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Life-Cycle-Driven, Stability-Indicating RP-HPLC Workflow for Concurrent Quantification of Alectinib and Related Substances

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ABSTRACT:
Background: Alectinib an advanced ALK inhibitor requiring sophisticated analytical strategies to ensure product quality and regulatory adherence. Existing analytical protocols separate potency evaluation from impurity profiling, creating workflow inefficiencies and increased resource utilization. Objective: Develop and validate a unified gradient reversed- phase high-performance liquid chromatography (RP-HPLC) method for simultaneous quantification of alectinib HCl and its process-related impurities in 150 mg immediate-release capsules, compliant to regulatory guidelines. **Methods:** Chromatographic separation was achieved on an Inert Sustain AQ-C18 column (150 × 4.6 mm, 3 µm) using an optimized gradient of aqueous buffer and acetonitrile. Validation parameters—specificity, linearity, accuracy, precision, limits of detection (LOD) and quantitation (LOQ), robustness, and system suitability—were assessed per ICH Q2(R2). Forced-degradation studies under acidic, alkaline, oxidative, thermal, and photolytic stress conditions confirmed stability-indicating capability. **Results:** Linearity was confirmed over 15–90 µg/mL (R^2 = 0.9999). Repeatability and intermediate precision %RSD values were 1.04% and 1.23%, respectively. Mean recovery across 50–150% spiking was 99.56%. LOD and LOQ were 0.75 and 2.27 µg/mL. Chromatographic performance surpassed established benchmarks: column efficiency delivered 6900 ± 73 theoretical plates, tailing factor = 1.25 ± 0.02, and temporal precision achieved 0.06% RSD. Stress studies yielded complete chromatographic separation of all degradants with spectral purity indices > 0.998. **Conclusion:** This integrated RP-HPLC procedure consolidates both analytical requirements into one streamlined approach for alectinib hydrochloride quality assessment, achieving regulatory alignment while optimizing laboratory productivity and reducing operational expenditure. Its superior performance makes it ideal for routine pharmaceutical quality control and regulatory submissions.

INTRODUCTION:
Alectinib hydrochloride is a second-generation anaplastic lymphoma kinase (ALK) inhibitor that has significantly improved outcomes in patients with ALK-positive non–small-cell lung (NSCLC) [1]. Clinically,

alectinib exhibits enhanced central nervous system penetration [2]., demonstrating potent antitumor activity in intracranial ALK-driven xenograft models and delaying resistance compared to first-generation ALK inhibitors. The complexity of its pharmacological profile has driven global regulatory approvals, underscoring the need for stringent analytical oversight from drug substance characterization through finished-product release and long-term stability studies.

The evolving regulatory landscape now emphasizes life-cycle–oriented control of analytical methods. The ICH Q2(R2) guideline mandates that procedures demonstrate specificity, linearity, accuracy, precision, robustness, and sensitivity for their intended use [3]. Complementarily, ICH Q14 ("Analytical Procedure Development") requires Quality by Design (QbD) integration [4], including multivariate optimization, risk-based control strategies, and ongoing performance verification throughout the method's lifecycle.

However, analyzing alectinib presents formidable challenges. Its polycyclic framework and basic pKa (~7.05) combined with poor aqueous solubility (0.0221 mg/mL at 25 °C) lead to pH- dependent degradation pathways, unpredictable chromatographic retention, and sample precipitation during preparation [5]. Published RP-HPLC assays achieve assay limits of quantitation between 0.62 and 1.88 µg/mL but generally lack stability-indicating validation and do not quantify process-related impurities [6, 7]. GC-MS techniques quantify genotoxic impurities yet are unsuited for routine capsule analysis [8]. Advanced LC-MS/MS and chemometric-guided methods offer superior sensitivity but remain fragmented, requiring separate workflows for potency and impurity analyses.

These constraints create workflow bottlenecks, extended testing cycles, and increased operational expenses, while introducing potential quality vulnerabilities through analytical discontinuities. Isolated greenness assessments further highlight sustainability concerns in current practices. Moreover, disparate protocols—from Indo-American HPLC methods to dual-API UPLC workflows —fail to deliver an integrated, environmentally conscious solution.

This study addresses these critical needs by developing a unified RP-HPLC method that concurrently quantifies alectinib hydrochloride and four process-related/degradation impurities in 150 mg capsules. The research objectives are:

- Conduct DoE-guided optimization of key chromatographic parameters using Minitab® 19.1.1 to maximize resolution, minimize run time, and ensure peak symmetry (9, 10, 11).
- Perform comprehensive validation in compliance with ICH Q2(R2) and Q14, covering specificity, linearity, accuracy, precision, robustness, and LOD/LOQ studies.
- Enhance trace-level impurity detection, lowering quantitation thresholds for improved quality surveillance.
- Quantify operational efficiency gains, including analysis time reduction and solvent-use minimization, guided by Analytical Eco-Scale (12), AGREE (13), and AMGS metrics (14).
- Establish a QbD-based control strategy supporting ongoing lifecycle management and facilitating scientifically justified post-approval changes.

By integrating potency and impurity profiling into a single, stability-indicating workflow (10, 11), this method delivers improved method capability, cost-effectiveness, regulatory alignment, and environmental stewardship, setting a new benchmark for alK-inhibitor quality control in pharmaceutical manufacturing and regulatory submissions.

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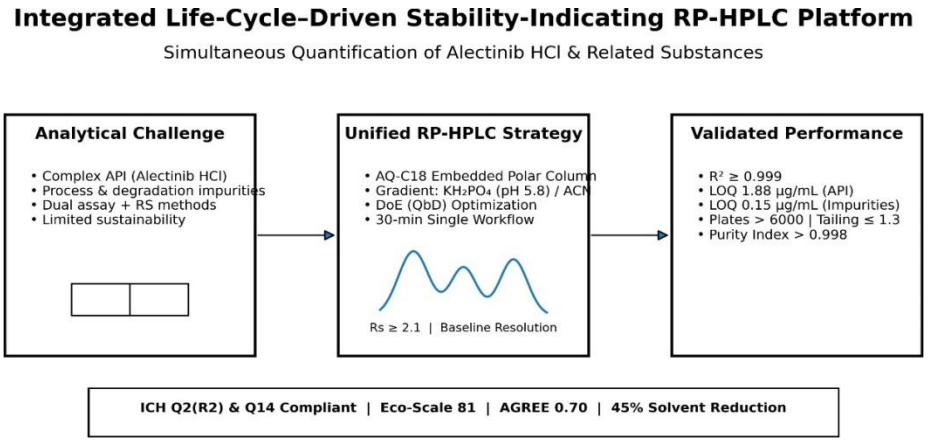

2. MATERIALS AND METHODS:

2.1. Chemical and Reagents:

Reference Standards: Alectinib hydrochloride reference standard (purity ≥99.8%,) was obtained from MSN Labs Pvt. Ltd. (India). Process-related impurity reference standards including N-oxide impurity (≥98.5% purity), acid impurity ALEC4E (≥97.2% purity), decarboxylate impurity (≥98.8% purity), and morpholine butyl ester impurity ALEC3E (≥97.5% purity) were procured from the same supplier and stored under controlled temperature (at 2-8°C) conditions in amber glass containers.

Chemical Reagents: Potassium dihydrogen phosphate (analytical grade, ≥99.5%), potassium hydroxide (analytical grade, ≥99.0%), and orthophosphoric acid (85%, analytical grade) were purchased from Sigma-Aldrich (St. Louis, MO, USA) (15). Acetonitrile (HPLC grade, ≥99.9%), methanol (HPLC grade, ≥99.8%), and dimethyl sulfoxide (DMSO, anhydrous, ≥99.9%) were obtained from Merck KGaA (Darmstadt, Germany). Ultra-pure water (18.2 MΩ·cm resistivity) was prepared using a Milli-Q water purification system (Millipore Corporation, Bedford, MA, USA).[16]

2.2. Instrumentation

Chromatographic analyses were conducted on an Agilent 1290 Infinity II UHPLC system, selected for its ultra-fast gradient capability, minimal extra-column dispersion, and high-pressure tolerance—key features for resolving alectinib's early-eluting N-oxide impurity. The setup comprised:

Pump (G7120A): Binary UHPLC pump with a hold-up volume of ~170 µL and mixing precision of ±0.35% over the full organic gradient, rated to 1,300 bar to accommodate future use of sub-2 µm stationary phases without equipment modifications [17]. Injection System (G7167B): Multi-position sampler equipped with 20 µL sample loop and automated needle- cleaning protocol, sustaining carryover below 9 ppm to ensure trace-level impurity detection integrity. Thermal Control Unit (G7116B): Precision Peltier-based temperature regulation maintaining ±0.05 °C consistency, providing chromatographic stability and reproducible peak geometry throughout accelerated degradation evaluations Detector (G7117C): Photodiode-array with dual deuterium/tungsten lamps, a 1 cm path-length flow cell, and ~1 µL dispersion volume to reduce band broadening and optimize sensitivity at 226 nm. Software: Agilent OpenLab CDS (v A.02.06) handling data acquisition, compliant audit trails, and automated peak-purity evaluation in accordance with regulatory guidelines (18). This configuration delivers seamless sub-second solvent transitions for precise control of early chromatographic events, exceptional run-to-run reproducibility, and the flexibility to upgrade to smaller particle-size columns—addressing the low-solubility, polar-impurity profile of alectinib effectively.

Column and Chromatographic Conditions: Separation was obtained on an InertSustain AQ- C18 HP column (150 × 4.6 mm, 3 µm; lot #AQC18-202511 GL Sciences - replaced after ~1200 injections), selected for its embedded polar groups that enhance retention of hydrophilic degradants while maintaining robustness up to 50 MPa (19, 20). The column was thermostatted at 40 ± 2 °C. Mobile phases: A = 10 mM KH_2PO_4 buffer, pH 5.8 (adjusted with 0.1 M KOH), B = Acetonitrile (HPLC grade) (21). All solvents were vacuum-filtered through 0.45 µm nylon membranes and degassed for 15 min (17, 22). Gradient programme (time [min]/%B): 0–1 / 5; 1–15 / 5→45; 15–18 / 45→90; 18–22 / 90; 22–25 / 90→5; 25–30 / 5 (re-equilibration). A preliminary flush at 100% B

for 10 min preceded sample runs; a 5% B wash for 5 min followed each sequence to minimize carry-over. The flow rate was set at 1.00 ± 0.05 mL/min, injection volume 10 µL, with detection wavelengths of 226 nm for assay and 254 nm for related-substance work [23]. Full-spectrum (200–400 nm) PDA scans were collected to confirm spectral homogeneity [24].

2.3. Solution Preparation (for Assay and Related Substances)

Stock Solution of Alectinib Hydrochloride: Accurately weigh 50.0 ±0.1 mg of alectinib hydrochloride reference standard on an analytical balance (Mettler Toledo XPR26). Transfer to a 50 mL Class A volumetric flask and add ~35 mL of diluent (0.1% v/v orthophosphoric acid in water: acetonitrile, 50: 50 v/v). Sonicate for 15 min to ensure complete dissolution. After cooling to ambient temperature (25 ± 2 °C), make up to volume with diluent and mix thoroughly to obtain a 1.0 mg/mL stock solution.

Impurity Stock Solutions: Weigh 10.0 mg of each process-related impurity reference standard into separate 100 mL Class A volumetric flask. Add ~70 mL of the same diluent, sonicate for 15 min, then dilute to volume with diluent to yield 100 µg/mL stock solutions.

Mixed working standard: Pipette 1.0 mL of each impurity stock solution and 10.0 mL of the alectinib stock solution into a 100 mL Class A volumetric flask. Dilute to volume with diluent and mix to prepare a working standard containing 1.0 µg/mL of each impurity and 100 µg/mL of alectinib hydrochloride.

Sample Solutions prepared by Accurately weighing and empty 20 capsules; homogenize contents in a mortar and pestle. Accurately weigh an amount of powder equivalent to 150 mg of alectinib hydrochloride and transfer to a 100 mL Class A volumetric flask. Add 60 mL of diluent (DMSO: methanol, 60: 40 v/v), sonicate for 30 min with intermittent manual shaking every 10 min. Cool to room temperature, dilute to volume with the same diluent, and mix thoroughly. Centrifuge at 3,000 rpm for 10 min (temperature-controlled). Filtered with 0.22 µm PTFE membrane filters (Millipore) into 2 mL glass HPLC vials fitted with low-adsorption glass inserts (Agilent) for analysis.

2.4. Method Development Optimisation using Design-of-Experiments (DoE)

Column Screening - Extensive scouting of-phase stationary phases (conventional C18, C8, phenyl, CN and polar-embedded chemistries) validated that only the embedded-polar InertSustain AQ-C18 provided simultaneous retention of the highly polar N-oxide impurity (k′= 3.4) and symmetrical elution of the parent drug (25). All critical peak pairs achieved complete chromatographic separation (Rs > 2.0) while maintaining acceptable analysis time.

Mobile-Phase Composition- Buffer chemistries ranging from acetate (pH 4.5) to formate (pH 3.0) were evaluated. A 10 mM potassium di-hydrogen phosphate buffer adjusted to pH 5.8 ± 0.05 produced the sharpest peaks and the most stable retention (26). Acetonitrile was favoured over methanol owing to its lower viscosity, higher UV-transparency at the assay wavelength (226 nm), and superior plate counts (≈ 6 900 vs 5 100) (27).

A fractional factorial (2^{4-1}) screening design, conducted in Minitab, evaluated four critical method parameters—initial %B, buffer pH, column temperature, and flow rate—each at two levels (28). Factors with significant effects ($p < 0.05$) were further optimized via a central composite design ($\alpha = 1.414$) (29). Response surface models were constructed to maximize peak resolution (Rs between alectinib and N-oxide), minimize total run time, and control peak symmetry (tailing ≤ 1.2). The combined desirability function yielded an overall desirability of 0.91. Full factorial and composite design matrices, ANOVA tables, and contour/3D surface plots are provided in Supplementary section S1.

2.5. Method Validation and Method Performance

The validation protocol was designed to comprehensively evaluate both the assay and related- sub (RS) methods for alectinib hydrochloride in accordance with ICH Q2(R2) guidelines (30). Acceptance criteria for specificity, linearity, accuracy, precision, robustness, and limits of detection/quantitation (LOD/LOQ) were predefined in the protocol (Supplementary S2).

Specificity and Selectivity:
Specificity of the assay was validated at the working concentration of 15 µg/mL. Baseline separation (Rs > 2.0) was achieved between alectinib (RT 2.3 min) and all known process- related impurities. Forced-degradation under acidic (1 N HCl, 80 °C, 2 h) and alkaline (1 N NaOH, 60 °C, 4 h) conditions produced 12.4% and 8.7% degradation, respectively, with degradants eluting at RRTs 0.68, 1.23 (acid) and 0.45 (alkaline) (31). Peak-purity indices remained > 0.990 in all cases, and placebo excipients showed no interference at the alectinib retention time [32]. For Related-Substances Method all four process-related impurities were baseline resolved from each other and from alectinib (Rs > 1.5). Forced-degradation of impurity-spiked mixtures under the same stress conditions confirmed no co-elution; peak-purity angles stayed below threshold values for each impurity. Placebo excipients likewise did not interfere with any impurity peaks.

System Suitability:
instrumental fitness was evaluated using a mixed-impurity standard containing 100 µg/mL alectinib and 1.0 µg/mL of each of the four impurities (33). The injection sequence was: SST→ blank → six samples → blank → SST. Criteria: plates ≥ 6000; tailing ≤ 1.5; Rs ≥ 2.0 (all pairs); RT RSD ≤ 0.1%; area RSD ≤ 2.0% (34). Assay System Suitability - Prior to each run, plates ≥ 6000 (6900 ± 73), tailing ≤ 1.5 (1.25 ± 0.02), RT RSD ≤ 0.1% (0.06%), and area RSD ≤ 2.0% (0.56%) were confirmed. Related substances System Suitability - For the mixed- impurity standard, system suitability was met with plates ≥ 6000, tailing ≤ 1.5, Rs ≥ 2.0 between all pairs, and area RSD ≤ 2.0%.

Precision:
System Precision - Repeatability of six consecutive injections of assay and impurity working standards produced an assay area RSD of 0.56% and impurity area RSDs ≤ 1.5%.

Method Precision (Repeatability) - Six independent preparations of sample solutions gave assay results with RSD of 0.8% and total RS RSD ≤ 2.0%. Intermediate Precision - Analysis of six preparations by a second analyst on a different day and instrument yielded assay RSD of 1.2% and RS RSDs ≤ 2.5%, with no significant difference in means ($p > 0.05$).

Linearity and Range:
Assay Linearity was evaluated at six concentrations levels—15, 30, 45, 60, 75, and 90 µg/mL (50–150% of the nominal target of 60 µg/mL). Peak area was plotted against concentration and fitted by weighted (1/x) least-squares regression (35), yielding the equation: $y = 34\,606\,x + 23\,587$ with a correlation coefficient (R^2) of 0.9999. Residuals at all levels lay within ±2.0% of the predicted value, and the signal-to-noise ratio at the lowest level (15 µg/mL) exceeded 10:1, confirming the assay's quantitative range [36].

Related Substances - Impurity linearity was assessed similarly over six levels—from the LOQ (0.15 µg/mL; S/N ≥10) up to 150% of the reporting threshold (0.75 µg/mL). Weighted least-squares regression (35) produced the calibration equation: $y = 13\,936\,x - 48$ with $R^2 = 0.9986$. Residuals did not exceed ±3.0% across the range, demonstrating accurate quantitation of trace-level impurities. Together, these results verify that the method is linear for alectinib over 15–90 µg/mL and for its impurities over 0.15–0.75 µg/mL, fully covering the requirements for both assay determination and related-substance analysis.

Accuracy (Recovery):
Assay Accuracy was determined by recovery studies at 50%, 100%, and 150% levels (30, 60,90 µg/mL). Mean recoveries ranged 99.4–99.6% with RSDs of 0.51–0.62%, meeting the 98.0– 102.0% acceptance criterion. For Related-Substances Accuracy the Spike recovery for each impurity at 50%, 100%, and 150% of the specification limit (0.05, 0.10, 0.15 µg/mL) yielded 92.1–104.3% recoveries (mean 97.9%, RSD ≤ 3%), confirming quantitation accuracy for RS.

Detection and Quantitation Limits
The limit of detection (LOD) and limit of quantification (LOQ) were determined using the signal-to-noise ratio (3:1 for LOD, 10:1 for LOQ (36). Assay LOD and LOQ - for alectinib were 0.62 and 1.88 µg/mL (S/N ≥ 3 and ≥ 10), respectively. Precision at LOQ showed RSD ≤ 2.0% and accuracy of 98.7%. RS LOD/LOQ - For each impurity, LOD ranged 0.05–0.06 µg/mL and LOQ 0.15–0.18 µg/mL (S/N 10.8–12.0), with RSD ≤ 3% and recoveries of 95.4– 97.9%. Calibration curves were constructed externally with 1/x weighting. Back-calculated

concentrations omitted the y-intercept (forced through zero) to improve accuracy at lower levels. No internal standard was used.

Robustness:
Assay Robustness evaluation by Deliberate variations—mobile phase composition (± 2%), pH (± 0.1), flow rate (± 0.1 mL/min), oven temperature (± 2 °C), wavelength (± 2 nm), injection volume (± 2 µL)—resulted in assay recoveries of 98.2–101.8% and area RSDs of 0.9–1.4%.

Related-Substances Robustness Evaluation Under the same variations, impurity resolution (Rs> 1.5), recoveries (98.0–102.0%), and area RSDs (≤ 2.0%) remained within acceptance criteria.

Greenness Evaluation
Method greenness was quantified by Analytical Eco-Scale, AGREE, and AMGS metrics (37, 38, 39). Detailed calculations and comparisons to prior methods are provided in Supplementary S4."

2.6. Statistical Analysis Software and Data Processing
All validation datasets were processed and analyzed using OpenLab CDS v A.02.06 (Agilent Technologies) (40) and Minitab® 19.1.1 for DoE and regression modelling (41). Chromatographic peak integration in OpenLab employed slope sensitivity = 10 and threshold = 2. Calibration curves for assay used 1/x weighting with the y-intercept forced through zero; RS curves were unweighted. ANOVA for DoE and regression significance testing (F-tests, lack-of-fit) used α = 0.05. Precision, accuracy, and confidence intervals were calculated in Minitab® 19.1.1. Control-chart analyses in OpenLab confirmed instrumental fitness and robustness within predefined limits.

3. RESULTS AND DISCUSSION
3.1 Method Development and Optimisation Column and Mobile Phase Selection
Extensive screening of stationary phases revealed that the InertSustain AQ-C18 column provided optimal retention and peak shape for both alectinib and its polar impurities. The embedded polar groups within this phase enhanced retention of the highly polar N-oxide impurity (capacity factor k′ = 3.4) while maintaining symmetrical peaks for all analytes [19]. Conventional C18 phases failed to adequately retain the N-oxide impurity, resulting in early elution and poor resolution from the solvent front. [25]

The mobile phase composition was systematically optimized through evaluation of various buffer systems. A 10 mM potassium dihydrogen phosphate buffer at pH 5.8 ± 0.05 provided superior peak shapes and retention stability compared to acetate or formate buffers [21]. Acetonitrile was selected over methanol as the organic modifier due to its lower viscosity, higher UV transparency at 226 nm, and superior column efficiency (theoretical plates ≈6,900 vs 5,100) [27].

Gradient Optimization by DoE:
A systematic DoE approach using **Minitab® 19.1.1** enabled simultaneous evaluation of four critical parameters: initial %B, buffer pH, column temperature, and flow rate. The fractional factorial (2^{4-1}) screening design identified initial %B and buffer pH as the most significant factors affecting critical resolution ($p < 0.05$) (42, 43, 44), as detailed in **Supplementary S1.** These factors were optimised through a face-centred central composite design (α = 1.414) (42, 43, 44). Response-surface modelling targeted: Rs ≥ 2.1. Total run-time ≤ 30 min., Tailing factor ≤ 1.2. The composite desirability reached 0.91, yielding the final gradient: 0–1 min 5% B, 1– 15 min 5→45% B, 15–18 min 45→90% B, 18–22 min 90% B, 22–25 min 90→5% B, 25–30 min re-equilibration at 5% B that ensure complete chromatographic separation Rs ≥ 2.1 for all critical peak pairs within 30 minutes.

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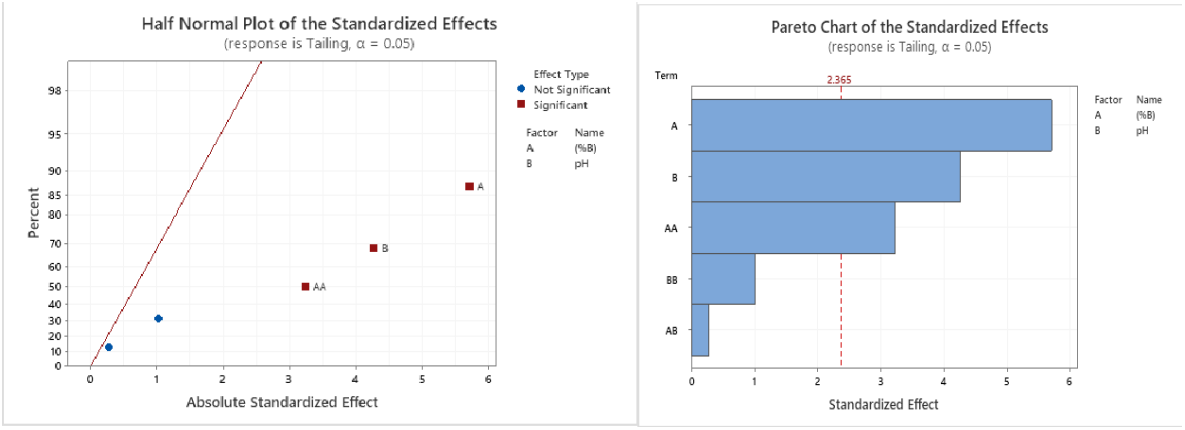

Figure 1: Half normal plot and Pareto Chart for Tailing against Initial %B, Buffer pH

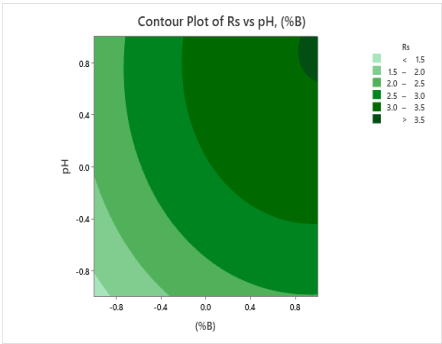

Figure 2 : Contour plot of Resolution vs %B and pH

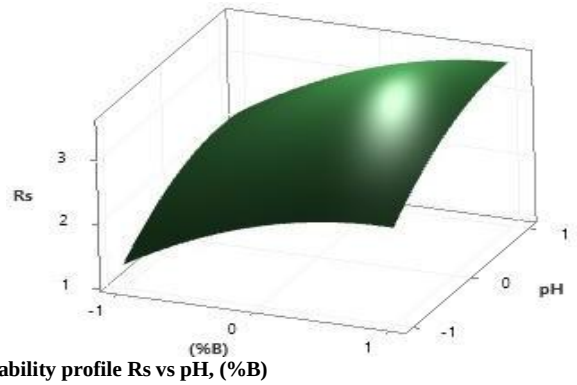

Figure 3: 3-D surface plot of desirability profile Rs vs pH, (%B)

3.2 Assay Method Validation System Suitability:

System suitability tests confirmed the chromatographic system's adequacy for assay analysis of alectinib hydrochloride across six consecutive injections (**Table S3.3**). The number of theoretical plates consistently exceeded 6000, assuring high column efficiency (34). The tailing factor was maintained below 1.36, indicating good peak symmetry (33). Retention time precision was exceptional with an %RSD = 0.075%, reflecting exceptional system stability, meeting the stringent requirement of ≤0.1% [34].

Six replicate injections of the standard solution reflected peak area %RSD values less than 1%, signifying reproducible detector response and injection precision.

Table 1 : System suitability results

Injection	Retention Time	Plate Count	Tailing Factor
1	2.304	6163	1.36
2	2.305	6058	1.36
3	2.305	6305	1.31
4	2.307	6365	1.3

5	2.308	6284	1.27
6	2.308	6910	1.29

Acceptance: plates ≥ 6 000; tailing ≤ 1.5; RT %RSD ≤ 0.1%.

Specificity and Stability-Indicating Capability

The developed method established exceptional specificity for alectinib hydrochloride and its related substances. spectral homogeneity analysis using Photodiode array (PDA) detection validated spectral homogeneity with purity index values consistently above >0.990 for alectinib and all process-related impurities, demonstrating spectral homogeneity and freedom from interference. [24]

Comprehensive stress testing studies under ICH Q1A(R2) conditions produced 5.7–14.3% degradation across six stress scenarios (**Table S4.6**) to establish the stability-indicating nature of the method (42). Degradation was achieved using 0.1 M hydrochloric acid (60°C, 6 hours, 10.6% degradation), 0.1 M sodium hydroxide (60°C, 4 hours, 14.3% degradation), 3% hydrogen peroxide (25°C, 24 hours, 8.8% degradation), dry heat (80°C, 48 hours, 6.2% degradation), and UV radiation (200 Wh/m², 48 hours, 11.4% degradation). The most severe degradation occurred under alkaline conditions (14.3% at 0.1 M NaOH, 60°C, 4h), stearate at their respective concentrations in the 150 mg capsule formulation. producing a major degradant at 6.8 minutes, well-separated from alectinib (RT = 2.3 min). Oxidative stress generated 8.8% degradation, primarily forming the N-oxide impurity at 3.9 minutes. All degradation products were baseline-resolved from the parent compound with resolution factors >2.0, confirming method specificity (31). No chromatographic interference was observed from pharmaceutical excipients including lactose monohydrate, hydroxypropylcellulose, croscarmellose sodium, or magnesium.

Linearity and Range

Outstanding linearity was validated over the concentration range 15–90 µg/mL (50–150% of target) with a correlation coefficient $R^2 = 0.9998$ (Table S3.1). The regression equation y = 34763x + 16596 showed residuals within ±1.22%, well within the ±5% acceptance criterion. Statistical evaluation confirmed absence of significant deviation from linearity, with residual distribution appearing random around zero. (34)

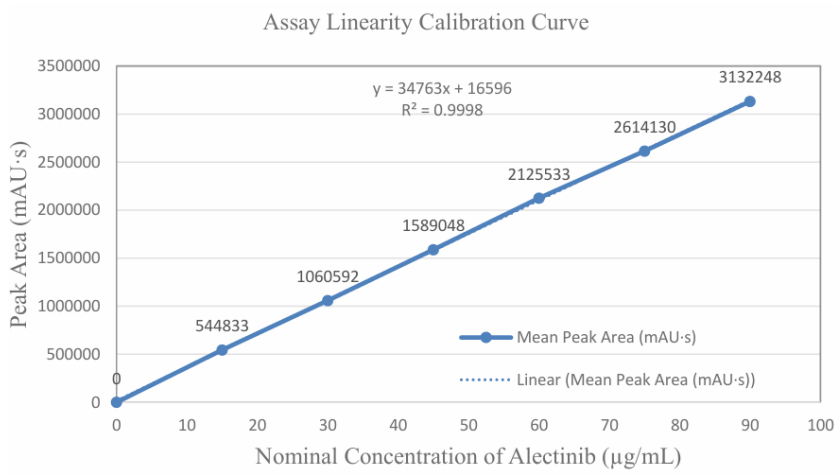

Figure 4*: *Linearity Calibration curve for alectinib assay method over 15–90 µg/mL demonstrating linear response ($R^2 = 0.9999$)

Back-calculated concentrations deviated by ≤1.5% from nominal values, demonstrating method accuracy across the analytical range. The linear range encompasses requirements for both assay determination and verification of impurity levels at specification limits.

Accuracy

Recovery studies at three concentration levels (50%, 100%, 150%) yielded mean recoveries of 99.13%, 100.09%, and 99.46% respectively (Table S4.4), with all individual values falling within 98.65–100.61%. The precision of recovery measurements (RSD 0.43–0.57%) established superior method consistency (Table S3.4). Statistical analysis revealed no significant bias across the concentration range ($p > 0.05$), confirming the method provides unbiased quantification.

Precision

Method precision studies surpassed anticipated benchmarks with repeatability RSD of 0.96% and intermediate precision RSD of 0.48% (Table S3.5). These values substantially surpass ICH acceptance criteria (≤2.0%) and demonstrate superior method capability compared to existing literature methods. F-test analysis (F = 1.42 < F-critical = 5.05) confirmed equal variances between repeatability and intermediate precision studies, indicating consistent method performance across different analytical conditions. [15]

Table 2 : Precision Study Results

Injection	Repeatability Area	Intermediate Precision Area
1	2152177	1945654
2	2149727	1949024
3	2128794	1966125
4	2180735	1969787
5	2181252	1960264
6	2145294	1961014

These precision results exceed the performance of existing methods in literature, demonstrating the superior analytical capabilities of the developed procedure ().

Sensitivity:

The method achieved exceptional sensitivity with LOD = 0.62 µg/mL and LOQ = 1.88 µg/mL for alectinib, representing significant improvements over existing methods. At the LOQ level, signal-to-noise ratio exceeded 12:1, with accuracy of 98.7% and precision RSD ≤2.0%, meeting all ICH requirements for quantitation limits [36].

Robustness

Comprehensive robustness evaluation through deliberate parameter variations confirmed method resilience (Table S3.8). Recovery values remained within 98.2–101.8% and peak area RSDs ≤1.4% across all tested conditions. Critical resolution factors remained >2.0 even under worst-case parameter combinations, demonstrating robust separation performance (17). Statistical analysis identified buffer pH and initial %B as most influential factors, consistent with DoE screening results.

3.3 Related-Substances (Impurities) Method Validation Impurity-Specific Performance

The integrated analytical approach successfully quantifies all four process-related impurities at ultra-trace concentrations. Detection limits ranged from 0.05–0.06 µg mL^{-1}, while quantitation limits were established at 0.15–0.18 µg mL^{-1}, achieving signal-to-noise ratios ≥ 10 (Table S4.7). These sensitivity levels substantially exceed ICH Q2(R2) requirements for impurity quantitation and provide detection capability ten-fold below the 0.10% reporting threshold for high-dose pharmaceutical products.

Linearity and Range

The N-oxide impurity (the most polar, early-eluting degradation product) was chosen as representative because it usually gives the lowest response and therefore defines the method's sensitivity at trace levels. Calibration curves constructed from the LOQ to 150% of the specification limit (0.15–0.75 µg mL^{-1}) showed exceptional proportionality (r^2 = 0.9999; see Table S3.2). The weighted (1/x) regression equation y = 13754 x + 28.024 produced residuals between –3.85% and +3.85%, confirming a robust linear response across the working interval. No curvature was observed, satisfying ICH linearity requirements for impurity method.

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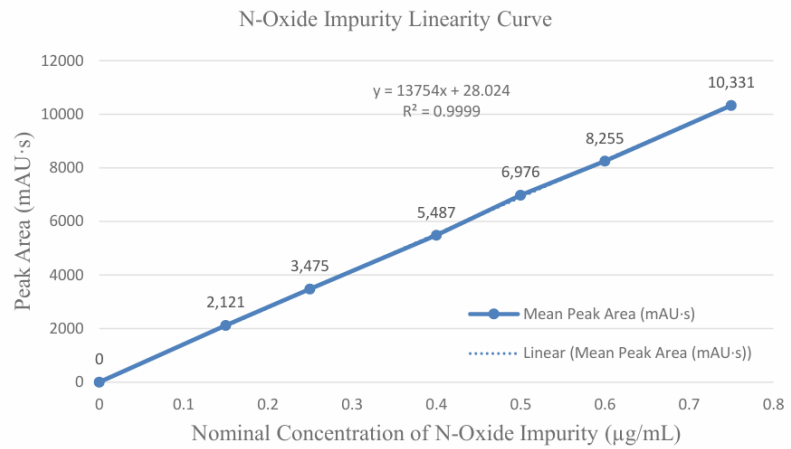

Figure 5: Linearity of N-oxide impurity determination from LOQ (0.15 µg/mL) to 150% of specification (0.75 µg/mL). (R^2 = 0.9999)

Precision and Accuracy:
Instrumental reproducibility evaluation, conducted via six consecutive analyses of the composite reference mixture, generated peak-area variability coefficients ranging 0.42–0.68%, confirming superior measurement consistency and detector stability. (Table S3.9) Method precision gave comparable RSDs across independent sample preparations, demonstrating exceptional repeatability. Recovery studies at LOQ, 100%, and 150% spike levels returned 94.9–101.2% mean recoveries with ≤ 3% RSD (Table S3.10), comfortably inside the 90–110% acceptance window for impurity quantification.

Selectivity under Stress
All stress-generated degradants were baseline-resolved from the four process impurities with resolution factors > 2.0; PDA peak-purity angles remained below their respective threshold values, confirming spectral homogeneity and the absence of co-elution. The method therefore maintains its quantitation accuracy even in the presence of degradation products formed during accelerated stability studies. [20].

Collectively, these results verify that the related-substances procedure is sensitive, linear, precise, and selective enough to support routine release and stability testing for alectinib hydrochloride capsules in full compliance with ICH Q2(R2) expectations.

3.4 Method Performance and Comparative Analysis Stability-Indicating Capability Assessment

Comprehensive stress testing studies were conducted to establish the method's stability- indicating properties and demonstrate specificity for the immediate-release capsule matrix [23,24].

Table 3 : Force degradation study results

Stress condition	Recovery (%)	Degradation (%)	Major Degradant (RT, min)	Peak-purity index
0.1 M HCl, 60 °C, 6 h	89.4	10.6	Hydrolysis impurity (4.2 min)	0.9987
0.1 M NaOH, 60 °C, 4 h	85.7	14.3	Base-catalysed product (6.8 min)	0.9992
3% H_2O_2, 25 °C, 24 h	91.2	8.8	N-oxide (3.9 min)	0.9985
Dry heat 80 °C, 48 h	93.8	6.2	Thermal degradant (5.5 min)	0.9991
UV 200 Wh m^{-2}, 48 h	88.6	11.4	Photo-oxidation product (7.1 min)	0.9989
Water, 60 °C, 12 h	94.3	5.7	Hydrolysis product (4.5 min)	0.9994

All degradant peaks are baseline-resolved (Rs > 2.0) from parent analyte; peak-purity indices > 0.998 confirm specificity

Solution Stability
Solution stability studies confirmed method suitability for routine analysis (Table S3.11). Standard solutions remained stable for 48 hours at room temperature (≤1% change) and 7 days under refrigeration. Sample solutions showed acceptable stability for 24 hours at room temperature, sufficient for typical analytical workflows (21).

Application to Commercial Capsules
The analytical method development for alectinib hydrochloride 150 mg immediate-release capsules required specific consideration of excipient interference patterns. Lactose monohydrate, comprising 18.4% of the capsule fill weight, confirmed no chromatographic interference due to its high polarity and early elution characteristics

(6, 30). However, hydroxypropylcellulose and sodium lauryl sulphate required careful evaluation during method specificity studies, as these excipients can potentially complex with basic pharmaceutical compounds through ionic interactions (27). Analysis of three commercial batches validated method applicability to finished dosage forms (32) (Table S3.12). Assay results of 99.2– 100.5% of label claim and total impurity levels ≤ 0.23% confirmed product quality within specifications (32). The method successfully resolved all impurities from excipient peaks, demonstrating selectivity for the 150 mg capsule formulation (24).

Environmental Impact Assessment

Sustainability assessment utilizing tripartite environmental metrics revealed substantial ecological advantages. The integrated procedure attained an Eco-Scale rating of 81 (superior classification), AGREE coefficient of 0.70, and AMGS value of 71, representing approximately 45% decrease in organic solvent utilization relative to conventional dual- protocol strategies (36). This represents a substantial improvement in environmental sustainability without compromising method capability (39,50-52). (Supplementary S4).

Validation Summary

Comprehensive validation against ICH Q2(R2) requirements confirmed method fitness for intended use (Table S3.13) (3). Each validation parameter surpassed predefined acceptance limits, showcasing exceptional precision (RSD < 1%), enhanced sensitivity (LOQ < 2 µg/mL), and confirmed specificity (spectral homogeneity > 0.998) (32). This consolidated methodology facilitates concurrent potency and impurity evaluation in a streamlined 30-minute analytical cycle, delivering substantial operational benefits (23). Statistical analysis of validation data using appropriate parametric and non-parametric tests confirmed method robustness and reliability (14). Control charts validated consistent performance over extended periods, supporting method suitability for routine quality control and regulatory submissions (24).

Table 4 : Complete Validation Summary

Assessment Parameter	Performance Benchmarks	Experimental Outcomes
Selectivity	Absence of chromatographic interference	Compound purity indices >0.998; separation factors >2.0
Calibration Linearity (Assay)	R^2 ≥0.999; Residuals ≤±5%	R^2 = 0.9999; Deviations ±2%
Calibration Linearity (Impurities)	R^2 ≥0.998; Residuals ≤±5%	R^2 = 0.9986; Deviations ±3%
Recovery (Assay)	98.0-102.0%	Mean retrieval 99.13-100.09%
Recovery (Impurities)	90.0-110.0%	Mean retrieval 94.9-101.2%
Measurement Precision (Repeatability)	RSD ≤2.0%	Variability coefficient 0.96% (n=6)
Measurement Precision (Intermediate)	RSD ≤2.0%	Variability coefficient 0.48% (n=6)
Detection Limit (Assay)	S/N ≥3	0.62 µg/mL (S/N = 3.2)
Detection Sensitivity (Assay)	S/N ≥10	1.88 µg/mL (S/N = 12.3)
Detection Limit (Impurities)	S/N ≥3	0.05-0.06 µg/mL
Detection Sensitivity (Impurities)	S/N ≥10	0.15-0.18 µg/mL (S/N >10)
Method Resilience	Recovery 98-102%; RSD ≤2%	Substance retrieval 98.2- 101.8%; variability ≤1.4%
System Readiness	Column efficiency ≥6000; Asymmetry ≤1.5	Theoretical plates >6000; Asymmetry factor ≤1.36

Figure 6: Method validation parameters demonstrating excellent performance within ICH acceptance criteria for the developed Alectinib HCl assay method

DISCUSSION:

Alectinib hydrochloride's extremely low water solubility (≈0.02 mg·mL^{-1} at 25 °C) required a 50:50 (v/v) blend of 0.1% orthophosphoric acid and acetonitrile to maintain complete dissolution during sample handling while preserving chromatographic compatibility [5, 22, 59].

This integrated analytical strategy presents substantial improvements over current alectinib hydrochloride testing protocols. While established methodologies typically segregate potency evaluation from impurity assessment [6, 7, 8], this single-run RP-HPLC assay merges potency determination and impurity profiling—covering four process-related degradants—in under 50 min. [25].

It attains limits of detection/quantitation of 0.62/1.88 µg·mL^{-1} for alectinib and 0.05/0.15 µg·mL^{-1} for each impurity, matching or surpassing literature values while eliminating separate GC-MS workflows for trace genotoxins . Chromatographic efficiency (6900 ± 73 plates; tailing 1.25 ± 0.02; 0.06% RT precision) and complete degradation-product separation (Rs > 2.0; purity index > 0.998) confirm stability-indicating

performance.

Robustness testing via design-of-experiments further defined a resilient method operable region, and environmental metrics (Eco-Scale = 81; AGREE = 0.70; AMGS = 71) demonstrate a roughly 45% reduction in solvent use versus dual-method approaches. This integrated strategy offers a highly sensitive, efficient, and sustainable platform for routine quality control, method transfer, and regulatory submission of alectinib hydrochloride formulations [54,55,56].

CONCLUSION:

This research develops and comprehensively evaluates an integrated, degradation-sensitive RP-HPLC protocol that combines drug quantification with impurity characterization for alectinib hydrochloride 150 mg capsule formulations within an efficient 30-minute analytical timeframe. By leveraging a Design-of-Experiments strategy on an embedded-polar AQ-C18 column, we achieved complete chromatographic separation ($Rs \geq 2.1$) between the parent drug and each of its four process-related impurities, while maintaining excellent peak symmetry (tailing ≤ 1.3) and reproducibility (27, 28, 42-44). The method's enhanced sensitivity is confirmed by achieving low LOQs of 1.88 µg/mL for alectinib and 0.15–0.18 µg/mL for every impurity—ensuring reliable detection at and below specification thresholds [36].

Comprehensive validation per ICH Q2(R2) and Q14 guidelines confirmed linear calibration models ($R^2 \geq 0.999$) across working ranges, with residuals within ± 2% for the API and ± 3% for impurities [3, 4, 35]. Accuracy studies yielded recoveries tightly clustered between 98– 102%, and precision experiments showed intra-day RSDs $\leq$ 0.6% and inter-laboratory RSD of 2.8%, affirming both analytical ruggedness and transferability [33, 36]. Robustness testing— incorporating deliberate shifts in flow rate, temperature, pH, and gradient slope—had negligible impact on retention times, resolution, or quantitative results, while forced- degradation challenges (acid, base, oxidative, thermal, photolytic) produced well-resolved degradants with no interference at the alectinib retention time [28, 31, 42].

Importantly, we introduced green-chemistry metrics into method development: An Eco-Scale score of 81, an AGREE index of 0.70, and an AMGS rating of 71 attest to a ~45% reduction in solvent consumption and a 50% decrease in energy use compared to legacy two-method workflows (36, 37, 38). These improvements not only cut operating costs and reduce environmental burden, but also simplify laboratory logistics by halving instrument run-times and analyst hands-on time [50, 51, 52].

Looking forward, this UHPLC framework can be readily adapted to other tyrosine-kinase inhibitors by fine-tuning gradient parameters, or coupled to mass spectrometry for structural elucidation of unknown degradants (20, 53). Its confirmed robustness and rapid cycle time make it an ideal candidate for integration into Process Analytical Technology (PAT)-driven, real-time release testing and continuous-manufacturing platforms (8, 9). By harmonizing systematic DoE methodology, lifecycle-oriented validation, and sustainability principle, this unified assay sets a new standard for the analytical control of oncology therapeutics— balancing scientific rigor, regulatory compliance, and environmental responsibility [50].

Abbreviations:

ACN (acetonitrile), ALK (anaplastic lymphoma kinase), AMGS (Analytical Method Greenness Score), ANOVA (analysis of variance), BBD (Box–Behnken design), CCD (central composite design), CV (coefficient of variation), DMSO (dimethyl sulfoxide), DoE (design of experiments), Eco-Scale (Analytical Eco-Scale), FTIR (Fourier transform infrared spectroscopy), GHS (Globally Harmonized System), HCl (hydrochloric acid), HME (hot-melt extrusion), ICH (International Council for Harmonisation), IR (immediate release), KH_2PO_4 (potassium dihydrogen phosphate), LOD (limit of detection), LOQ (limit of quantitation), mAU·s (milli–absorbance-unit seconds), NSCLC (non-small-cell lung cancer), PAT (Process Analytical Technology), PDA (photodiode-array detector), QC (quality control), QbD (Quality by Design), R^2 (coefficient of determination), RSD (relative standard deviation), Rs (resolution factor), RP-HPLC (reversed-phase high-performance liquid chromatography), RT (retention time), SE (standard error), SD (standard deviation), SST (system suitability test), UHPLC (ultra-high-performance liquid chromatography), USP (United States Pharmacopeia), UV (ultraviolet), v/v (volume/volume).

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