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A QBD-BASED STABILITY-INDICATING RP-HPLC METHOD FOR LAROTRECTINIB: DEGRADATION KINETICS AND INTEGRATED WHITE, GREEN, AND BLUE ANALYTICAL ASSESSMENT

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ABSTRACT
Background: Larotrectinib, a selective TRK inhibitor, received FDA approval on April 10, 2025, for treating solid tumors with NTRK gene fusions. Despite its therapeutic significance, no RP-HPLC method using a Quality-by-Design (QbD) framework has been reported. This study aimed to develop and validate a QbD-based RP-HPLC method for larotrectinib estimation. **Methodology:** Critical Analytical Parameters (CAPs) were identified using a Plackett–Burman Design and optimized via a Central Composite Design (CCD). Separation was achieved on a Sunfire C18 column (250 × 4.6 mm, 5 µm) with a mobile phase of 0.1% OPA and acetonitrile (70:30, v/v), flow rate 1.0 mL/min, injection volume 10 µL, and detection at 262 nm. Optimized conditions from the Method Operable Design Region (MODR) gave a desirability value of 1. **Results and Discussion:** The method achieved sharp separation with a retention time of 2.2 min in a 5-minute runtime. Validation per ICH Q2(R1) confirmed linearity (12.5–75 µg/mL, R^2 = 0.9998), intra- and inter-day precision (%RSD < 2%), mean recovery of 99.29%, and sensitivity with DL 0.30 µg/mL and QL 0.92 µg/mL. Forced degradation studies revealed zero-order kinetics under 0.1 N HCl, 0.5 N NaOH, and thermal stress, and first-order kinetics under 0.5 N HCl, 0.1N NaOH, 3% and 5% H_2O_2, and water. Greenness, blueness, whiteness, and sustainability were assessed using AMGS, AGREE, ComplexMoGAPI, BAGI, RGB, and EVG tools, yielding favourable outcomes. **Conclusion:** The developed QbD-based RP-HPLC method is robust, validated, and stability-indicating, suitable for quality control, regulatory submissions, and bioanalysis of larotrectinib.

INTRODUCTION

Larotrectinib (LTB) is a selective tropomyosin receptor kinase (TRK) inhibitor that received complete Food and Drug Administration (FDA) approval on April 10, 2025 [1]. It is used to treat tumors harboring Neurotrophic Tyrosine Receptor Kinase (NTRK) gene fusions, which are rare genetic alterations observed in cancers such as soft tissue sarcomas, thyroid, and lung tumors. As the first tissue-agnostic NTRK inhibitor, LTB marks a significant milestone in precision oncology by targeting specific molecular pathways that drive tumor growth. Compared

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to conventional chemotherapy, LTB offers a highly targeted, tumor-agnostic treatment with promising clinical outcomes in both pediatric and adult patients [2,3]. It is marketed by Bayer and Loxo Oncology [4] as an amorphous off-white to pinkish powder that is freely soluble in water and common organic solvents, such as methanol. Its chemical structure ($C_{21}H_{22}F_2N_6O_2$, 428.44 g/mol) features pyrazole and indazole rings, enabling potent and selective inhibition of TRK A, B, and C by blocking aberrant signaling in cancer cells with NTRK fusions [5,6,7]. Despite the therapeutic significance of LTB, it is not yet included in any major pharmacopoeia, highlighting the need for a validated in-house analytical method. The existing literature on LTB is limited to a few LC–MS/MS techniques for bioanalytical applications in biological matrices [8–10] & a single reversed-phase High Performance Liquid Chromatography (RP-HPLC) method [11] employing conventional C18 columns with binary mobile phases under gradient conditions. These approaches were developed using the traditional one-factor-at-a-time (OFAT) method, which can restrict method robustness, prolong development timelines & compromise reproducibility [12]. A stereoselective normal-phase LC method has also been reported for separating and quantifying LTB and its isomeric impurities using an immobilized cellulose tris-(3-chloro-5-methyl phenyl carbamate) chiral stationary phase [13]; however, its application is confined to chiral impurity profiling. Notably, none of these reported methods apply an Analytical Quality-by-Design (AQbD) framework to systematically optimize method parameters, conduct detailed kinetic degradation profiling of LTB under varied stress conditions, an essential aspect for formulation development and determination of optimal storage conditions, and integrate environmental sustainability assessment to align with green analytical chemistry principles. To address these gaps, the present study developed the first AQbD-based stability-indicating RP-HPLC method for LTB. The method incorporates a risk-based design, comprehensive forced degradation studies (acidic, basic, oxidative, thermal, and neutral), and kinetic modeling to elucidate degradation behavior. Additionally, environmental sustainability was quantitatively evaluated using Analytical Greenness Metric (AGREE), ComplexModelGAPI (ComplexMoGAPI), Blue Applicability Grade Index **(**BAGI), and whiteness metrics, including Red, Green, Blue (RGB) and Efficient, Valid, Green (EVG), ensuring the method is robust, regulatory-compliant, and eco-conscious. This approach offers a practical solution for routine quality control in the absence of an official pharmacopoeial monograph for LTB.

MATERIALS AND METHODS

Chromatographic analysis was performed using a Waters Alliance e2695 separation module equipped with a Waters 2998 PDA detector. A Sunfire C18 column (250 × 4.6 mm, 5 µm) was used for separation.0.45 µm HPLC-grade membrane filters were used for mobile phase and sample filtration (Millipore Sigma, St. Louis, MO, USA). The experimental design was carried out using Design Expert software (v13.1.0, Stat-Ease 360). Data acquisition & system control were performed using Empower 2 software (Waters Corporation). Acetonitrile (ACN)- HPLC grade was obtained from Merck Ltd. (Mumbai, India). Pure LTB (99.8%) reference standard was procured from Akrivis Pharma, Ltd. (Hyderabad, India). Analytical grade hydrochloric acid (HCl), sodium hydroxide (NaOH), orthophosphoric acid (OPA) & hydrogen peroxide (H_2O_2) were procured from Rankem (Gurugram, India). HPLC-grade water was used throughout the study for all dilutions & mobile phase preparations.

Preparation of Mobile Phase and Diluent

To prepare the buffer, a 1 mL solution of OPA was diluted with HPLC water to a total volume of 1000 mL, resulting in a 0.1% OPA solution. The mobile phase consisted of a 70:30 volume ratio of ACN and 0.1% OPA. A 50:50 volume mixture of ACN and water was used as diluent.

Sample and Standard Preparation

A standard stock was prepared by dissolving 25 mg of LTB in a diluent and making up to 50 mL (500 µg/mL). For the working standard, 1 mL of the stock solution was diluted to 10 mL with diluent to obtain a 50 µg/mL solution. To prepare a sample stock, a sample portion equivalent to 100 mg was transferred to a 100 mL volumetric flask. Then, 50 mL of diluent was added, and the volume was made up to 100 mL with diluent. (1000 µg/mL). 0.5 mL of the filtered sample stock solution was diluted to 10 mL with diluent to obtain a 50 µg/mL sample working solution

Method Development

Method development for the RP-HPLC assay was conducted in accordance with the ICH Q14 guidelines for analytical procedure development [14,15], with risk management principles aligned with those outlined in ICH Q9 [16]. The first step in this approach involved defining the Method Analytical

Target Profile (MATP), which outlines the expected performance characteristics of the analytical method. Subsequently, an initial risk assessment using Failure Mode and Effects Analysis (FMEA) was conducted to identify and rank the potential factors that affect the method's performance. The FMEA highlighted several Critical Analytical Parameters (CAPs), including organic solvent composition, injection volume, flow rate, column temperature, and detection wavelength, as having the most significant potential impact on method performance criteria. To screen these variables efficiently, a Plackett–Burman Design (PBD) was employed, allowing multiple factors to be evaluated simultaneously with a minimal number of runs [17]. Each factor was tested at +1 and -1 levels, and its influence on Critical Analytical Attributes (CAAs), specifically retention time (Rt), tailing factor (Tf), and number of theoretical plates (NTP), was assessed. Pareto analysis was employed to identify high-impact variables [18].

Optimization and Establishment of Design Space

Method optimization was performed within a QbD framework using Response Surface Methodology (RSM) to refine the selected CAPs systematically [19,20]. Based on the screening results, flow rate (0.9–1.1 mL/min), organic phase composition (25-35%), and column temperature (27–33°C) were identified as the most influential variables. A CCD with 20 experimental runs was used to model the combined and interactive effects of these factors on CAAs. The factor levels were coded as low (−1), high (+1), and center (0) points, with the axial points included to capture the curvature in the design space. A second-order polynomial regression model was developed to describe the relationships between the factors and responses, and the model's fit was confirmed using ANOVA (R^2, adjusted R^2, and p-values). Contour plots and 3D response surface plots were generated to visualize the main effects and interactions. Numerical optimization using a desirability function was applied to identify the Method Operable Design Region (MODR).

The range of operating conditions where the method reliably meets the MATP criteria is as follows: Rt < 3 min, Tf between 1.0 and 1.5, and NTP > 2000. In line with QbD principles, confirmatory experiments under optimized conditions validated the predictive capability and robustness of the model. The final method was developed to minimize solvent usage and maintain an eco-friendly approach, thereby supporting the sustainability goals of modern analytical science.

Validation of the Proposed Analytical Method

As per ICH (2023) and CDER (1994) guidelines, the method was validated against established acceptance criteria to ensure reliability and reproducibility. System suitability was confirmed with %RSD of peak area and retention time ≤ 2.0%, theoretical plates ≥ 2000, and tailing factor ≤ 2.0. Accuracy was demonstrated by mean recoveries within 98–102% across all levels, while precision showed intra- and inter-day %RSD ≤ 2.0%. Linearity was excellent with $R^2 \geq 0.999$. LOD and LOQ were determined from signal-to-noise ratios of approximately 3:1 and 10:1, respectively. Specificity testing confirmed no interfering peaks at analyte or impurity retention times, and robustness trials showed no significant changes in retention time, resolution, or peak shape upon minor, deliberate method variations.

Stress-Induced Degradation Studies of Larotrectinib

The stability of LTB was evaluated through forced degradation studies carried out in accordance with ICH Q1A(R2) guidelines [23]. A stock solution of LTB (2000 µg/mL) was prepared and serially diluted to obtain working solutions for the stress testing. After exposure to the specified stress conditions, the samples were neutralized as required, diluted with the mobile phase, and analyzed using the validated RP-HPLC method [24]. The applied stress conditions and overall experimental workflow are summarized in Figure 1.

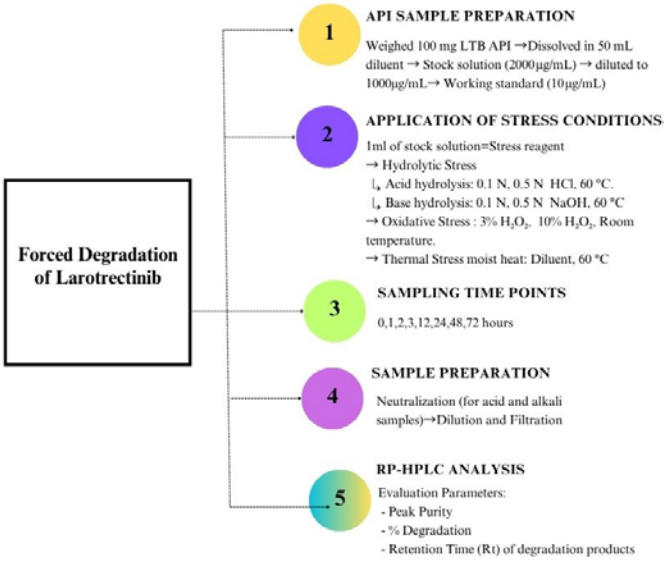


Figure 1: Stepwise Protocol for Forced Degradation of Larotrectinib

The workflow includes API sample preparation, application of stress conditions (acidic, basic, oxidative, and thermal), sampling at specified time points, sample neutralization and preparation, followed by RP-HPLC analysis.

RESULTS AND DISCUSSION

Method Development

LTB is a selective TRK inhibitor featuring aromatic rings, UV-absorbing chromophores, balanced polarity, and chemical stability, making it suitable for RP-HPLC with UV detection [25]. A C18 column was selected because of its strong hydrophobic retention characteristics, which make it ideal for LTB with a moderately non-polar aromatic structure [26]. An AQbD-based RP-HPLC method was essential for LTB because existing analytical reports primarily rely on traditional OFAT method development without statistically driven optimization, robustness prediction, or integrated greenness assessment. Such approaches can be inefficient, may not fully capture the Critical Quality Attributes (CQAs) fully capture the Critical Quality Attributes (CQAs), and often lack predictive capability for method performance under minor variations in parameters. Various mobile phases, including methanol, ACN, phosphate, acetate buffers & OPA (pH 3.0–4.0, were screened. ACN with OPA yielded sharp symmetrical peaks with minimal tailing. In contrast to previously reported buffer-based mobile phases, OPA was chosen to simplify mobile phase preparation while providing effective pH control, good peak shape, and minimal column fouling [27]. The method was developed using a risk-based QbD framework in line with ICH Q14 for analytical procedure development, ICH Q8(R2) for systematic pharmaceutical design, and ICH Q9 for risk management. MATP was defined to ensure appropriate Rt, TF, and NTP levels. CQAs, including organic phase percentage, flow rate, and column temperature, have been identified as key factors influencing MATP. An initial FMEA (Table 1) was used to prioritize the method variables, followed by a PBD to screen five parameters. (Table 2) These factors were selected based on preliminary trials that focused on peak parameters & minimizing run time. A PBD was employed to screen the factors influencing the CAAs, namely, Rt (Y1), NTP (Y2), and Tf (Y3). The influence of these factors was visualized using a Pareto chart (Figure 2), which facilitated the identification of statistically significant effects above the t-value & Bonferroni limit. The results indicated that the flow rate, mobile phase composition & temperature had positive effects on the retention time, while the flow rate also positively impacted NTP and Tf.

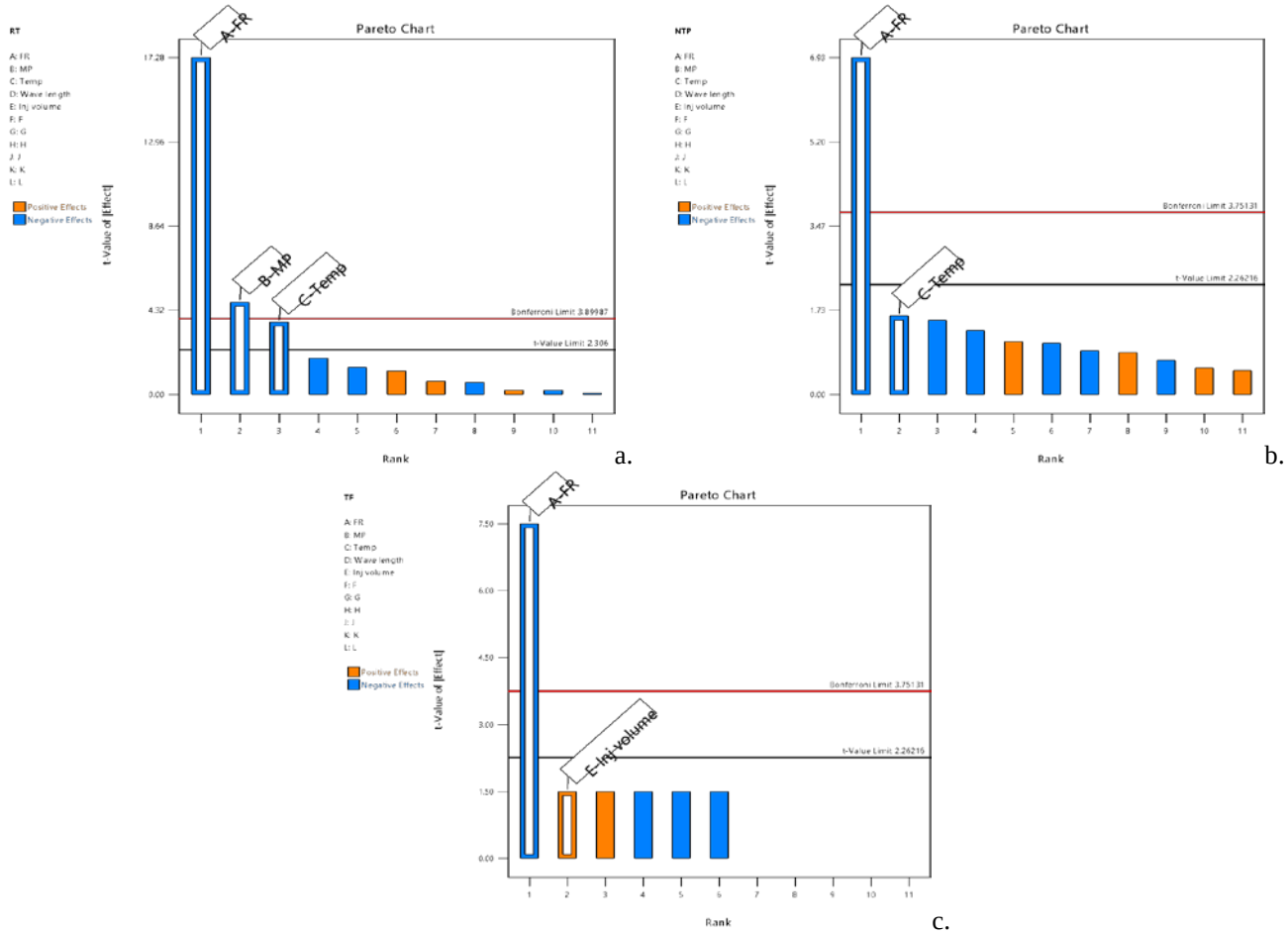


Figure 2: Pareto Analysis of a. Retention time of LTB b. Number of Theoretical Plates c. Tailing factor

Table 1: Failure Mode and Effects Analysis (FMEA) for Method Variables

Potential Method Variable	Potential Failure Mode	Implication	Severity (S)	Occur-rence (O)	Detect-ability (D)	RPN = S×O×D	Decision
Organic Solvent Proportion	Inappropriate mobile phase composition	Poor resolution, altered *Rt*	8	7	4	224	Selected for screening by PBD
Injection Volume	Overloading or insufficient signal	Peak broadening or low response	7	6	5	210	Selected for screening by PBD
Flow Rate	Unstable flow or deviation from set rate	Varies retention time and affects resolution	8	5	5	200	Selected for screening by PBD
Column Temperature	Inconsistent or inappropriate temperature control	Variability in peak shape and retention	7	5	5	175	Selected for screening by PBD
Detection Wavelength	Inaccurate wavelength setting	Reduced sensitivity, incorrect peak quantification	9	4	5	180	Selected for screening by PBD
pH of Mobile Phase	Improper pH adjustment	Minor shift in retention time	4	4	4	64	Low impact – not screened
Organic modifier Conc.	Incorrect preparation	Slight changes in peak symmetry or efficiency	3	3	5	45	Low impact – not screened

Optimization and Establishment of Design Space

CCD was utilized to generate a quadratic polynomial model to examine the individual and interactive effects of the variables. CCD was chosen because of its efficiency in constructing a second-order model without the complexity of a full three-level factorial design while also minimizing variability in regression estimates. Following screening, three variables–flow rate (X1), mobile phase ratio (X2), and temperature (X3)–were identified as having the most substantial impact on CAAs and were thus selected for optimization. These variables were studied at three coded levels (low, medium, and high) using a fixed analyte concentration across 20 experimental runs, including eight factorial, six axial, and six center points (Table 3). Ranges for X1, X2, and X3 were set from preliminary trials and practical method constraints to balance speed, efficiency, and peak shape for LTB. Design Expert® software was employed to systematically explore the primary, interaction & quadratic effects of these parameters on the responses of Rt(Y1), NTP(Y2) & tailing factor(Y3). ANOVA confirmed the significance of the model, with high R^2, adjusted R^2, and non-significant lack-of-fit values, as shown in Table 4. The response (Y) was modeled using a second-order polynomial equation:

$$Y = \alpha 0+\alpha 1X1+\alpha 2X2+\alpha 3X1X2+\alpha 4X12+\alpha 5X22 \quad \ldots\ldots\ldots\ldots (1)$$

where Y is the observed response; α0 is the intercept; α1 & α2 are linear coefficients; X1X2 denotes the interaction; & X12 & X22 are the quadratic terms of the independent variables [28].

Table 2: Experimental Design for Screening

Std	Run	A	B	C	D	E	F	G	H	J	K	L	RT	NTP	TF
		ml/min	%	0 C	nm	ul	num	num	num	num	num	num	min	num	num
2	10	0.8	40	35	257	15	1	1	-1	-1	-1	1	2.561	4352.6	1.3
4	12	0.8	40	25	267	15	-1	1	1	1	-1	-1	2.899	3966.8	1.3
5	7	0.8	20	35	257	15	1	-1	1	1	1	-1	2.882	4444.8	1.3
6	4	0.8	20	25	267	5	1	1	-1	1	1	1	3.260	4904.8	1.3
10	2	0.8	40	35	267	5	-1	-1	1	-1	1	1	2.895	3815.4	1.2
12	9	0.8	20	25	257	5	-1	-1	-1	-1	-1	-1	3.265	5187.7	1.3
1	8	1.2	40	25	267	15	1	-1	-1	-1	1	-1	1.908	2720.8	1.2
3	5	1.2	20	35	267	5	1	1	1	-1	-1	-1	1.997	1154.9	1.1
7	11	1.2	20	25	257	15	-1	1	1	-1	1	1	2.166	1360.8	1.1
8	6	1.2	40	25	257	5	1	-1	1	1	-1	1	1.903	2789.5	1.1
9	3	1.2	40	35	257	5	-1	1	-1	1	1	-1	1.763	2474.7	1.1
11	1	1.2	20	35	267	15	-1	-1	-1	1	-1	1	2.009	1198.0	1.1

A Flow rate, B-Mobile phase C-Temperature D- Wavelength E-Injection volume F,G,H,J,K,L-Dummy Variables, RT-Retention time, NTP- Number of theoretical Plates, TF- Tailing factor

Table 3: Central Composite Design (CCD) matrix with coded and actual factor levels

Factors and coded levels used in the CCD							
Factor	**Name**	**Units**	**Coded Low(–1)**	**Coded Low(0)**	**Coded High(+1)**	**Responses**	
X1	Flow Rate	mL/min	0.90	1.00	1.10	Retention time (Y1)	
X2	Mobile Phase	%	25.00	30.00	35.00	Number of theoretical Plates (Y2)	
X3	Temperature	°C	27.00	30.00	33.00	Tailing factor (Y3)	
	Central Composite Design matrix						
Std	Run	X1	X2	X3	Y1	Y2	Y3
1	18	0.9	25	27	2.755	3821.1	1.08
2	16	1.1	25	27	2.245	3124.9	1.08
3	5	0.9	35	27	2.595	3437.1	0.98
4	7	1.1	35	27	2.123	3000.2	0.88
5	1	0.9	25	33	2.472	3419.9	1.08
6	20	1.1	25	33	2.049	2734.6	1.08
7	11	0.9	35	33	2.333	3214.8	0.98
8	17	1.1	35	33	1.947	2720.1	0.88
9	9	0.831821	30	30	2.724	3594.4	1.18
10	12	1.16818	30	30	1.962	2657	1.08
11	6	1	21.591	30	2.427	3437.7	1.08
12	2	1	38.409	30	2.202	3006.8	0.83
13	15	1	30	24.9546	2.53	3535.9	0.95
14	4	1	30	35.0454	2.087	2898.5	1
15	13	1	30	30	2.269	3071	1.13
16	3	1	30	30	2.271	3077	1.15
17	10	1	30	30	2.272	3103	1.15
18	14	1	30	30	2.28	3095	1.16
19	19	1	30	30	2.283	3132	1.13
20	8	1	30	30	2.289	3126	1.13

X1 – Flow Rate (mL/min), X2 – Mobile Phase Composition (% v/v), X3 – Column Temperature (°C), Y1 – Retention Time (min), Y2 – Number of Theoretical Plates, Y3 – Tailing Factor.

Perturbation plots and the corresponding 3D response surface graphs were employed to explore and visualize the relationship between the process parameters and CAAs. Complete quadratic regression models were developed for each CAA, as presented in Equations 2-4.

$$\mathbf{Y1(Rt)} = +\mathbf{2.28} - \mathbf{0.2250X_1} - \mathbf{0.0660X_2} - \mathbf{0.1217X_3} + \mathbf{0.0211X_1^2} + \mathbf{0.0111X_2^2} + \mathbf{0.0089X_3^2} + \mathbf{0.0094X_1X_2} + \mathbf{0.0216X_1X_3} + \mathbf{0.0051X_2X_3} \quad \text{.....(2)}$$

For Y1, the negative linear coefficients for all three factors indicate that increasing any of them tends to reduce retention time. However, the positive quadratic terms suggest that this effect is not purely linear; beyond a certain point, further increases may slightly raise retention time. The positive interaction terms imply that combinations of factors, particularly X_1 with X_3, have a synergistic effect in increasing retention time.

$$\boldsymbol{Y2(NTP)} = \mathbf{3100.98} - \mathbf{284.81}\boldsymbol{X_1} - \mathbf{106.39}\boldsymbol{X_2} - \mathbf{173.24}\boldsymbol{X_3} + \mathbf{6.82}\boldsymbol{X_1^2} + \mathbf{40.96}\boldsymbol{X_2^2} - \mathbf{39.17}\boldsymbol{X_3^2} + \mathbf{56.24}\boldsymbol{X_1X_2} - \mathbf{5.86}\boldsymbol{X_1X_3} - \mathbf{36.14}\boldsymbol{X_2X_3} \quad \text{.....(3)}$$

The number of theoretical plates (Y_2) is affected by the three factors (X_1, X_2, X_3). The large negative linear coefficients indicate that increasing any of the three factors generally reduces column efficiency. The positive quadratic terms for X_1^2 and X_2^2 suggest that moderate increases in these factors can improve efficiency after an initial decline. In contrast, the negative quadratic term for X_3^2 indicates a consistent drop in efficiency at higher levels. The strong positive interaction between X_1 and X_2 enhances efficiency, while the interactions involving X_3 are negative, meaning that combining high levels of X_3 with other factors tends to reduce performance. Overall, the results suggest that maintaining X_3 at lower levels and optimising X_1 and X_2 in combination can yield the highest number of theoretical plates.

$$\boldsymbol{Y3(Tf)} = +\mathbf{1.14} - \mathbf{0.0270}\boldsymbol{X_1} - \mathbf{0.0747}\boldsymbol{X_2} + \mathbf{0.0062}\boldsymbol{X_3} - \mathbf{0.0250}\boldsymbol{X_1} + \mathbf{0.00}\boldsymbol{X_1X_3} + \mathbf{0.00}\boldsymbol{X_2X_3} - \mathbf{0.0057}\boldsymbol{X_1^2} - \mathbf{0.0676}\boldsymbol{X_2^2} - \mathbf{0.0605}\boldsymbol{X_3^2} \quad \text{.....(4)}$$

For Y3, the negative linear coefficients for X_1 and X_2 indicate that increasing these factors tends to reduce peak tailing, which

is desirable for better peak symmetry. In contrast, X_3 has a small positive linear effect, slightly increasing tailing. All three quadratic terms are negative, meaning that very high levels of any factor will eventually worsen tailing. The interaction effects are negligible here, suggesting that each factor mainly acts independently. Overall, controlling X_1 and X_2 at optimal mid-range values while avoiding excessive X_3 can help maintain sharp, symmetrical peaks. In practical terms, X_1 and X_2 most strongly influence robustness and selectivity, especially when optimised together at mid-range levels. X_3 should be kept moderate, as high levels reduce efficiency and peak shape. Positive X_1–X_2 interaction improves separation, while interactions involving high X_3 are detrimental. Quadratic effects show that extremes of any factor can harm performance. For Y_1, Y_2, and Y_3, the normal probability plots show residuals closely following a straight line, confirming normality. In contrast, the residuals vs. predicted plots display random scatter around zero, indicating homoscedasticity and absence of bias.

The Box–Cox plots suggest no power transformation is needed (λ within the 95% CI), and the predicted vs. actual plots show points lying near the 45° line, demonstrating strong agreement between experimental and predicted values, thereby confirming model adequacy and predictive reliability. Figures 3, 4, and 5 present the normal probability plot of residuals, residuals vs. predicted plot, Box–Cox plot for power transformations, and predicted vs. actual plot for Y_1, Y_2, and Y_3, respectively. The response surface methodology (RSM) plots (Figure 6 (a-f)) were generated to study the interactive effects of critical method variables on chromatographic responses.

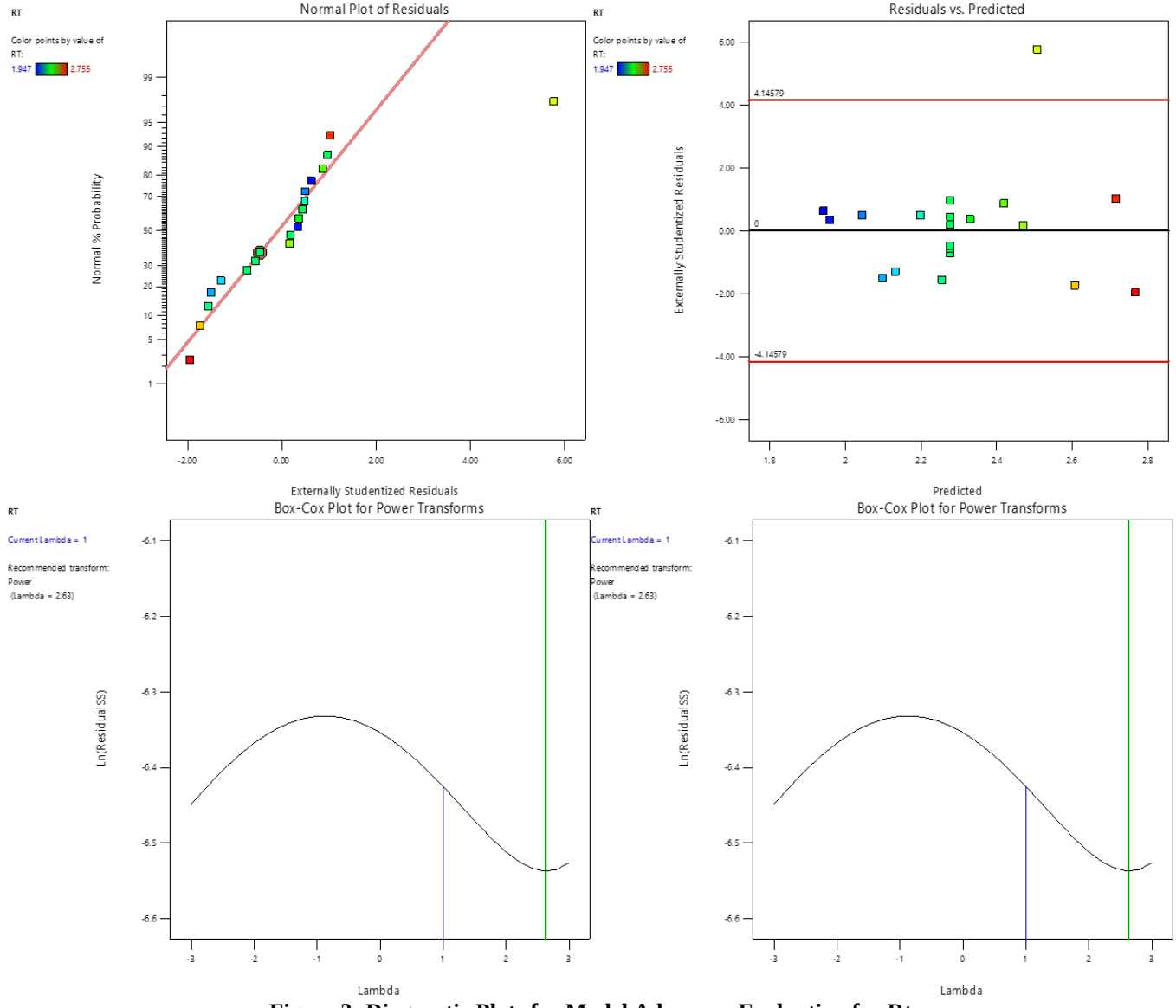


Figure 3: Diagnostic Plots for Model Adequacy Evaluation for Rt

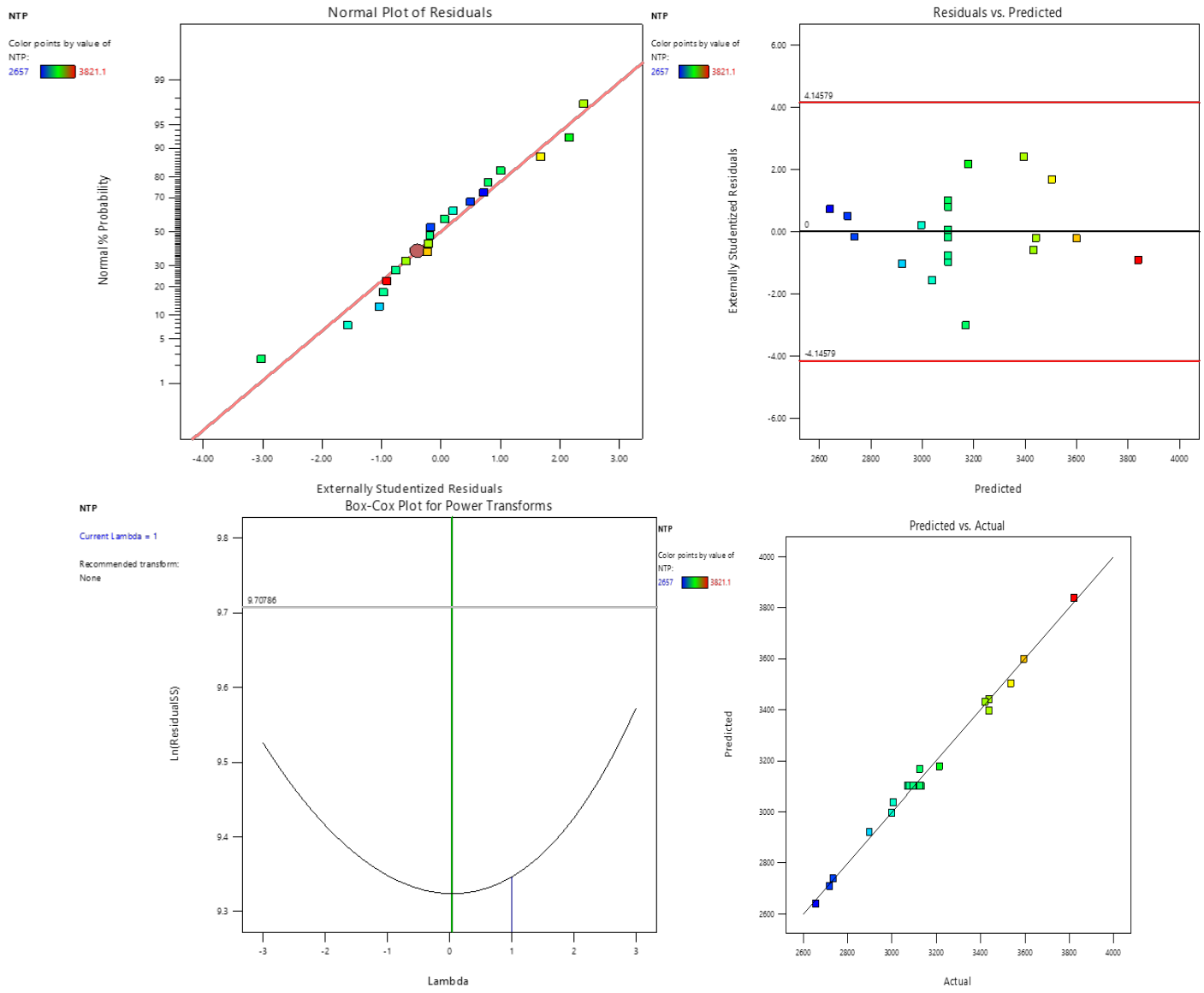
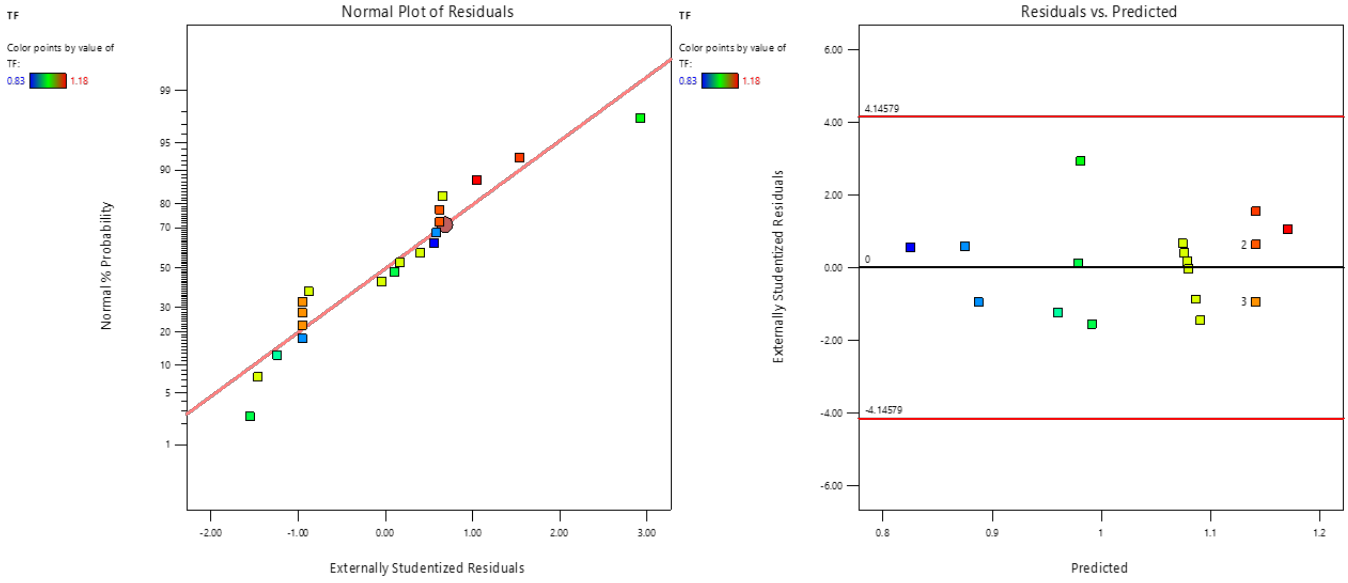


Figure 4: Diagnostic Plots for Model Adequacy Evaluation for NTP



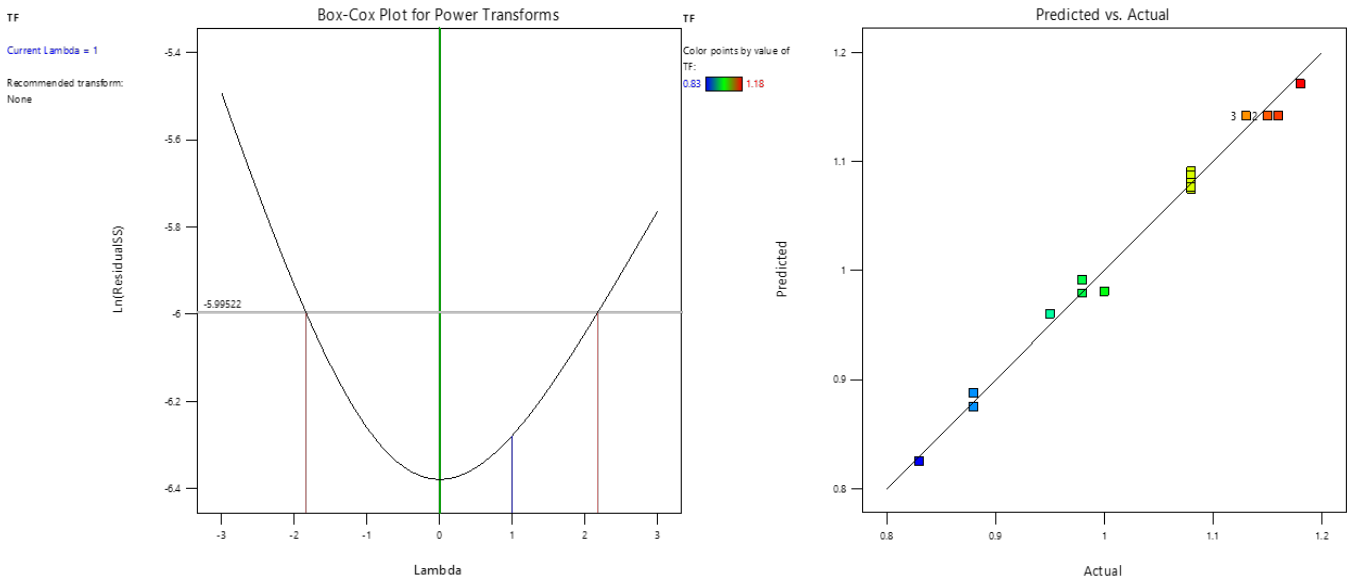


Figure 5: Diagnostic Plots for Model Adequacy Evaluation for Tf

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These optimal conditions are displayed graphically as an overlay plot (Figure 6g), representing the analytical design space region within which MATP could be consistently met, supporting lifecycle management and regulatory flexibility. Numerical optimization using the desirability function (D = 1.000) confirmed that a 30% aqueous phase, flow rate of 1.0 mL/min & column temperature of 30°C provided optimal chromatographic performance with good alignment between the predicted and observed values. The selected parameter ranges were practical, robust & consistent with sustainable analytical practice for routine QC of LTB.

Table 4: ANOVA results for Larotrectinib

Source	Sum of Squares	df	Mean Square	F-value	p-value	Significance
Response 1: Retention Time						
Model	0.9658	9	0.1073	662.33	<0.0001	Significant
X1 – Flow Rate	0.6913	1	0.6913	4266.38	<0.0001	Significant
X2– Mobile Phase Ratio	0.0595	1	0.0595	367.20	<0.0001	Significant
X3– Temperature	0.2023	1	0.2023	1248.38	<0.0001	Significant
X1X2	0.0007	1	0.0007	4.34	0.0639	Not Significant
X1X3	0.0037	1	0.0037	23.09	0.0007	Significant
X2X3	0.0002	1	0.0002	1.30	0.2813	Not Significant
$X1^2$	0.0064	1	0.0064	39.75	<0.0001	Significant
$X2^2$	0.0018	1	0.0018	10.89	0.0080	Significant
$X3^2$	0.0012	1	0.0012	7.11	0.0236	Significant
Residual	0.0016	10	0.0002	–	–	–
Lack of Fit	0.0013	5	0.0003	4.17	0.0715	Not Significant
Pure Error	0.0003	5	0.0001	–	–	–
Cor Total	0.9674	19	–	–	–	–
Response 2: Number of Theoretical Plates						
Model	1.750E+06	9	1.945E+05	169.77	<0.0001	Significant
X1 – Flow Rate	1.108E+06	1	1.108E+06	967.01	<0.0001	Significant
X2 – Mobile Phase Ratio	1.546E+05	1	1.546E+05	134.94	<0.0001	Significant
X3 – Temperature	4.099E+05	1	4.099E+05	357.77	<0.0001	Significant
X1X2	25301.25	1	25301.25	22.09	0.0008	Significant
X1X3	274.95	1	274.95	0.24	0.6348	Not Significant
X2X3	10447.35	1	10447.35	9.12	0.0129	Significant
$X1^2$	670.55	1	670.55	0.59	0.4619	Not Significant

Source	Sum of Squares	df	Mean Square	F-value	p-value	Significance
$X2^2$	24174.40	1	24174.40	21.10	0.0010	Significant
$X3^2$	22112.65	1	22112.65	19.30	0.0013	Significant
Residual	11456.00	10	1145.60	–	–	–
Lack of Fit	8354.67	5	1670.93	2.69	0.1504	Not Significant
Pure Error	3101.33	5	620.27	–	–	–
Cor Total	1.762E+06	19	–	–	–	–
Response 3: Tailing Factor						
Model	0.2002	9	0.0222	118.54	<0.0001	Significant
X1 – Flow Rate	0.0099	1	0.0099	52.89	<0.0001	Significant
X2 – Mobile Phase Ratio	0.0762	1	0.0762	406.30	<0.0001	Significant
X3 – Temperature	0.0005	1	0.0005	2.76	0.1277	Not Significant
X1X2	0.0050	1	0.0050	26.64	0.0004	Significant
X1X3	0.0000	1	0.0000	0.00	1.0000	Not Significant
X2X3	0.0000	1	0.0000	0.00	1.0000	Not Significant
$X1^2$	0.0005	1	0.0005	2.52	0.1432	Not Significant
$X2^2$	0.0659	1	0.0659	350.97	<0.0001	Significant
$X3^2$	0.0528	1	0.0528	281.39	<0.0001	Significant
Residual	0.0019	10	0.0002	–	–	–
Lack of Fit	0.0010	5	0.0002	1.12	0.4503	Not Significant
Pure Error	0.0009	5	0.0002	–	–	–
Cor Total	0.2021	19	–	–	–	–

X1 – Flow Rate (mL/min); X2 – Mobile Phase Ratio (% v/v); X3 – Column Temperature (°C); X1X2, X1X3, X2X3 – Interaction terms; $X1^2$, $X2^2$, $X3^2$ – Quadratic terms. The table presents the ANOVA summary for the three responses: Retention Time (Y1), Number of Theoretical Plates (Y2), and Tailing Factor (Y3). A p-value less than 0.05 indicates statistical significance. "Significant" terms contributed meaningfully to the model, while "Not Significant" terms had limited impact. "Lack of Fit" tests confirm the model's suitability, with non-significant values indicating good fit.

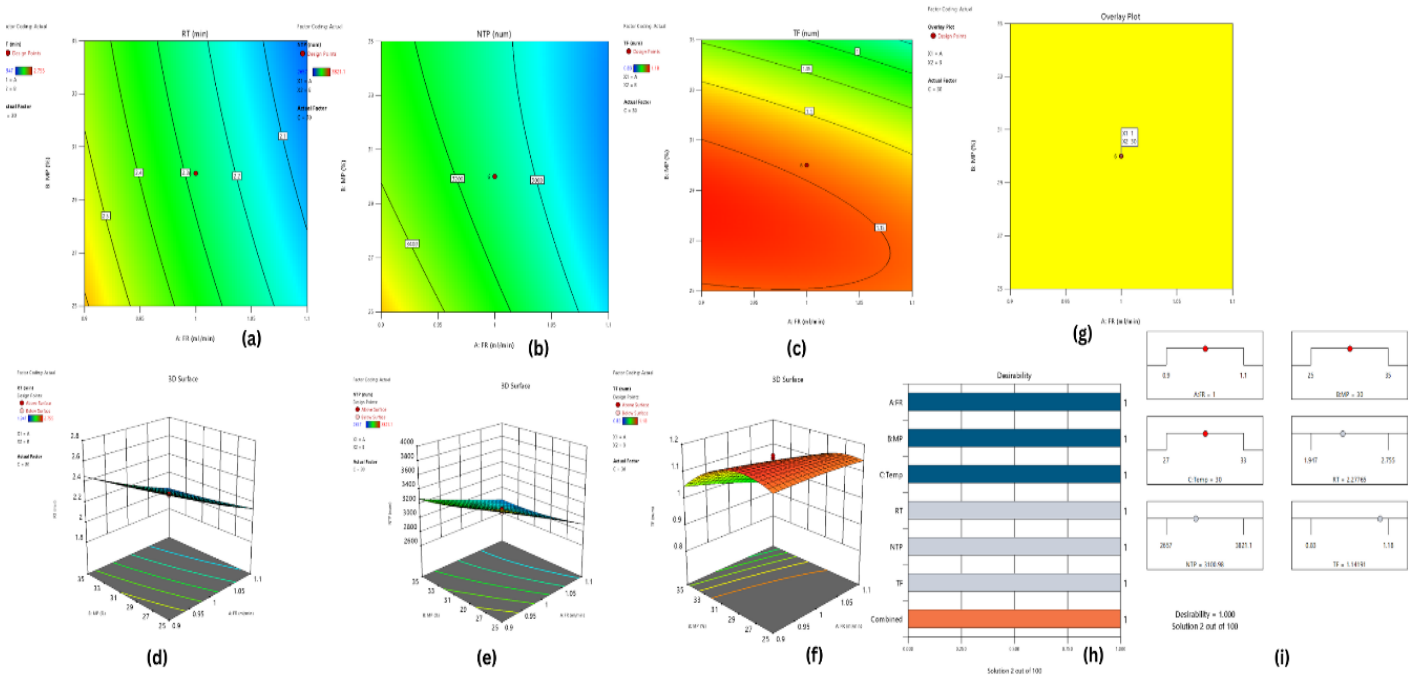

Figure 6 Illustration of 2D contour plots (a–c), 3D surface plots (d–f), overlay plot (g), desirability plot (h), and ramp plots (i) for optimization of RP-HPLC method using response surface methodology.

In Figure 6, Contour plots (a–c) depict the interactive effects of the selected critical method parameters on Rt, NTP & Tf, respectively. The 3D surface plots (d–f) further visualize these interactions, highlighting the trends in each response. The overlay plot (g) shows the optimal design space, represented in yellow, where all responses meet the desired criteria. The desirability plot (h) confirms that the selected conditions achieved a composite desirability of 1.0. The perturbation plot (i) indicates the relative influence of each factor on the responses, confirming the robustness of the optimized method.

METHOD VALIDATION RESULTS

System Suitability: System suitability was verified before each sequence to ensure reliable chromatographic performance. Critical parameters, including Rt, NTP, and TF, consistently met the acceptance limits across six replicate injections of the standard solution (50 µg/mL), demonstrating a stable system performance.

Specificity: Specificity was confirmed by injecting blank, degraded sample, and LTB working standard solutions. No co-eluting peaks or interferences were observed in analyte retention times. The LTB peak was well-resolved and showed a purity angle below the purity threshold, confirming the selectivity of the method. (Figure 7).

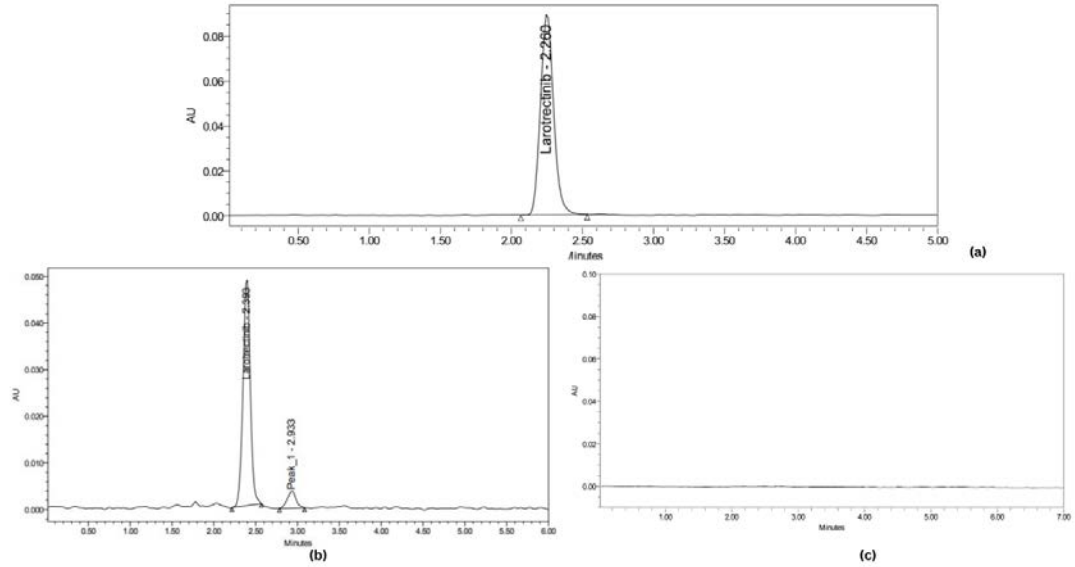

Figure 7: Representative Chromatograms of Larotrectinib: (a) Standard API, (b) Degraded Sample, (c) Blank

Table 5: Summary of Method Validation Parameters for RP-HPLC Analysis of Larotrectinib

Validation Parameter	Test Details	Observed Values
System Suitability	Retention Time (min)	2.257, 2.262, 2.264, 2.268, 2.270, 2.273
	Peak Area	599029, 608805, 607646, 607894, 609769, 596978
	Plate Count	3123, 3161, 3115, 3197, 3122, 3122
	Tailing Factor	1.15, 1.18, 1.17, 1.15, 1.16, 1.14
	Mean Peak Area	605020
	Standard Deviation (Area)	5524.5
	% RSD (Area)	0.91%
Precision	Peak Area (n=6)	592581, 589734, 598773, 588675, 586840, 586297
	Mean Peak Area	590483
	Standard Deviation	4642.2
	% RSD	0.8%
Linearity	Concentration (µg/mL)	0, 12.5, 25, 37.5, 50, 62.5, 75
	Peak Area	0, 150462, 302337, 451662, 608207, 754725, 896564
	Range	12.5–75 µg/mL
	Regression Equation	y = 11987x + 2904.3
	Correlation Coefficient (R^2)	0.9998
Accuracy (% Recovery)	50% Level (25 µg/mL)	Recovered: 24.8, 25.0, 24.8 → %Recovery: 99.31, 99.92, 99.34
	100% Level (50 µg/mL)	Recovered: 49.6, 49.6, 49.8 → %Recovery: 99.18, 99.25, 99.51
	150% Level (75 µg/mL)	Recovered: 74.3, 74.3, 74.3 → %Recovery: 99.07, 99.03, 99.04
	Overall Mean Recovery	99.29%
Robustness	Flow Rate Variation (±0.1 mL/min)	0.9 mL/min: %RSD = 0.31.1 mL/min: %RSD = 0.2
	Mobile Phase Composition (±5%)	25:75 – %RSD = 0.535:65 – %RSD = 0.3
	Column Temperature Variation (±3°C)	27°C – %RSD = 0.733°C – %RSD = 0.4

The table presents validation data for the RP-HPLC method for Larotrectinib, including system suitability, precision, linearity (12.5–75 µg/mL, R^2 = 0.9998), accuracy (mean recovery = 99.29%), and robustness (%RSD < 2% under varied conditions). Results confirm the method's reliability and reproducibility.

Linearity and Range: Linearity was established over the range of 12.5–75 µg/mL by plotting the peak area against concentration. The calibration curve exhibited an excellent correlation coefficient ($r^2 \geq 0.999$), demonstrating the ability of the method to produce results that were directly proportional to the analyte concentration within the validated range (Table 5).

Accuracy: Accuracy was assessed through recovery studies at three concentration levels (50, 100, and 150% of the target assay levels) by spiking known amounts of LTB into the matrix. The mean recoveries ranged from 98 to 102%, meeting the acceptance criteria for assay accuracy (Table 5).

Precision: Repeatability (intraday precision) and intermediate precision (different days, analysts, and instruments) were evaluated. The results showed RSD values below 2% for both sets of measurements, confirming the method's reproducibility under various conditions (Table 5).

Detection and Quantitation Limits: The sensitivity of the method was evaluated using the standard formulas for detection and quantification limits: DL = 3.3 × (standard deviation/slope) and QL = 10 × (standard deviation/slope). Based on these calculations, the method demonstrated a detection limit (DL) of 0.30 µg/mL and a quantification limit (QL) of 0.92 µg/mL. These values were further confirmed by experimental injections at the respective concentrations.

Robustness: Robustness was verified by introducing minor, deliberate variations in key method parameters: flow rate (1.0 mL/min ± 0.2 mL/min), organic phase composition (70% ACN ± 5%), and column temperature (30°C ± 1°C). These changes did not produce significant effects on retention time, theoretical plates, or peak shape deformation; tailing or fronting was observed under the tested robustness conditions. The baseline remained stable, showing no significant noise or drift, confirming the method's resilience to minor operational changes (Table 5).

Forced Degradation and Kinetic Study

LTB was subjected to hydrolytic (acidic, alkaline, and neutral), oxidative, and thermal stress conditions as illustrated in Figure 1, following ICH Q1A(R2) guidelines, to establish its degradation behavior [29,30,31]. LTB showed pronounced degradation under oxidative (5 % H_2O_2 up to 51.73%) and alkaline (0.5 N NaOH upto 49.81%) conditions, indicating its susceptibility to oxidative and base-induced hydrolysis. Moderate degradation occurred under acidic (up to 40.99%), thermal (40.45%), and neutral hydrolytic stress (39.33%). Significant reductions in peak area confirmed degradation under stress; however, chromatograms showed no interfering peaks, and peak purity remained within acceptable limits, demonstrating the specificity of the method, even under stress conditions (Figure 8 (a-d)). Degradation kinetics were evaluated to understand the chemical stability of LTB better. For degradation kinetic modeling, both zero-order and first-order models were evaluated, and the selection of the appropriate model for each stress condition was based on the highest coefficient of determination (R^2), ensuring the best fit to the experimental data. The half-life (t1/2) and shelf life(t90) were determined using Equations 5 and 6 for zero-order kinetics and Equations 7 and 8 for first-order kinetics.

$$t\,½ = \frac{0.5Co}{k} \quad \text{.....(5)}$$

$$t_{90} = \frac{0.1Co}{k} \quad \text{.....(6)}$$

$$t\,½ = \frac{0.693}{k} \quad \text{.....(7)}$$

$$t_{90} = \frac{0.105}{k} \quad \text{.....(8)}$$

where, C_0 is the initial concentration, and k is the slope.

The combined zero- and first-order plots are shown in Figure 8 (e, f, g). A summary of the kinetic parameters is presented in Table 6.

Under milder acid (0.1 N HCl), strong alkaline (0.5 N NaOH), and thermal conditions, the degradation followed zero-order kinetics, confirmed by linear plots of percentage remaining versus time: where C_0 is the initial concentration and C_t is the concentration at time *t*. All the remaining conditions followed first-order kinetics. The observed kinetic profiles highlight LTB's susceptibility to degradation in oxidative, strongly acidic, or basic environments, whereas its stability improved near neutral pH. Based on these findings, the formulations should be buffered appropriately to maintain a stable pH range.

Further stabilization approaches, such as antioxidants, protective excipients, and microencapsulation, may help protect labile groups from oxidative or thermal degradation. Suitable packaging, such as moisture-proof blister packs, may also extend the shelf life by minimizing environmental exposure. These results provide crucial evidence to guide formulation design, establish shelf life & support regulatory submissions with clear scientific justification for the recommended storage conditions.

Table 6: Summary of kinetic modeling data for degradation

Stress Condition	Best Fit Model	R^2 Value	t1/2 (days)	t 90 (days)	Degraded %	k
0.1 N HCl	Zero-order	0.9930	4.23	0.85	36.07	1.183E+01
0.5 N HCl	First-order	0.9955	1.45	9.56	40.99	1.67E-01
0.1 N NaOH	First-order	0.9959	1.25	8.23	46.60	1.94E-01
0.5 N NaOH	Zero-order	0.9915	3.39	0.68	49.81	1.477E+01
3% H_2O_2	First-order	0.9929	1.20	7.94	47.55	2.01E-01
5% H_2O_2	First-order	0.9942	1.10	7.25	51.73	2.20E-01
Thermal	Zero-order	0.9924	3.88	0.78	40.45	1.29E+01
Water	First-order	0.9942	1.50	9.93	39.33	1.61E-01

The table summarizes degradation kinetics of Larotrectinib under various stress conditions, indicating the best-fit kinetic model, R^2 values, half-life ($t_{1/2}$), time for 10% degradation (t_{90}), percentage degradation, and rate constant (k). Both zero- and first-order kinetics were observed depending on the condition.

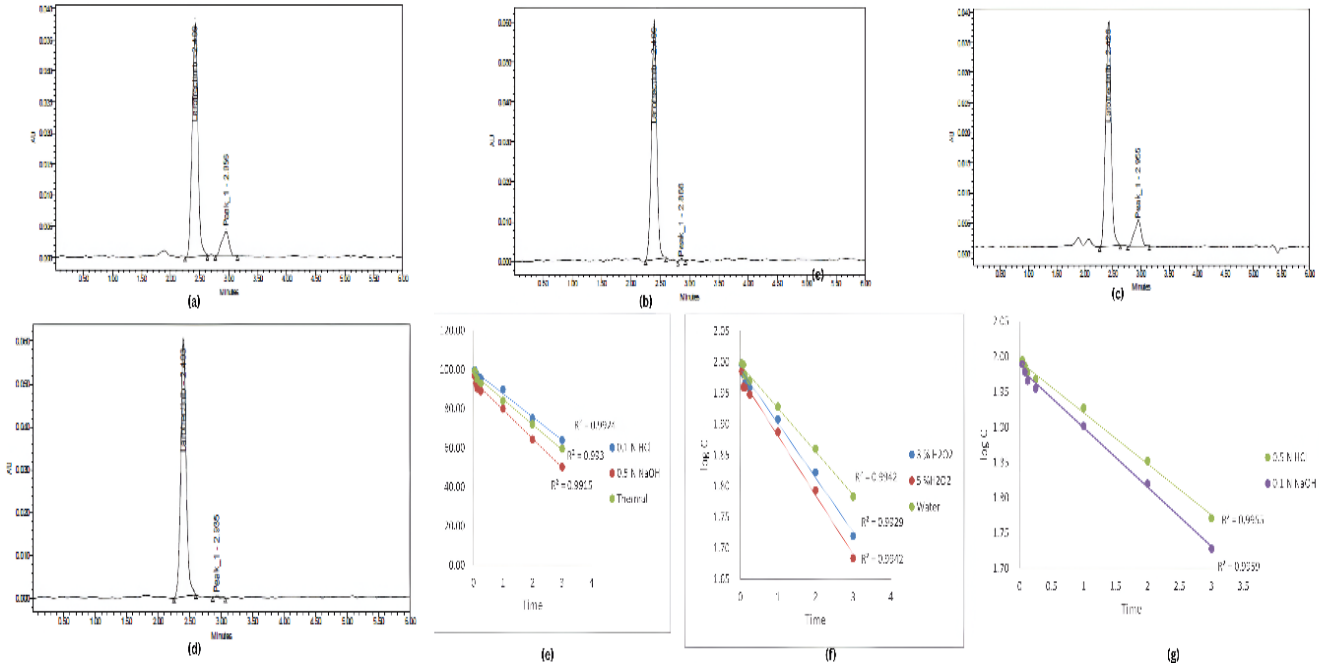

Figure 8: Degradation Chromatograms(a-d) and Kinetics plots of Larotrectinib: e) Zero order plot, f, g) First Order plots

Table 7: AGREE Method

Criteria	Proposed Method
Sampling procedure	At-line
Sample amount in g/mL	0.1
Analytical device positioning	On-line
How many major steps involved in sample preparation	3 or fewer
Automation and sample miniaturization	Automatic / None
Select CAS no. of derivatizing agents	-
Amount of waste in g or mL	5
No. of analytes determined per run / and samples analysed per hour	1 / 12
Technique used in analysis / and its total power consumption in kWt	HPLC / 0.1
Select CAS no. of derivatizing agents	-
Does the method involve toxic reagents? / and amount in g/mL	Yes / 1
Select threats which are not avoided?	- Corrosive, highly flammable, Toxic to aquatic life
Overall Score	0.66

Greenness assessment of the developed method

The environmental impact of the final method was rigorously assessed using modern greenness assessment tools, including AGREE, AMGS, ComplexMoGAPI, BAGI, RGB, and the EVG framework. By integrating these tools at the method design stage, this work demonstrates a practical application of green analytical chemistry principles, ensuring that the method is not only precise and reliable but also environmentally responsible. Such an approach sets a strong foundation for future innovations in green method development and supports a broader sustainability agenda in pharmaceutical analysis [32].

Analytical Greenness Metric (AGREE)

AGREE is a comprehensive and user-friendly tool that generates a pictogram showing the method's overall greenness and performance for each criterion [33,34]. The score of the developed method was 0.66 (Table 7), indicating that the method was environmentally acceptable (Figure 9a).

Analytical Method Greenness Score (AMGS)

AMGS metric evaluates not only solvent toxicity and waste volumes but also cumulative energy demand, including instrument power usage [35,36]. The developed HPLC method showed good greenness with a score of 425.8. While ACN contributes to most of the environmental impact, the short run time and efficient conditions help to keep the method fairly sustainable. There is still room for improvement using greener solvents; however, overall, this method is environmentally acceptable(Table 8).

ComplexModelGAPI

ComplexMoGAPI builds on the original GAPI index by extending its scope beyond the analytical procedure to include pre-analytical steps, such as sample collection, transport, and reagent preparation. It utilizes a hexagonal pictogram to visually highlight areas of strength or concern throughout the workflow [37,38]. This method achieved a score of 77 (Figure 9b), reflecting good overall greenness (Table 9).

Table 8: Detailed AMGS Spreadsheet output

Parameter	Value
Method Number	2025-06-21-21:22:40.220
Technique	HPLC
Greenness Score	425.80
Instrument Energy Score	111.63 (26.22%)
Solvent Energy Score	199.76 (46.91%)
Solvent EHS Score	114.41 (26.87%)
Number of Analytes	1
Number of Injections/Runs	14
Flow Rate (mL/min)	1
Run Time (min/injection)	5
Gradient	5 min: 70% A, 30% B
Mobile Phase A - Solvent 1	Acetonitrile – 70%
Mobile Phase A - Solvent 2	Water – 30%
Sample Diluent	Water:Acetonitrile (50:50)
Sample Prep Volume (mL)	10
Number of Sample Preps	2
Stock Standard Diluent	Water:Acetonitrile (50:50)
Stock Standard Prep Volume	50
Number of Stock Standard Preps	1
Working Standard Diluent	Water:Acetonitrile (50:50)
Working Standard Prep Volume	10
SST Diluent	Water:Acetonitrile (50:50)
SST Prep Volume (mL)	10
Number of SST Preps	1
Sensitivity Solution Diluent	Water:Acetonitrile (50:50)
Sensitivity Solution Prep Volume (mL)	10
Number of Sensitivity Preps	2

Table 9: ComplexMoGAPI Method

Category	Proposed Method
Collection	At-line
Preservation	Chemical or physical
Transport	None
Storage	Under special conditions
Type of Method	Simple procedures
Scale of extraction	Not Applicable
Solvents / Reagents used	Non-green solvents/ reagents
Additional treatments	None
Reagents and solvents	
Amount	<10 mL
Health hazard	Moderately toxic (NFPA= 2 or 3)
Safety hazard	Highest NFPA inflammability of instability score 2 or 3
Instrumentation	
Energy	< 0.1 kWh per sample
Occupational hazard	Hermetic sealing of the analytical process
Waste	1-10 mL (1-10 g)
Waste treatment	No treatment
Quantification	Yes
Overall Score	77

Table 10: BAGI Evaluation

Category	Proposed method
Type of analysis	Quantitative and confirmatory
Multi- or single-element analysis	Single Element
Analytical technique	Simple instrumentation available in most labs (UV, HPLC-UV, HPLC-DAD, UHPLC, FAAS, ETAAS, ICP-OES, GC-FID etc.)
Simultaneous sample preparation	1
Sample preparation	Simple, lowcost sample preparation required(eg.protein precipitation)
Samples per h	>10
Reagents and materials	Common commercially available reagents (methanol, acetonitrile, HNO3, nitrogen or other common gasses, etc.)
Preconcentration	No preconcentration required. Required sensitivity and /or legislation criteria are met directly.
Degree of automation	Semi-automated with common devices (e.g. HPLC autosampler)
Amount of sample	<100 µL(or mg) bioanalytical samples; <10 mL (or g) food/environmental
Overall Score	77.5

Table 11: EVG of the proposed Method

	Efficiency	Validation	Greenness
A	3	3	3
B	2	3	3
C	2	3	2
D	2	2	2
E	0	2	2
Average score	1.8	2.6	2.4

Table 12: Whiteness Assessment

Category	Parameter	Metric/Value	Score (0–100)
R1: Scope	Scope of application	0–100	100
R2: LOD & LOQ	LOD (µg/mL)	0.3	
	LOQ (µg/mL)	0.92	100
R3: Precision	RSD% (repeatability)	≤ 2.0%	
	RSD% (reproducibility)	≤ 2.0%	100
R4: Accuracy	Relative Error (%)	0.69	
	Recovery (%)	99.29	100
G1: Toxicity	Total pictograms (hazard)	5	50
G2: Reagents/Waste	Reagent consumption	≤ 400 mL	
	Waste production	≤ 400 mL	100
G3: Energy/Media	Energy/media consumption	9 hr/100 runs	100
G4: Direct Impact	Occupational hazards	5	
	Safety of users	100	100
	Use of animals	No	0
	Use of GMO	No	0
B1: Cost-efficiency	Total cost	₹2000 / 100 runs	100
B2: Time-efficiency	Speed of analysis	9 hr / 100 runs	100
B3: Requirements	Sample consumption	1 mL / 100 runs	100
	Other needs (advanced skills/tools)	None	0
B4: Simplicity	Miniaturization	No	0
	Integration/Automation	Yes	100
	Portability	No	0
Method name	Greenness (%)	Blueness (%)	Whiteness (%)
HPLC-PDA	87.5	70.8	86.1

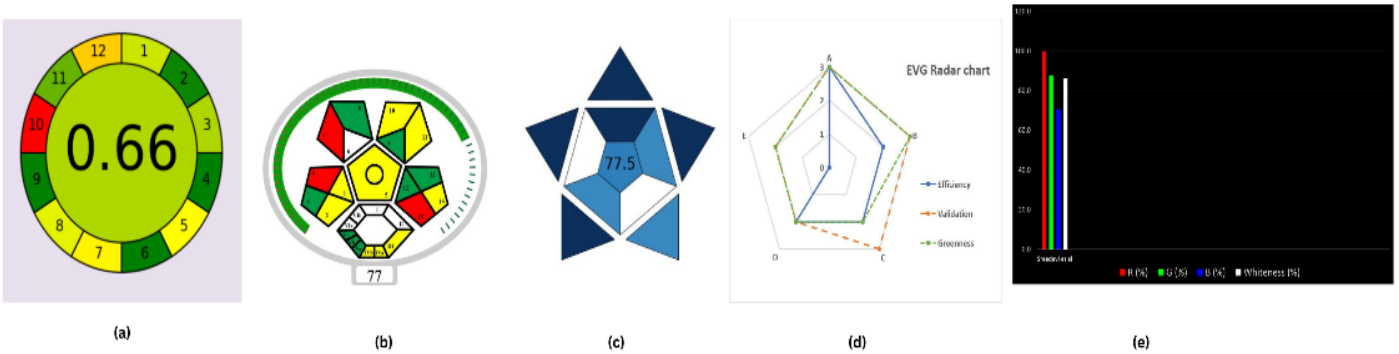

Figure 9: Comprehensive Evaluation of the Developed RP-HPLC Method Using Greenness, Performance, and Sustainability Metrics. a) AGREE (b) ComplexMoGAPI pictogram (c) BAGI (d) EVG radar chart (e) Whiteness score bar graph.

Blue Applicability Grade Index (BAGI)

BAGI was designed to complement greenness tools by focusing specifically on practicality. It assesses aspects such as sample throughput, automation, reagent handling, and preconcentration steps, generating an easily interpreted pictogram to help evaluate the real-world applicability of the method [39]. The BAGI score of the developed method was 77.5 (Figure 9c), indicating that it is environmentally sound and has good practicality and applicability (Table 10).

Efficient, Valid, Green framework

The method demonstrated excellent performance in terms of validation and greenness, with average scores of 2.6 and 2.4, respectively, placing both in the first quartile.

Efficiency received a slightly lower average score of 1.8 (Figure 9d), putting it in the second quartile and earning a rating of very good. Overall, the method showed a strong and reliable performance across all key evaluation parameters [40] (Table 11).

WHITENESS OF THE DEVELOPED METHOD

The whiteness was determined using an RGB model. (Figure 9e) The method showed a high whiteness score (~85%), reflecting excellent overall sustainability, balance, and eco-friendliness [41,42,43] (Table 12)

CONCLUSION

In this study, we developed a QbD-based stability-indicating RP-HPLC method for LTB that is robust, selective, and reproducible. Using a CCD, we systematically optimized flow rate, mobile phase composition, and column temperature, modeling their effects on retention time, theoretical plates, and tailing factor. This approach established a design space compliant with ICH Q8(R2), ensuring consistent performance and sharper peaks with efficient separation of the drug from impurities and degradation products. The method also incorporates comprehensive degradation kinetics under acidic, basic, oxidative, photolytic, and thermal stress, confirming true stability-indicating capability not reported earlier. Beyond method development, we performed a detailed evaluation of greenness, whiteness, and blueness, adding a sustainability dimension absent in previous studies. While acetonitrile was used as the mobile phase, greener alternatives can be explored in the future. The resulting method is statistically optimized, eco-conscious, and regulatory-ready, with applications in routine quality control, stability testing, dosage form analysis, and bioanalytical studies.

FINANCIAL ASSISTANCE

NIL

CONFLICT OF INTEREST

The Authors Declare No Conflict Of Interest.

AUTHOR CONTRIBUTION

Syamala P.N.S. conceptualized and designed the study, conducted the experimental work, including method development, degradation kinetics, and greenness assessment, analyzed the data, and drafted the manuscript. Sreedevi Adikay provided supervision and expert guidance throughout the study and critically reviewed and revised the manuscript. Both authors read and approved the final version of the manuscript.

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