimization and Validation of the Developed Models

Box-Behnken Design (BBD) was applied in this study as a statistical method for optimization of the proposed HPLC method for the determination of Nilotinib (NL). The effect of three factors, such as the composition of the mobile phase ( $X_1$ , % v/v), column temperature ( $X_2$ , °C) and flow rate ( $X_3$ , mL/min), was examined at three levels (−1, 0, and +1). The effect of these independent factors on the dependent analytical responses, which include the tailing factor ( $Y_1$ ), retention time ( $Y_2$ ), and theoretical plates ( $Y_3$ ), was evaluated using Design-Expert® software. The data obtained from the experiments conducted using different combinations of the independent factors were entered into the design matrix for statistical analysis in order to

derive the optimal mathematical models. The significance of the models was assessed using the Analysis of Variance (ANOVA) at 5% level of significance. A  $p$ -value below 0.05 indicated a statistically significant model (Table 2). Majority of the model terms were found to be statistically significant ( $p < 0.05$ ) indicating the significance of their contribution to the respective response functions. The values of the regression coefficients were used for interpreting the effect direction of the factors on the response, whereby positive coefficients indicated synergy effect on the response while negative coefficients indicated antagonism effect on the response. The model proposed is A, B, and C for flow rate (mL/min), mobile phase composition, and column temperature ( $^{\circ}\text{C}$ ), respectively.<sup>21</sup>

### Impact of Variables in the Chromatographic Process on Peak Tailing

The impact of chosen independent variables on the tailing factor was evaluated for all 15 experimental runs of the designed HPLC method. The minimum value of tailing factor was found to be  $1.04 \pm 0.03$  (Run 9), while the maximum one was  $1.40 \pm 0.05$  (Run 4). According to ANOVA, the differences in the values of the tailing factor among different experimental runs were statistically significant ( $p < 0.05$ ). It was concluded that **flow rate (A), mobile phase composition (B), and column temperature (C)** significantly influenced the process of peak tailing. In order to establish a quantitative relationship between independent variables and tailing response, the obtained experimental data were approximated by means of a polynomial equation of the form:

$$\text{Tailing Factor (Y}_1\text{)} = 1.06 + 0.12A - 0.08B + 0.04C \text{ (Equation 1)}$$

The equation highlights that increasing the flow rate (A) exacerbated tailing, as evidenced by its positive coefficient (+0.12). For instance, reducing the flow rate from 1.3 mL/min (Run 4: 1.40) to 0.7 mL/min (Run 9: 1.04) significantly improved peak symmetry. This improvement is attributed to prolonged analyte-stationary phase interactions at lower flow rates, which enhance equilibration and reduce peak distortion. Mobile phase composition (B) exhibited an inverse relationship with tailing, as indicated by its negative coefficient (-0.08). Elevating the acetonitrile:buffer ratio from 80:20 (Run 7: 1.19) to 90:10 (Run 2: 1.08) minimized secondary interactions, likely due to the enhanced elution strength of the organic-rich mobile phase. Column temperature (C), though less impactful, showed a marginal positive coefficient (+0.04), suggesting that higher temperatures slightly increased tailing. Lowering the temperature from  $30^{\circ}\text{C}$  (Run 3: 1.10) to  $25^{\circ}\text{C}$  (Run 5: 1.12) stabilized analyte diffusion rates, balancing retention and symmetry. Model validation confirmed the robustness of this relationship, with a high coefficient of determination ( $R^2 = 0.912$ ) and a statistically significant  $f$ -value of 38.14 ( $p < 0.0001$ ). Three-dimensional response surface plots (Figure 3) further elucidated the synergistic effects of these variables. For example, combining a low flow rate (0.7 mL/min) with a high acetonitrile ratio (90:10) yielded minimal tailing (1.06-1.08), demonstrating the method's tunability. Mechanistically, the reduced tailing at lower flow rates aligns with extended interaction times for equilibration, while higher organic content weakens hydrophobic interactions, sharpening peak profiles. Temperature adjustments, though secondary, fine-tuned these effects by modulating analyte mobility. This systematic optimization underscores the efficacy of the Quality by Design (QbD) approach in achieving precise

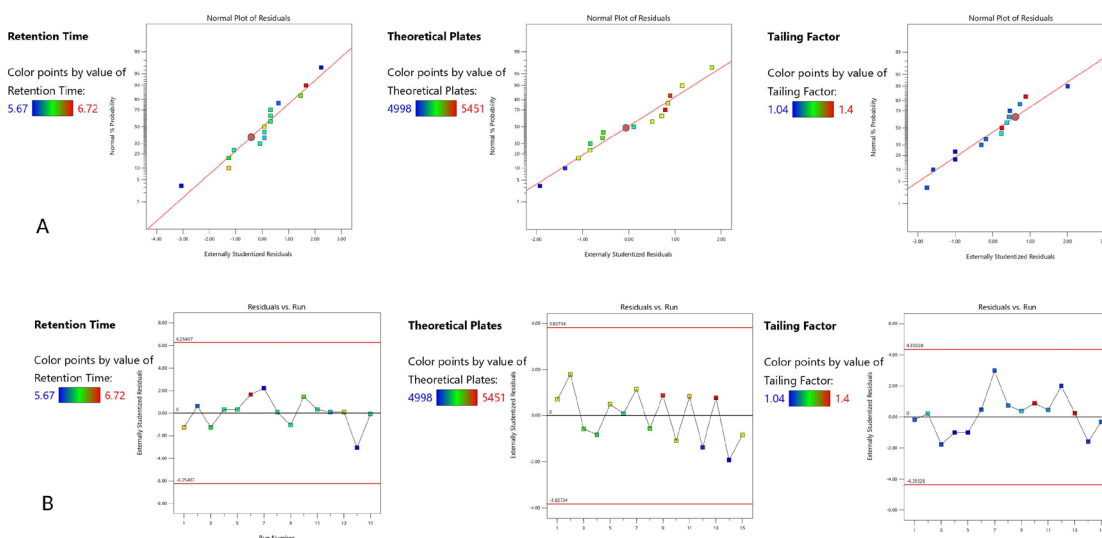


**Figure 2:** Model diagnosis graphs (A) Normal Plot of Residuals and (B) residuals versus run graphs for retention time, tailing factor, and theoretical plate count.

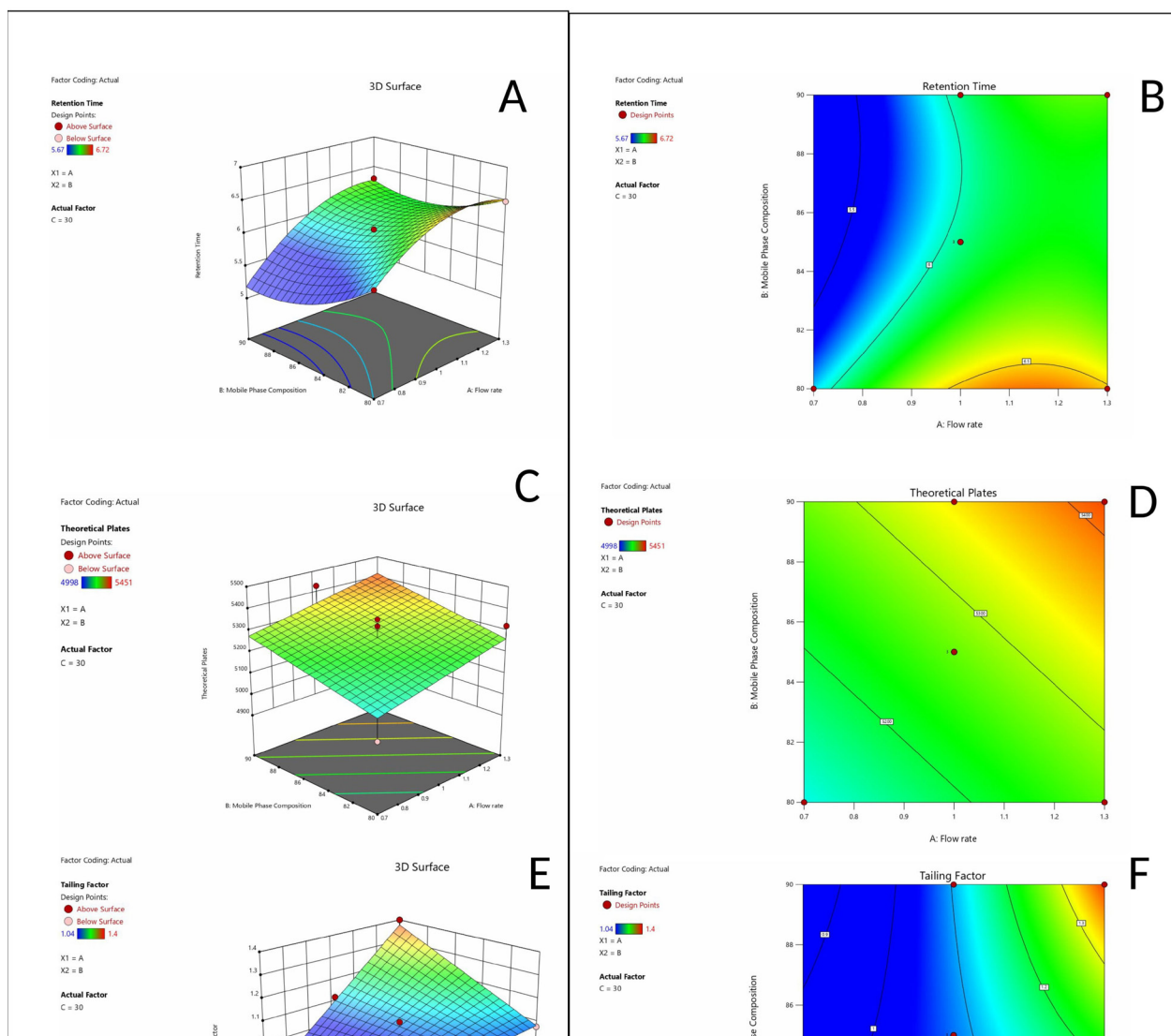


**Figure 3:** 3D surface plots (A, C, and E) and corresponding contour plots (B, D, and F) depicting the effects of mobile phase composition (% acetonitrile) and flow rate (mL/min) on chromatographic performance parameters. (A, B) Retention Time (min), (C, D) Theoretical Plates, and (E, F) Tailing Factor.

control over chromatographic performance, ensuring robust separation efficiency for the estimation of Nilotinib.

### Effect of Critical Chromatographic Parameters on Retention Time

The HPLC method that was successfully developed demonstrated retention times of  $5.67 \pm 0.13$  min (Run 15)\*\* and  $6.72 \pm 0.20$  min (Run 14)\*\*. The statistical analysis through ANOVA proved that variations in retention time were statistically significant ( $p < 0.05$ ). Effects of critical process parameters such as \*\*flow rate (A), composition of mobile phase (B)\*\*, and \*\*column temperature (C)\*\* on retention time ( $Y_2$ ) were studied next. The correlation between these independent variables and the response variable of retention time was determined by the following polynomial equation:

$$\text{Retention Time} = 6.07 + 0.32A - 0.08B + 0.12C - 0.21AB + 0.06AC + 0.14BC \text{ (Equation 2).}$$

Flow rate (A) exhibited a dominant positive influence (+0.32), where increasing it from 0.7 mL/min (Run 2: 5.67 min) to 1.3 mL/min (Run 7: 6.49 min) unexpectedly prolonged retention by ~14%, likely due to turbulent flow dynamics or reduced equilibration efficiency at elevated flow rates. Conversely, mobile phase composition (B) showed an inverse relationship (-0.08), with higher acetonitrile ratios (e.g., 90:10 in Run 6: 6.00 min vs. 80:20 in Run 14: 6.72 min) shortening retention by weakening hydrophobic interactions. Column temperature (C) contributed modestly (+0.12), as raising it from 25°C (Run 5: 5.78 min) to 30°C (Run 3: 6.07 min) extended retention, potentially due to altered stationary phase porosity or analyte solubility. Interaction effects further modulated responses: the antagonistic AB term

(-0.21) highlighted how combining high flow rates with low acetonitrile ratios (e.g., Run 7: 6.49 min) amplified retention, while synergistic BC (+0.14) interactions balanced these effects. The model's robustness was validated by a high  $R^2$  value (0.976) and significant  $f$ -value (95.22,  $p < 0.0001$ ), with 3D response surface plots (Figures 3A and 3B) illustrating optimal retention time control at intermediate flow rates (~1.0 mL/min) and acetonitrile ratios (~85:15). Mechanistically, retention time elongation at higher flow rates may arise from incomplete analyte-stationary phase equilibration under turbulent conditions, while elevated acetonitrile levels reduce buffer ionic strength, accelerating elution. These findings underscore the method's adaptability, enabling precise retention time tuning for robust separation of Nilotinib under QbD-guided chromatographic conditions.

### The Effect of Critical Parameters on Theoretical Plate Count

The HPLC method developed had theoretical plate count varying between  $4998 \pm 12.35$  (Run 15)\*\* and  $5489 \pm 27.33$  (Run 10)\*\* for the 15 experimental runs conducted. ANOVA indicated a statistically significant difference in theoretical plate count for the experimental runs conducted ( $*p < 0.05$ ), meaning that the critical process parameters chosen had a significant effect on the column efficiency. The effect of flow rate (A), mobile phase composition (B), and column temperature (C) on theoretical plate count ( $Y_3$ ) was analyzed. The mathematical equation relating the above independent variables to the theoretical plate count is shown below:

$$\begin{aligned} \text{Theoretical Plates} = & 5325.80 + 142.50A + 68.75B + 45.30 \\ & C - 85.20AB - 32.50AC + 28.75BC - 105.40A^2 - 40.15 \\ & B^2 + 18.90C^2 \text{ (Equation 3).} \end{aligned}$$

Flow rate (A) exhibited the strongest positive influence (+142.50), with theoretical plates increasing from  $5156 \pm 10.44$  (Run 14, 0.7 mL/min) to  $5451 \pm 20.13$  (Run 4, 1.3 mL/min) as flow rates rose. This trend aligns with reduced band broadening at higher flow rates, which minimizes longitudinal diffusion and enhances peak sharpness. Mobile phase composition (B) also contributed positively (+68.75), where elevating the acetonitrile: buffer ratio from 80:20 (Run 14: 5156 plates) to 90:10 (Run 10: 5489 plates) improved partitioning efficiency, likely due to weakened hydrophobic interactions and accelerated analyte elution. Column temperature (C) showed a moderate positive effect (+45.30), as increasing temperature from 25°C (Run 5: 5300 plates) to 30°C (Run 3: 5352 plates) enhanced diffusion rates, optimizing mass transfer between phases. Interaction terms further refined responses: the antagonistic AB term (-85.20) revealed that combining high flow rates with low acetonitrile ratios (e.g., Run 7: 5322 plates) marginally reduced efficiency, while the synergistic BC term (+28.75) demonstrated that pairing elevated temperatures with high acetonitrile ratios (Run 6: 5325 plates) maximized plate counts. Quadratic terms ( $A^2$ :

$-105.40$ ;  $B^2$ :  $-40.15$ ) highlighted nonlinear effects, emphasizing the need for balanced parameter optimization. Model validation confirmed exceptional robustness, with a near-perfect  $R^2$  value of 0.996 and a highly significant  $f$ -value of 427.84 ( $p < 0.0001$ ). Three-dimensional response surface plots (Figures 3C and 3D) illustrated peak column efficiency at intermediate flow rates (~1.0 mL/min), high acetonitrile ratios (~90:10), and moderate temperatures (~30°C). Mechanistically, higher flow rates reduce analyte residence time, curbing longitudinal diffusion and sharpening peaks, while elevated acetonitrile levels weaken secondary interactions, improving partitioning. Temperature adjustments fine-tuned these effects by modulating stationary phase dynamics and analyte solubility.

### Validation of the Developed Model

The theoretical values of  $Y_1$ ,  $Y_2$ , and  $Y_3$  in the developed technique were obtained by putting the correct values of A, B, and C in their respective equations obtained from the Design Expert® software. See Table S3 for a numerical summary of the experimental and theoretical values for the reactions. There was an excellent match between the observed and theoretical values. These results validated the established Response Surface Methodology (RSM) Model.<sup>22</sup>

### Optimization and Selection of the Optimum HPLC Method

The systematic optimization of the HPLC method, guided by Quality by Design (QbD) principles, integrated screening (Plackett-Burman design) and refinement (Box-Behnken design) phases to identify chromatographic conditions balancing speed, efficiency, and precision. Numerical optimization, employing desirability function analysis, prioritized simultaneous minimization of retention time and tailing factor alongside maximization of theoretical plates. Among four candidate solutions (Table S4), Solution 1-comprising a flow rate of 1.0 mL/min, mobile phase composition of 80:20 (methanol: orthophosphoric acid), and column temperature of 25°C emerged as optimal, achieving a desirability score of 1.000. This configuration delivered a retention time of 5.952 min, theoretical plates of 5245.625, and a tailing factor of 1.077, fulfilling predefined acceptance criteria (retention time < 4 min, tailing factor < 1.1, theoretical plates > 5000). Comparative analysis revealed that alternative solutions, while effective in specific attributes, exhibited trade-offs: Solution 2 (85:15 mobile phase, 30°C) prioritized plate count (5396.667) at the expense of prolonged retention (6.111 min), while Solution 4 (35°C) maximized efficiency (5413.125 plates) but extended analysis time (6.244 min). Solution 3 (1.2 mL/min flow rate) suffered from reduced efficiency (5151 plates) and elevated tailing (1.103) due to turbulent flow effects. Mechanistically, the selected method's success stemmed from balanced laminar flow at 1.0 mL/min, minimizing band broadening without inducing turbulence; a 90:10 mobile phase ratio, which enhanced elution strength

to sharpen peaks; and a 25°C column temperature, stabilizing analyte diffusion and stationary phase interactions. Validation via the chromatogram of Run 9 (Figure 4) confirmed robust performance, with symmetrical peaks, baseline resolution, and reproducibility. This optimization underscores the efficacy of QbD in harmonizing competing chromatographic variables, ensuring a robust, high-throughput method for precise quantification of Nilotinib in bulk formulations (Table S5).

### Validation of the Analytical Method

The analytical method developed for the quantitative determination of Nilotinib (NL) has been systematically validated as per the requirements of ICH Q2(R1) guidelines. The validation experiments have been carried out in order to ensure that the method is reliable, reproducible and suitable for the quantitation of NL in the bulk drug as well as its nanoformulations. This was achieved by analyzing various parameters, which include linearity, accuracy, precision, specificity, robustness, Limit of Detection (LOD), and Limit of Quantitation (LOQ).<sup>5</sup>

The linearity of the established analytical method was estimated using 1-25 µg/mL of Nilotinib concentration. The calibration graph was plotted by correlating the values of the peak area to the corresponding drug concentrations. The obtained calibration data were analyzed using the least-square regression approach, giving the value of correlation coefficient equal to  $R^2=0.999$ . Such high value of correlation coefficient means the good linear dependence of Nilotinib concentration and peak response.<sup>23</sup>

Accuracy of the newly developed method was checked by applying standard addition technique at three different concentrations, which are equal to 80% (8 µg/mL), 100% (10 µg/mL), and 120% (12 µg/mL) of the desired concentration. The values of % recovery

for Nilotinib were calculated as 97.65-98.14%, whereas those of the test formulation were determined to be 97.21-97.50%. Small values of SD and % RSD, as provided in Table S5, showed close results from different replicates. These results proved the accuracy of the newly developed method for the quantitative estimation of Nilotinib in spite of the presence of an additional amount of drug.<sup>24</sup>

All the values are expressed as mean±SD,  $n=6$ .

The accuracy of the proposed analytical technique was studied using Nilotinib at three different concentration levels: 30, 40, and 50 µg/mL. Intra-day accuracy was performed by the repeat analysis of the chosen concentration levels on the same day, while the inter-day accuracy was carried out through the analysis performed on different days. The recorded mean percentage recovery value ranged between 98 and 100%, with the related %RSD value remaining under 2%. This low variability indicated that the analytical method provided good precision and reproducibility. These results confirmed the validity of the analytical method for Nilotinib, which is presented in Table 3.<sup>23</sup>

The sensitivity of the proposed analytical method was assessed in terms of its Limit of Detection (LOD) and Limit of Quantification (LOQ) based on the slope of the calibration curve and the standard deviation of the blank response. According to calculations, LOD and LOQ equalled 3.02 µg/mL and 7.35 µg/mL, correspondingly. Thus, one can claim that the proposed method demonstrates sufficiently high sensitivity for the detection and accurate determination of Nilotinib even at relatively low concentrations.

The robustness of the method was also tested by deliberately making certain changes in the selected chromatographic conditions. Specifically, the concentration of acetonitrile in the mobile phase and column temperature were changed by  $\pm 2^\circ\text{C}$

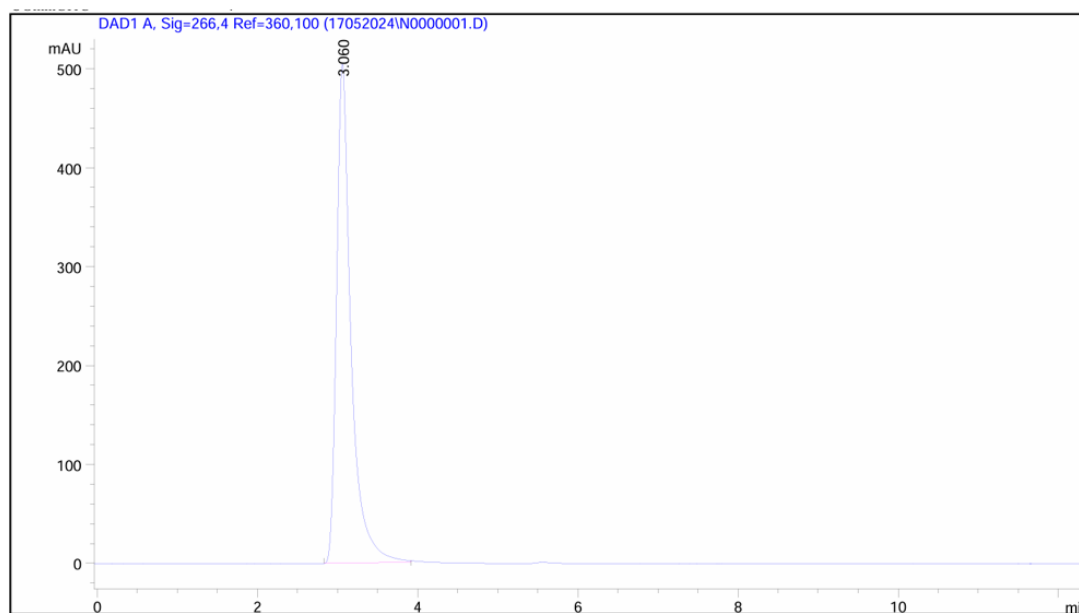


Figure 4: HPLC Chromatogram of optimized run (R9).

**Table 3: Repeatability and intermediate precision.**

| Study Type | Formulation     | Time Interval/<br>Day | Sample<br>Weight<br>(mg) | AUC ( $\mu$ V) | % Label<br>Claim | Mean % Label<br>Claim $\pm$ SD | % RSD |
|------------|-----------------|-----------------------|--------------------------|----------------|------------------|--------------------------------|-------|
| Intraday   | Bulk            | 1 hr                  | 202.4                    | 221487         | 99.93            | 100.26 $\pm$ 0.415             | 0.41  |
|            |                 | 2 hr                  | 202.4                    | 447212         | 100.26           |                                |       |
|            |                 | 3 hr                  | 202.4                    | 689356         | 100.40           |                                |       |
|            |                 | 4 hr                  | 202.4                    | 948452         | 100.69           |                                |       |
|            | Nanoformulation | 1 hr                  | 202.4                    | 100180         | 99.97            | 100.26 $\pm$ 0.415             | 0.41  |
|            |                 | 2 hr                  | 202.4                    | 100478         | 100.26           |                                |       |
|            |                 | 3 hr                  | 202.4                    | 100752         | 100.40           |                                |       |
|            |                 | 4 hr                  | 202.4                    | 100689         | 100.69           |                                |       |
| Interday   | Bulk            | Day 10                | 202.4                    | 1167580        | 120.27           | 120.27 $\pm$ 0.0986            | 0.08  |
|            |                 | Day 10                | 201.7                    | 1167580        | 120.21           |                                |       |
|            |                 | Day 10                | 201.8                    | 1167580        | 120.39           |                                |       |
|            | Nanoformulation | Day 10                | 202.4                    | 120443         | 120.23           | 120.27 $\pm$ 0.0986            | 0.08  |
|            |                 | Day 10                | 201.7                    | 120113         | 120.21           |                                |       |
|            |                 | Day 10                | 201.8                    | 120254         | 120.39           |                                |       |

All the values are expressed as mean $\pm$ SD,  $n=6$ .

correspondingly. The results of robustness tests are shown in Table S6.

System suitability was done by conducting six replicates of injection of Nilotinib standard at a concentration of 20  $\mu$ g/mL. Suitability of the system was checked using standard deviation (SD) and percentage Relative Standard Deviation (% RSD) for peak area and retention time of Nilotinib. The recorded SD and %RSD for both were observed to be less than 2%, suggesting that the system is well suited and shows good reproducibility of responses.

### Application of the Proposed Method for Pharmaceutical Nanoformulation of Nilotinib (NL)

In order to evaluate the practical utility of the proposed method, the quantitative analysis of Nilotinib in the developed nanoformulation was performed<sup>\*\*</sup>. According to the results of the assay, the concentration of Nilotinib in the nanoformulation was found to be 101.15 $\pm$ 0.245% of the claimed value, which indicates the satisfaction of the quality criteria of the nanoformulation. Furthermore, low values of %RSD (<2%) obtained during the replicate assays indicate the accuracy of the method. Finally, the proposed method demonstrated good specificity for Nilotinib determination, with no interfering substances detected for the excipients and other components of the drug-loaded nanocarrier (Figure 5).

### Forced Degradation Studies for Stability Indication

The forced degradation studies on Nilotinib bulk powder and its nanosuspension formulation<sup>\*\*</sup> were carried out as per the

guidelines of ICH Q1A(R2) to evaluate the stability indicating ability of the developed RP-HPLC method. The samples were subjected to various stressed conditions, including acidic, basic, oxidative, thermal, and photochemical to check the stability of Nilotinib under these conditions. The extent of degradation of the drug along with characterization of major degradation products was assessed using the developed HPLC method. The findings of these experiments are listed in Table 4. The method successfully separated Nilotinib from all stress-induced degradants, confirming its suitability for comprehensive stability profiling. Acidic hydrolysis (0.1 N HCl, 2 hr at room temperature) resulted in ~12% degradation of the bulk drug, whereas the nanoformulation degraded to a lesser extent (~9%). Hydrolyzed Nilotinib was identified as the predominant degradant, appearing at a Retention Time (RT) of 4.504 min, with partial hydrolysis products detected at RT 3.216 min (Figure 6A). The lower degradation in the nanoformulation can be attributed to the protective matrix of the nanocarrier, which limits direct acid attack and thereby reduces amide bond cleavage.

Under alkaline conditions (0.1 N NaOH, 2 hr), bulk Nilotinib exhibited ~14% degradation, compared to ~11% for the nanoformulation. Dealkylated derivatives were observed as major degradants (RT 4.503 and 14.058 min), accompanied by a minor ring-opened product at RT 7.5 min (Figure 6B). The degradation pathway predominantly involved quinoline ring fragmentation and amide cleavage. The nanoformulation again demonstrated improved resistance, possibly due to buffering effects and stabilization conferred by excipients.<sup>25</sup>

Oxidative stress (3% H<sub>2</sub>O<sub>2</sub>, 1 hr) was found to be the most severe condition, with ~17% degradation in the bulk drug and ~13% in the nanoformulation. The main degradant was identified as the N-oxide of Nilotinib at RT 8.058 min, while a dimeric species was detected at RT 14.048 min (Figure 6C). The proposed pathway involves radical-mediated C-N bond scission and subsequent oxidative transformations. Despite the protective effect of the nanocarrier, oxidative degradation was pronounced in both cases, highlighting this as the most critical stressor (Figure 5).<sup>26</sup>

Thermal degradation (80°C, 72 hr) was relatively mild, producing ~4.5% degradation in bulk drug and ~2% in the nanoformulation. The major degradant was a rearranged derivative (RT 4.471 min), along with a minor decarboxylated form at RT 14.041 min (Figure 6D). The pathway likely involved demethylation, decarboxylation, and partial polymerization. The reduced degradation in the nanoformulation suggests that the excipient matrix provided thermal shielding, stabilizing Nilotinib at elevated temperatures.<sup>27</sup>

Photolytic degradation (UV 254 nm, 24 hr) induced ~5% degradation in bulk drug and ~4% in the nanoformulation.

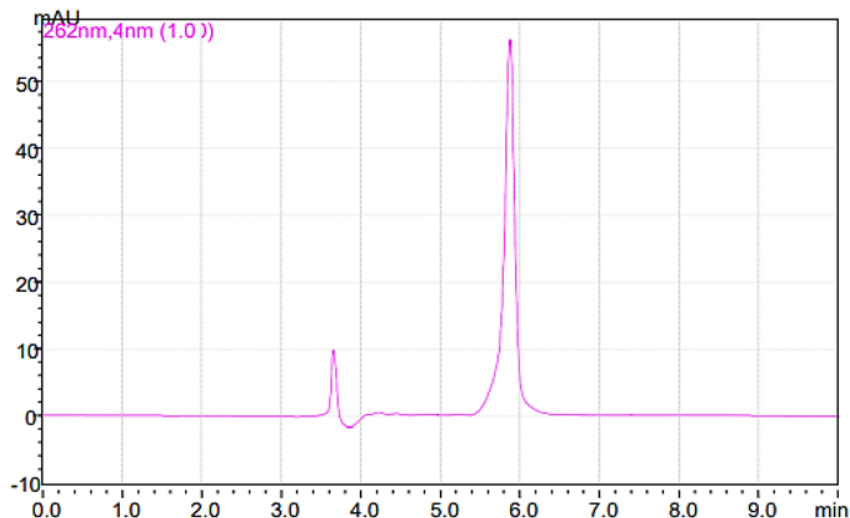


**Figure 5:** HPLC Chromatogram of NL nanoformulation.

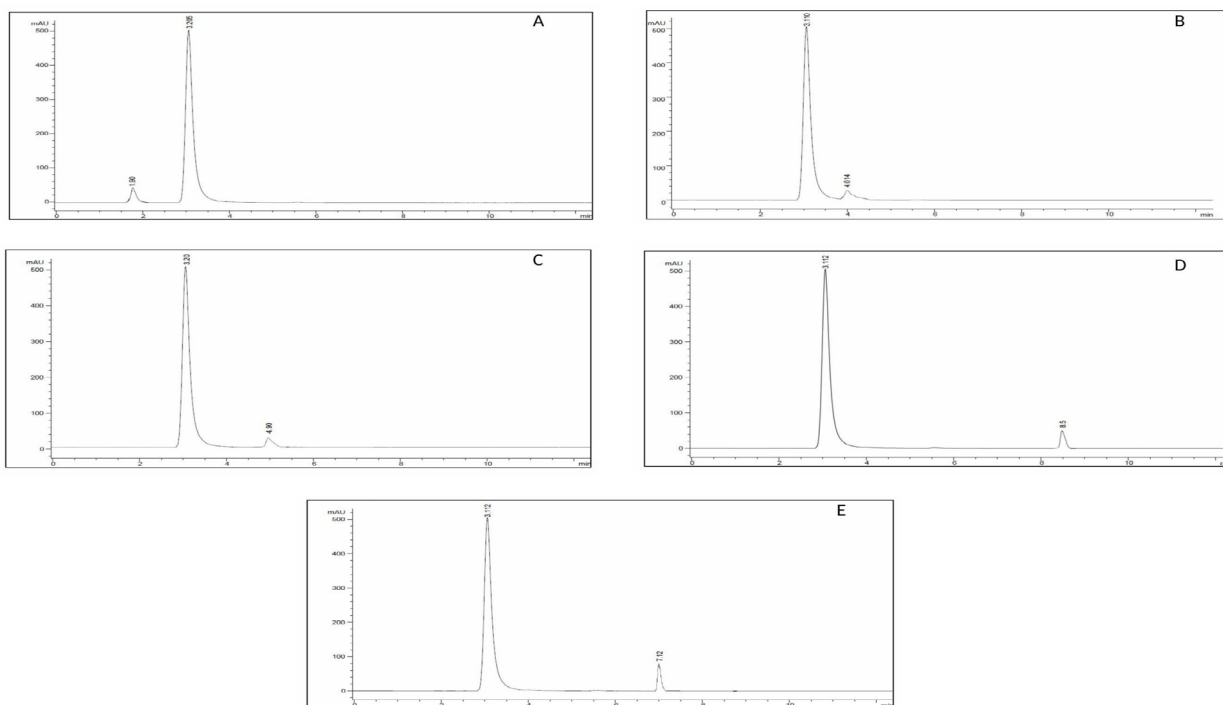


**Figure 6:** HPLC chromatograms of Nilotinib degradants (A) Acidic (0.1 N HCl), (B) Alkaline (0.1 N NaOH), (C) Oxidative (3% H<sub>2</sub>O<sub>2</sub>), (D) Thermal (80°C, 72 hr) and (E) Photolytic (UV 254 nm).

Photofragments F<sub>1</sub>-F<sub>5</sub> were observed, with a radical adduct identified at RT 3.8 min (Figure 6E). The degradation pathway involved C-F bond cleavage followed by radical recombination and partial defluorination. The slightly enhanced stability of the nanoformulation may be due to light absorption and scattering by the nanocarrier matrix, thereby reducing direct drug exposure to UV radiation.<sup>28</sup>

## FTIR analysis

Fourier-Transform Infrared Spectroscopy (FTIR) was employed to further characterize the structural modifications of Nilotinib

under different stress conditions, providing bond-specific insights complementary to the chromatographic data. In the case of acidic hydrolysis (0.1 N HCl, 2 h), the bulk drug exhibited clear attenuation of the N-H stretching vibration at 3309 cm<sup>-1</sup>, reflecting protonation of the amine group, along with a reduction in the amide C=O band at 1635 cm<sup>-1</sup>, consistent with partial cleavage of the amide bond. Additionally, the emergence of distinct CO<sub>2</sub> absorption bands at 2360 and 2339 cm<sup>-1</sup> indicated decarboxylation. These alterations were less pronounced in the nanoformulation, where the nanocarrier matrix appeared to confer measurable protection against acid-induced structural breakdown (Figure 7A).

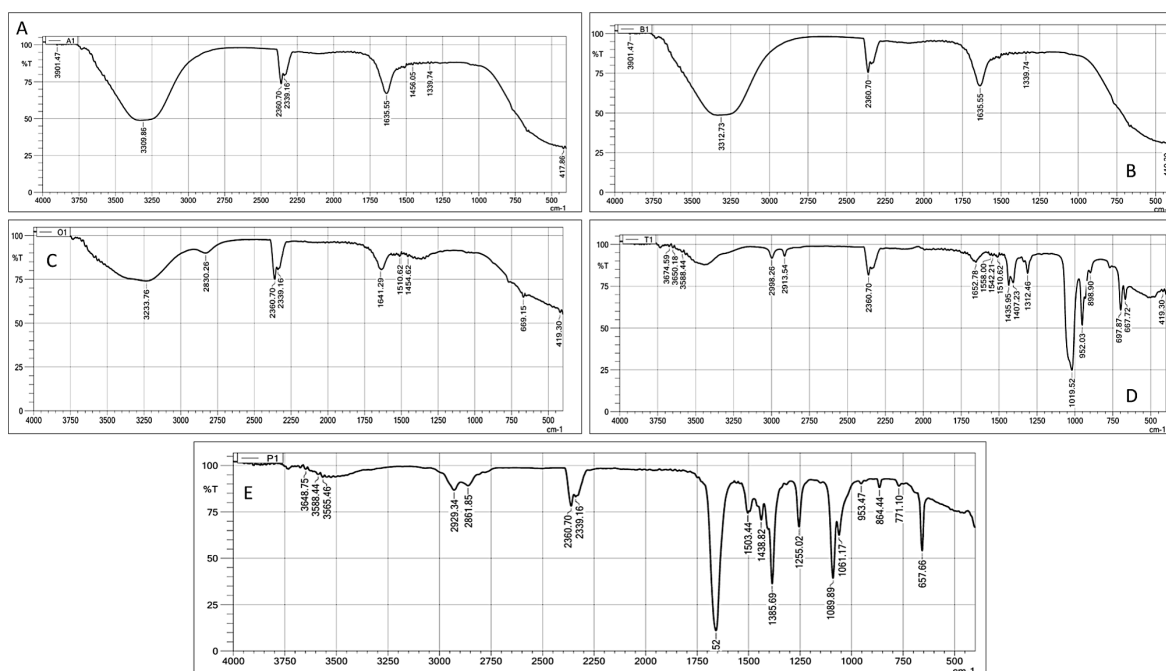


**Figure 7:** FTIR spectra (A) Acidic degradation, (B) Alkaline degradation, (C) Oxidative degradation, (D) Thermal degradation, and (E) Photolytic degradation.

**Table 4: Stress Degradation Results for Nilotinib in Bulk Drug and Nanoformulation.**

| Stress Condition                                    | Bulk Degradation (%) | Nanoformulation Degradation (%) | Major Degradants (RT)                             | Identified Degradants                    | Proposed Degradation Pathway                   |
|-----------------------------------------------------|----------------------|---------------------------------|---------------------------------------------------|------------------------------------------|------------------------------------------------|
| Acidic (0.1 N HCl, 2 hr)                            | 12±1                 | 9±1.5                           | Hydrolyzed (4.504 min)                            | 3.216 min (partial hydrolysis)           | Amide bond cleavage, amine protonation         |
| Alkaline (0.1 N NaOH, 2 hr)                         | 14±0.5               | 11±0.5                          | Dealkylated (4.503, 14.058 min)                   | 7.5 min (ring-opened product)            | Quinoline ring fragmentation, dealkylation     |
| Oxidative (3% H <sub>2</sub> O <sub>2</sub> , 1 hr) | 17±2                 | 13±2                            | N-Oxide (8.058 min)                               | 14.048 min (dimer)                       | Radical-mediated C-N scission, hydroxylation   |
| Thermal (80°C, 72 hr)                               | 4.5±0.2              | 2±0.5                           | Rearranged (4.471 min)                            | 14.041 min (decarboxylated form)         | Demethylation, decarboxylation, polymerization |
| Photolytic (UV 254 nm, 24 hr)                       | 5±0.7                | 4±0.3                           | Photo-fragments (F <sub>1</sub> -F <sub>5</sub> ) | F <sub>3</sub> (3.8 min, radical adduct) | C-F bond cleavage, radical recombination       |

Under alkaline conditions (0.1 N NaOH, 2 h), FTIR spectra of bulk Nilotinib showed marked decreases in both N-H ( $3312\text{ cm}^{-1}$ ) and C=O ( $1635\text{ cm}^{-1}$ ) vibrational intensities, accompanied by broadening of the C-N stretching region around  $1339\text{ cm}^{-1}$ . These spectral features suggest a degradation pathway involving amide cleavage, dealkylation, and quinoline ring fragmentation. In comparison, the nanoformulation retained sharper vibrational bands, indicating reduced susceptibility to base-catalyzed transformations (Figure 7B).

Oxidative stress with 3%  $\text{H}_2\text{O}_2$  (1 hr) produced the most distinctive spectral modifications. Bulk Nilotinib exhibited broadening in the O-H/N-H region at  $3233\text{ cm}^{-1}$  and attenuation of the carbonyl peak at  $1641\text{ cm}^{-1}$ , alongside the appearance of a bending vibration at  $1510\text{ cm}^{-1}$ . These signatures correspond to N-oxide formation, hydroxylation, and oxidative modification of the carbonyl group. The nanoformulation spectrum, however, preserved much of the original drug's vibrational profile, suggesting that encapsulation mitigated the extent of radical-mediated oxidation (Figure 7C).

Thermal degradation ( $80^\circ\text{C}$ , 72 hr) also generated unique FTIR fingerprints. The bulk drug spectrum showed evidence of C-H bond scission with decreased intensity at  $2913\text{ cm}^{-1}$ , while  $\text{CO}_2$ -associated peaks at  $2360\text{ cm}^{-1}$  indicated decarboxylation. Despite these changes, the amide C=O band at  $1662\text{ cm}^{-1}$  remained relatively stable, suggesting partial preservation of backbone structure. The nanoformulation displayed only minor deviations from the control spectrum, highlighting the stabilizing role of excipients at elevated temperatures (Figure 7D).

Photolytic exposure (UV 254 nm, 24 hr) resulted in scission of aliphatic C-H bonds, with characteristic absorptions between  $2861$  and  $2929\text{ cm}^{-1}$ , and the appearance of a new band at  $1568\text{ cm}^{-1}$ , consistent with radical adduct formation. These findings imply C-F bond cleavage, radical recombination, and partial defluorination as the dominant photodegradation pathways. Once again, the nanoformulation demonstrated attenuated changes, which may be attributed to light scattering or absorption by the nanocarrier matrix that reduced direct UV exposure of the encapsulated drug (Figure 7E).

## CONCLUSION

The present study provides an integrated degradation profile of Nilotinib and its nanoformulation through complementary HPLC and FTIR analyses. Nilotinib was found to be most vulnerable to oxidative stress, followed by acidic and alkaline hydrolysis, while thermal and photolytic conditions induced comparatively minor degradation. Chromatographic data clearly differentiated Nilotinib from its degradation products, while FTIR elucidated bond-level structural modifications, together confirming the reliability of the analytical approach. In all cases, the nanoformulation exhibited lower degradation percentages and less pronounced spectral alterations than the bulk drug,

confirming that the nanocarrier matrix confers significant protection against stress-induced chemical transformations. These findings not only establish a validated stability-indicating method but also demonstrate that nanoformulation enhances the resilience of Nilotinib, thereby supporting its development for safer and more stable therapeutic applications.

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## ABBREVIATIONS

**NL:** Nilotinib; **HPLC:** High-Performance Liquid Chromatography; **AQbD:** Analytical Quality by Design; **ICH:** International Council for Harmonisation; **RP-HPLC:** Reverse-phase High-Performance Liquid Chromatography; **CQAs:** Critical Quality Attributes; **CMs:** Critical Method Parameters; **FTIR:** Fourier-transform infrared spectroscopy; **PLGA:** Poly-lactic-co-glycolic acid; **PEG:** Polyethylene glycol; **FA:** Folic Acid; **ATP:** Analytical Target Profile; **CAAs:** Critical Analytical Attributes; **BBD:** Box-Behnken Design; **MLR:** Multiple Linear Regression; **RSM:** Response Surface Methodology; **LOD:** Limit of Detection; **LOQ:** Limit of Quantification; **SD:** Standard Deviation; **RSD:** Relative Standard Deviation; **OPA:** Ortho-phosphoric acid; **KBr:** Potassium bromide.

## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

## AUTHORS CONTRIBUTIONS

All authors contributed to the study's conception and design. Methodology, data collection, analysis, and writing of the original draft were performed by R A U.Z, S, S and D.Z, While supervision, material, and facility support were provided by V. B. All authors read and approved the final manuscript.

## SUMMARY

This study focused on the development, optimization, and validation of a stability-indicating RP-HPLC method for the quantitative estimation of Nilotinib in bulk drug and nanoformulated systems using an Analytical Quality by Design (AQbD) approach. Critical analytical attributes, including retention time, peak tailing, and theoretical plate count, were systematically evaluated through Plackett-Burman screening followed by Box-Behnken optimization to establish a robust analytical design space.

The optimized chromatographic conditions enabled efficient separation of Nilotinib from its degradation products and demonstrated excellent linearity, accuracy, precision, sensitivity,

robustness, and system suitability in compliance with ICH Q2 (R1) guidelines. The validated method was successfully applied to quantify Nilotinib in nanoformulations without interference from excipients.

Forced degradation studies under acidic, alkaline, oxidative, thermal, and photolytic stress conditions confirmed the stability-indicating nature of the method. Complementary FTIR analysis provided molecular-level insights into stress-induced structural modifications. Across all stress conditions, the nanoformulation exhibited reduced degradation compared to the bulk drug, indicating enhanced stability imparted by the nanocarrier system.

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**Table S1: Experimental Factors and Responses in Plackett-Burman Design.**

| Run | Factor    |                          |           |                    |                  | Response         |                |                |                         |
|-----|-----------|--------------------------|-----------|--------------------|------------------|------------------|----------------|----------------|-------------------------|
|     | Flow Rate | Mobile Phase Composition | Buffer pH | Column Temperature | Injection Volume | Injection Volume | Retention Time | Tailing Factor | Theoretical Plate Count |
| 1   | 1.3       | 85                       | 3         | 30                 | 20               | 12               | 1.6            | 2490           | 756000±1362.03          |
| 2   | 1.3       | 80                       | 3         | 25                 | 20               | 7                | 1.45           | 2435           | 755000±1258.67          |
| 3   | 1.3       | 90                       | 4         | 30                 | 20               | 15               | 1.4            | 2552           | 760200±1475.89          |
| 4   | 0.7       | 85                       | 3         | 25                 | 20               | 20               | 1.3            | 2552           | 770000±1354.22          |
| 5   | 1.3       | 80                       | 4         | 30                 | 10               | 12               | 1.3            | 2475           | 748000±1421.56          |
| 6   | 0.7       | 90                       | 3         | 30                 | 10               | 7                | 1.15           | 2540           | 757000±1289.34          |
| 7   | 0.7       | 80                       | 4         | 30                 | 20               | 12               | 1.19           | 2552           | 757000±1310.45          |
| 8   | 1.3       | 85                       | 4         | 25                 | 10               | 5                | 1.14           | 2470           | 766000±1502.78          |
| 9   | 0.7       | 85                       | 3         | 30                 | 10               | 15               | 1.23           | 2580           | 768200±1198.34          |
| 10  | 1.3       | 80                       | 3         | 25                 | 10               | 20               | 1.17           | 2490           | 756700±1387.12          |
| 11  | 0.7       | 80                       | 4         | 25                 | 20               | 19               | 1.11           | 2540           | 752000±1456.90          |
| 12  | 0.7       | 85                       | 4         | 25                 | 10               | 5                | 1.27           | 2520           | 776000±1324.67          |

**Table S2: Analysis of variance along with p-value.**

| Tailing Factor             |         | Retention Time             |          | Theoretical Plate          |         |
|----------------------------|---------|----------------------------|----------|----------------------------|---------|
| Factor                     | p Value | Factor                     | p Value  | Factor                     | p Value |
| A-Flow Rate                | 0.0009  | A-Flow Rate                | < 0.0001 | A-Flow Rate                | 0.0011  |
| B-Mobile Phase Composition | 0.0042  | B-Mobile Phase Composition | 0.0006   | B-Mobile Phase Composition | 0.0036  |
| C-Buffer pH                | 0.0032  | C-Buffer pH                | 0.0034   | C-Buffer pH                | 0.0028  |
| D-Column Temperature       | 0.0002  | D-Column Temperature       | 0.0034   | D-Column Temperature       | 0.0008  |
| E-Injection Volume         | 0.0025  | E-Injection Volume         | 0.0013   | E-Injection Volume         | 0.0028  |

**Table S3: Comparison between expected and experimental values.**

| Response Parameter                   | Predicted Value | Observed Value | Units  | % Error Deviation |
|--------------------------------------|-----------------|----------------|--------|-------------------|
| Tailing Factor (Y <sub>1</sub> )     | 1.15            | 1.12           | -      | 2.61%             |
| Retention Time (Y <sub>2</sub> )     | 3.6             | 3.06           | min    | 0.46%             |
| Theoretical Plates (Y <sub>3</sub> ) | 5700            | 5675           | plates | 0.44%             |

**Table S4: Numerical optimization solutions for developed HPLC method.**

| Sl. No. | Flow rate (mL/min) | Mobile Phase Composition | Column Temperature (°C) | Retention Time | Theoretical Plates | Tailing Factor | Desirability |          |
|---------|--------------------|--------------------------|-------------------------|----------------|--------------------|----------------|--------------|----------|
| 01.     | 1.000              | 80.00                    | 25.000                  | 3.060          | 5245.625           | 1.077          | 0.9982       | Selected |
| 02.     | 1.000              | 85.000                   | 30.000                  | 6.111          | 5396.667           | 1.094          | 0.9520       |          |
| 03.     | 1.200              | 90.000                   | 30.000                  | 6.099          | 5151.250           | 1.103          | 0.8902       |          |

**Table S5: Recovery study.**

| Concentration<br>( $\mu\text{g/mL}$ ) | Standard NL       | NL Nano<br>formulation |
|---------------------------------------|-------------------|------------------------|
|                                       | % Recovery        | % Recovery             |
| 8                                     | 97.65 $\pm$ 0.12% | 97.50 $\pm$ 0.17%      |
| 10                                    | 95.15 $\pm$ 0.21% | 96.07 $\pm$ 0.25%      |
| 12                                    | 98.14 $\pm$ 0.13% | 97.21 $\pm$ 0.15%      |

**Table S6: Robustness studies.**

| Sl. No. | Conditions           | Retention Time (min) | AUC             | Tailing Factor   |
|---------|----------------------|----------------------|-----------------|------------------|
| 01.     | Standard Condition   | 3.745 $\pm$ 0.10     | 28432 $\pm$ 115 | 0.771 $\pm$ 0.06 |
| 02.     | Flow rate 0.8 mL/min | 3.058 $\pm$ 0.21     | 28789 $\pm$ 144 | 0.921 $\pm$ 0.04 |
| 03.     | Flow rate 1.2 mL/min | 2.324 $\pm$ 0.07     | 28658 $\pm$ 84  | 0.811 $\pm$ 0.05 |
| 04.     | Wavelength 265       | 3.068 $\pm$ 0.415    | 22698 $\pm$ 446 | 0.974 $\pm$ 0.03 |
| 05.     | Wavelength 257       | 4.353 $\pm$ 0.71     | 22478 $\pm$ 209 | 0.79 $\pm$ 0.07  |
| 06.     | Mobile phase 50:50   | 7.647 $\pm$ 0.7      | 21456 $\pm$ 304 | 0.745 $\pm$ 0.05 |
| 07.     | Mobile phase 80:20   | 3.066 $\pm$ 0.22     | 24789 $\pm$ 67  | 0.861 $\pm$ 0.10 |

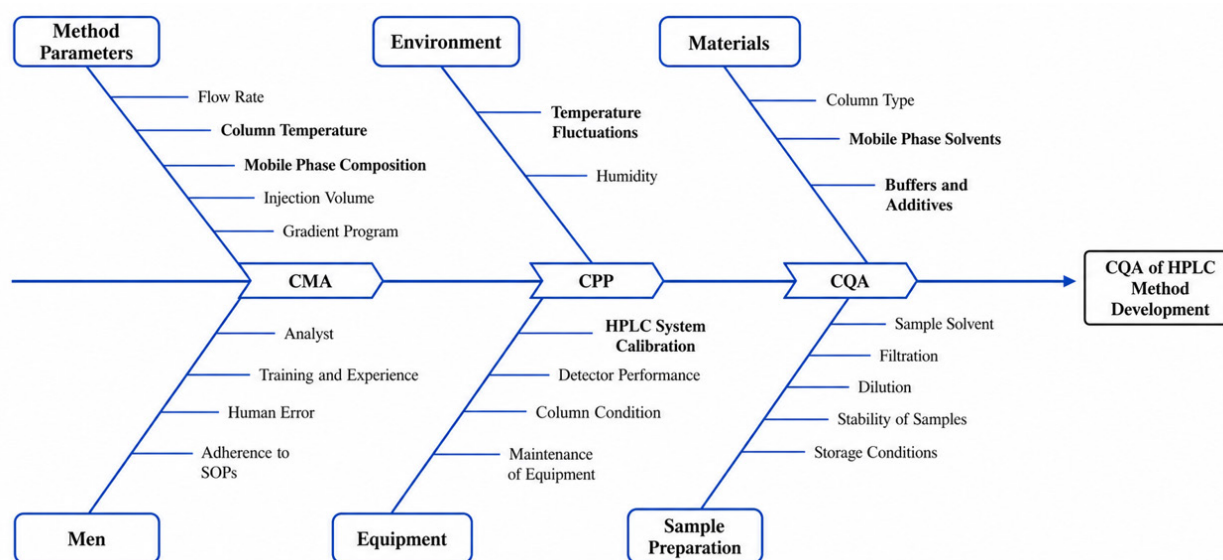


**Figure S1:** The Ishikawa Diagram for Risk Assessment in HPLC Method Development identifies critical factors influencing the method's robustness and ruggedness. It highlights Critical Method Attributes (CMAs), Critical Process Parameters (CPPs), and Critical Quality Attributes (CQAs) to ensure consistent and reliable performance across varying conditions.<sup>3,2</sup>