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RESEARCH ARTICLE

Development and Validation of a Stability-Indicating RP-HPLC Method for Gilteritinib Using a Quality by Design

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Abstract: A robust, stability-indicating reversed-phase high-performance liquid chromatography method was developed and validated for the quantitative determination of the dual FLT3/AXL inhibitor gilteritinib in pharmaceutical dosage forms utilizing an analytical Quality by Design framework. Separation was achieved on a Phenomenex C18 column (150 × 4.6 mm, 5 µm) with a mobile phase composed of 0.01 M potassium dihydrogen phosphate buffer (adjusted to pH 3.0 containing 0.1% v/v triethylamine) and acetonitrile in a 59:41 v/v ratio. Chromatographic conditions were systematically optimized utilizing a three-factor, three-level Central Composite Design to evaluate the impact of flow rate, column temperature, and organic modifier concentration on critical quality attributes, including retention time, theoretical plates, and peak asymmetry. Under optimal conditions of 0.93 mL/min flow rate and 27°C column temperature, gilteritinib eluted as a symmetrical peak at 2.66 min. The method showed a linear response over the concentration range of 10 to 60 µg/mL with a high correlation coefficient of 0.999. The limits of detection and quantitation were determined to be 0.17 µg/mL and 0.53 µg/mL, respectively. Forced degradation studies conducted under hydrolytic, oxidative, photolytic, and thermal stress conditions showed that the method effectively resolved gilteritinib from its primary degradation products eluting at 1.65 and 2.99 min, confirming its stability-indicating capability. Application to commercial formulations resulted in a quantitative drug recovery of 100.25%, showing excellent precision and accuracy for high-throughput pharmaceutical quality control.

Keywords: Gilteritinib; Quality by Design; HPLC; Stability-Indicating; Central Composite Design.

1. Introduction

Acute myeloid leukemia (AML) is a rapidly progressing hematological malignancy characterized by the clonal expansion of abnormal myeloid progenitor cells within the bone marrow and peripheral blood, leading to severe hematopoiesis failure [1]. Approximately 30% of newly diagnosed acute myeloid leukemia patients exhibit mutations in the FMS-like tyrosine kinase 3 receptor gene, which are categorized as internal tandem duplications or mutations within the tyrosine kinase domain [2]. These mutations lead to constitutive activation of the tyrosine kinase receptor, triggering downstream proliferative pathways such as the signal transducer and activator of transcription 5, extracellular signal-regulated kinase, and protein kinase B cascades [3].

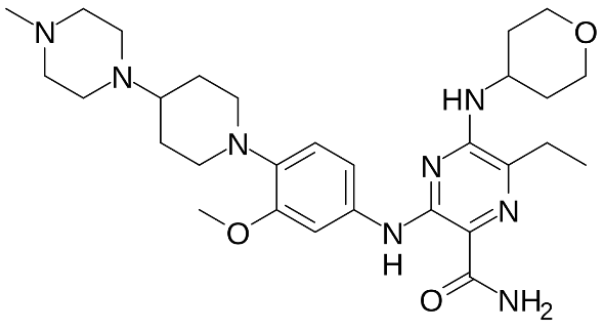

Figure 1. Structure of Gilteritinib

Gilteritinib, chemically designated as 2-pyrazinecarboxamide, 6-ethyl-3-[[3-methoxy-4-[4-(4-methyl-1-piperazinyl)-1-piperidinyl]phenyl]amino]-5-[(tetrahydro-2H-pyran-4-yl)amino], is a highly selective, small-molecule dual inhibitor of both the

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internal tandem duplication and tyrosine kinase domain mutations of the FMS-like tyrosine kinase 3 receptor [3,4]. It also targets the AXL tyrosine kinase receptor, which has been implicated in facilitating resistance mechanisms against conventional chemotherapies and other tyrosine kinase inhibitors [4,5]. Gilteritinib halts cellular proliferation and induces apoptosis in leukemia cells overexpressing mutant kinase isoforms by inhibiting these target kinases. Due to its therapeutic efficacy, gilteritinib received clinical approval for the treatment of adult patients presenting with relapsed or refractory acute myeloid leukemia harboring FMS-like tyrosine kinase 3 mutations, representing a critical advancement in targeted oncology [1,5]. The quality, therapeutic safety, and efficacy of pharmaceutical dosage forms depend on the purity and stability of the active pharmaceutical ingredient. Analytical methods deployed during drug development and commercial release must be capable of distinguishing the therapeutic entity from potential process impurities and degradation products generated throughout the shelf life of the product [6,7]. Standard analytical protocols require chromatographic techniques to achieve selective separation of compound matrices. Liquid chromatography remains the cornerstone of quantitative pharmaceutical analysis due to its high separation power, sensitivity, and adaptability [8].

The chemical structure of gilteritinib, with a molecular formula of $C_{29}H_{44}N_8O_2$ and a molecular mass of 552.72 g/mol, features basic nitrogen centers on the piperazine and piperidine ring systems. These functional groups impart a basic character to the molecule, with physiological pKa values typically falling in the range of 9.1 to 9.6 for the tertiary aliphatic amines, while the aromatic nitrogen systems show weaker basicity [9]. Consequently, standard reversed-phase high-performance liquid chromatography method development requires precise control over mobile phase pH to prevent unwanted interactions between protonated amine centers and active silanol groups on the silica surface of the stationary phase, which typically cause severe peak tailing and loss of column efficiency [10].

Conventional trial-and-error approach to chromatographic method development, often termed One-Factor-at-a-Time, frequently results in analytical methods that lack robustness when exposed to minor variations in operational parameters such as pH, mobile phase composition, or column temperature [11]. To overcome these limitations, regulatory agencies such as the International Conference on Harmonisation (ICH) recommend the implementation of Quality by Design principles as outlined in the Q8(R2) and Q9 guidelines [12,13]. Quality by Design involves a systematic approach starting with predefined objectives, emphasizing product and process understanding, and establishing control based on sound science and quality risk management.

Applying Quality by Design to chromatographic method development requires defining the Analytical Target Profile and identifying Critical Quality Attributes such as retention time (t_R), column efficiency (theoretical plates, N), and peak tailing or asymmetry (T_f) [14]. Potential risk factors, or Critical Method Parameters, are screened and optimized using experimental design tools. A multidimensional Design Space is established by exploring the multidimensional interactions between parameters such as mobile phase organic modifier concentration, buffer pH, and column temperature [11,14]. Operating within this validated design space ensures that minor, inadvertent changes in chromatographic conditions will not compromise the resolution, reproducibility, or accuracy of the analytical procedure, thereby facilitating life-cycle management and regulatory compliance.

2. Materials and Methods

2.1. Chemical Reagents

An active pharmaceutical ingredient reference standard of pure gilteritinib was obtained from Spectrum Pharma Labs (Hyderabad, India). Analytical reagent grade hydrochloric acid, sodium hydroxide pellets, and hydrogen peroxide (30% v/v) were purchased from Merck India Private Limited and Qualigens [10]. Monobasic potassium dihydrogen phosphate (KH_2PO_4), triethylamine, and orthophosphoric acid were sourced from S.D. Fine-Chem Limited. Acetonitrile and methanol of HPLC grade were purchased from Fisher Scientific [10,11]. High-purity water utilized for buffer preparation and dilution was obtained from a Milli-Q water purification system (Merck Millipore).

2.2. Instrumentation, Software, and Calibration Protocols

Chromatographic investigations were performed on a Waters Alliance 2965 chromatographic system equipped with an autosampler, a quaternary solvent delivery pump, and a Photo Diode Array (PDA) detector set at a detection wavelength of 225 nm [15]. Separation optimization was evaluated using various stationary phases, including an Agilent C18 column (150 × 4.6 mm, 5 µm), Discovery C18 column (150 × 4.6 mm, 5 µm), Zodiac C18 column (150 × 4.6 mm, 5 µm), BDS C18 column (150 × 4.6 mm, 5 µm), and a Phenomenex C18 column (150 × 4.6 mm, 5 µm) [11].

Instrument control, data acquisition, and chromatogram integration were managed using Empower 3 software. Design-Expert software (version 11.0.0, Stat-Ease Inc., Minneapolis, USA) was employed to construct the experimental design, generate response surface 3D plots, and execute multi-criteria optimization [14]. Structural confirmation and authentication of the drug sample were conducted using a Shimadzu UV-1800 spectrophotometer, a Bruker Alpha Fourier Transform Infrared spectrometer configured with an Attenuated Total Reflection module, a Buchi melting point apparatus, and a Waters LC-MS/MS (Q-ToF) mass spectrometer

[15]. Instrument calibration for pump flow rate, injection volume, wavelength accuracy, and detector response was verified prior to initiation of experimental runs in accordance with established laboratory guidelines [12,15].

2.3. Preparation of Standard and Buffer Solutions

A standard stock solution of gilteritinib was prepared by accurately weighing 10 mg of the drug substance, transferring it into a 25 mL volumetric flask, dissolving it in 15 mL of mobile phase diluent, and sonicating the mixture for 10 min to ensure complete dissolution. The flask was filled to volume with diluent to yield a final concentration of 400 µg/mL [15]. A working standard solution containing 40 µg/mL was prepared by diluting 1 mL of the stock solution to 10 mL with the same diluent.

The aqueous buffer was prepared by dissolving 1.36 g of monobasic potassium dihydrogen phosphate in 900 mL of Milli-Q purified water. After dissolution, the solution was sonicated to degas, and 1 mL of triethylamine was added. The pH of the buffer solution was adjusted to 3.0 by the dropwise addition of diluted orthophosphoric acid, and the volume was completed to 1000 mL with purified water. The resulting 0.01 M phosphate buffer was filtered through a 0.45 µm membrane filter under vacuum before use [10,11].

2.4. Method Optimization via Central Composite Design (CCD)

To explore the chromatographic behavior of gilteritinib and optimize the analytical parameters systematically, a three-factor, three-level Central Composite Design was implemented [14]. Preliminary trials guided the selection of three Critical Method Parameters: column temperature (ranging from 24.9˚C to 35.1˚C), mobile phase flow rate (ranging from 0.83 to 1.17 mL/min), and organic phase ratio (ranging from 49.9% to 70.1% acetonitrile) [11]. The design generated 20 experimental runs with varying combinations of these parameters, which were executed in a randomized sequence to minimize systemic bias. The experimental responses monitored as Critical Quality Attributes were defined as retention time (t_R, min), column theoretical plates (N), and peak tailing or asymmetry factor (T_f) [14]. Quadratic polynomial equations were fitted to the experimental response data to mathematically model the main effects, two-factor interactions, and quadratic terms [11,14].

2.5. Forced Degradation Studies (Stress Testing)

To determine the stability-indicating capability of the optimized HPLC method, gilteritinib was subjected to forced degradation studies under various stress conditions in accordance with the ICH Q2(R1) regulatory guidelines [12,13]. All stress experiments were executed using a Radleys carousel reaction station equipped with continuous stirring and digital temperature control [15].

Acid-catalyzed hydrolysis was conducted by adding 1 mL of 2N hydrochloric acid to 1 mL of gilteritinib stock solution, and the mixture was heated under reflux at 70˚C for 8 hours [15]. Following the stress period, the solution was cooled to room temperature, neutralized to pH 7.0 using an equivalent volume of 2N sodium hydroxide, and diluted to the appropriate analytical concentration before injection [15].

Base-catalyzed hydrolysis was performed in an identical manner by treating 1 mL of the stock solution with 1 mL of 2N sodium hydroxide and refluxing at 70˚C for 8 hours [15]. The stressed sample was neutralized with 2N hydrochloric acid and diluted prior to chromatographic analysis.

Neutral hydrolytic degradation was evaluated by dissolving 10 mg of gilteritinib in water and refluxing the solution at 70˚C for 24 hours in the reaction station.

Oxidative stress was induced by mixing 1 mL of gilteritinib stock solution with 1 mL of a 20% v/v hydrogen peroxide solution. The reaction mixture was kept in the dark at room temperature for 24 hours to prevent photolytic activation [15].

Thermal degradation of the solid state was evaluated by spreading 10 mg of gilteritinib drug substance uniformly in a Petri dish and placing it in a hot air oven maintained at 70˚C for 24 hours [15].

Photolytic degradation was studied by exposing a thin layer of solid gilteritinib drug substance directly to natural sunlight for 24 hours. The stressed solid samples from both thermal and photolytic studies were subsequently dissolved and diluted to obtain a concentration of 40 µg/mL for chromatographic analysis. Corresponding blanks were prepared under identical stress conditions to rule out any chromatographic interference from reagents or container materials.

2.6. Method Validation

The optimized chromatographic method was validated in compliance with the ICH Q2(R1) guidelines, assessing the parameters of system suitability, specificity, linearity, precision, accuracy, limit of detection, limit of quantitation, and robustness [12].

2.6.1. System Suitability

System suitability was verified by injecting six replicate injections of the working standard solution (40 µg/mL) of gilteritinib [12]. The relative standard deviation (% RSD) of the peak area and retention time was calculated, along with system suitability indicators such as column theoretical plates (N) and the peak asymmetry factor (T_f) [15].

2.6.2. Specificity

Specificity was established by comparing the chromatograms of blank diluent, placebo mixtures, standard drug solutions, and stressed drug solutions [12]. The resolution between gilteritinib and any generated degradation peaks was evaluated to confirm that no chromatographic co-elution occurred, ensuring accurate quantitative determination of the active pharmaceutical ingredient.

2.6.3. Linearity and Range

Linearity was established by preparing and analyzing seven calibration standards over the concentration range of 10 to 60 µg/mL (including the lower limit of quantitation) [12]. The calibration curve was constructed by plotting the mean peak area against the respective concentration of gilteritinib. The parameters of the linear regression equation, $y = mx + c$, and the correlation coefficient (r^2) were determined by the method of least squares [12,15].

2.6.4. Accuracy

Accuracy was evaluated via recovery experiments using the standard addition technique [12]. Known quantities of gilteritinib standard drug substance were spiked into pre-analyzed sample matrices at three concentration levels corresponding to 50%, 100%, and 150% of the nominal assay concentration (40 µg/mL) [15]. Triplicate injections were analyzed at each concentration level, and the percentage recovery and statistical deviation were calculated.

2.6.5. Precision

Method precision was evaluated through repeatability (intra-day precision) and intermediate precision (inter-day precision) [12]. Repeatability was evaluated by analyzing six replicate preparations of the standard solution (40 µg/mL) within a single day. Intermediate precision was assessed by performing the same analytical procedure on separate days using different analytical standard preparations [15]. The results are expressed as the percentage relative standard deviation (% RSD) of the peak areas.

2.6.6. Detection and Quantitation Limits

The Limit of Detection (LOD) and Limit of Quantitation (LOQ) were calculated using mathematical methods based on the standard deviation of the analytical response (σ) and the slope of the calibration curve (S), according to the following formulas [12]:

$$\mathrm{LOD} = \frac{3.3\sigma}{S}$$

$$\mathrm{LOQ} = \frac{10\sigma}{S}$$

where sigma represents the standard deviation of the intercept of the regression line, and S is the slope of the calibration curve.

2.6.7. Robustness

The robustness of the method was verified by introducing small, deliberate modifications to critical chromatographic parameters [12]. The investigated parameters included mobile phase flow rate (pm 10%, 0.837 to 1.023 mL/min), organic modifier ratio (pm 10%, 37% to 45% acetonitrile), and column temperature (pm 10%, 24.3˚C to 29.7˚C) [15]. The impact of these intentional modifications on system suitability parameters and the final quantitative assay results was assessed.

3. Results and Discussion

3.1. Identification of Gilteritinib

The identity and purity of the procured gilteritinib reference standard were verified through analytical characterization. Under UV-visible spectrophotometric scanning from 200 to 400 nm in methanol, gilteritinib showed an absorption maximum (λ_{max}) at 213 nm. FTIR confirmed the presence of characteristic functional groups, displaying prominent absorption bands at 3285 cm^{-1} (N-H amide stretching), 2931 cm^{-1} (aliphatic C-H stretching), 1658 cm^{-1} (C=O amide carbonyl stretching), 1587 cm^{-1} (aromatic C=C stretching), and 1224 cm^{-1} (C-O-C ether stretching). Mass spectrometric analysis using LC-MS/MS in positive electrospray ionization mode yielded a protonated molecular ion $[M^+H]^+$at m/z 553.3, confirming the nominal molecular weight of 552.72 g/mol [3]. The melting point of the drug substance was determined to range from 153˚C to 155˚C, consistent with values reported in the literature [16]. These analytical observations confirmed the identity and high purity of the active pharmaceutical ingredient.

3.2. Optimization by Response Surface Methodology

Preliminary trials were conducted to select an appropriate stationary phase and organic modifier. The Phenomenex C18 column (150 × 4.6 mm, 5 µm) exhibited superior performance compared to the Agilent, Discovery, Zodiac, and BDS columns, yielding high column efficiency and minimal peak tailing. Acetonitrile was selected as the organic modifier due to its favorable elution strength, lower viscosity, and superior solubility profile for gilteritinib compared to methanol, which produced broader peak widths and higher backpressures.

The three critical chromatographic variables (flow rate, acetonitrile concentration, and column temperature) were systematically optimized using Central Composite Design [11,14]. The experimental design and the resulting chromatographic responses are summarized in the table below.

Table 1. Experimental design and the chromatographic responses

Run	Flow Rate (A, mL/min)	% Acetonitrile (B, % v/v)	Temperature (C, ˚C)	Retention Time (t_R, min)	Asymmetry (T_f)	Theoretical Plates (N)
1	1.00	66.0	33.0	2.598	1.20	3728.2
2	1.00	60.0	35.0	2.516	1.30	3150.8
3	1.00	70.1	30.0	3.252	1.40	3989.5
4	1.00	60.0	30.0	2.469	1.20	4899.3
5	1.10	54.0	33.0	2.098	1.20	3810.4
6	1.17	60.0	30.0	1.738	1.20	3041.2
7	1.00	60.0	30.0	2.448	1.20	4621.4
8	1.00	60.0	30.0	2.423	1.20	4818.7
9	1.00	60.0	30.0	2.434	1.20	4869.9
10	0.90	66.0	33.0	3.130	1.40	2547.1
11	0.83	60.0	30.0	3.106	1.30	2943.3
12	0.90	54.0	33.0	2.566	1.20	3994.9
13	1.00	49.9	30.0	2.229	1.30	4075.6
14	1.00	60.0	30.0	2.421	1.20	4861.1
15	1.00	60.0	25.0	2.513	1.30	3339.4
16	0.90	66.0	27.0	3.143	1.40	2223.2
17	1.10	66.0	27.0	2.563	1.30	3527.1
18	1.00	60.0	30.0	2.443	1.20	4734.7
19	0.90	54.0	27.0	2.570	1.20	3616.2
20	1.10	54.0	27.0	2.092	1.20	3447.5

The results were statistically evaluated using Analysis of Variance (ANOVA) [14]. For retention time (t_R), a quadratic response model was selected, showing significance with an F-value of 35.21 and a p-value < 0.0001. The flow rate (A) and acetonitrile ratio (B) were determined to be highly significant model terms. The mathematical relationship modeling the retention time is expressed by the following quadratic regression equation in terms of coded factors:

$$t_R = 2.44 - 0.4095A + 0.3592B + 0.0033C - 0.0205AB - 0.0074AC + 0.0016BC - 0.0002A^2 + 0.2520B^2 + 0.0730C^2$$

Similarly, the column theoretical plates (N) response fit a quadratic model (F-value = 17.15, p-value < 0.0001), showing that both flow rate and temperature exert a parabolic influence on column efficiency. This behavior is represented by the following model equation:

$$N=4786.13+120.35A+156.49B-49.75C-354.71AB-17.33AC+27.08BC-624.78A^2-262.88B^2-536.43C^2$$

The peak asymmetry (T_f) was found to be dependent on the organic phase concentration, which is modelled by the following equation:

$$T_f = 1.20 - 0.0250\ A + 0.0357\ B + 0.0053\ C - 0.0375\ AB + 0.0125\ AC - 0.0125\ BC + 0.0085\ A^2 + 0.0426\ B^2 + 0.0256\ C^2$$

The interaction between critical method parameters were shown in the contour and 3D response surface plots.

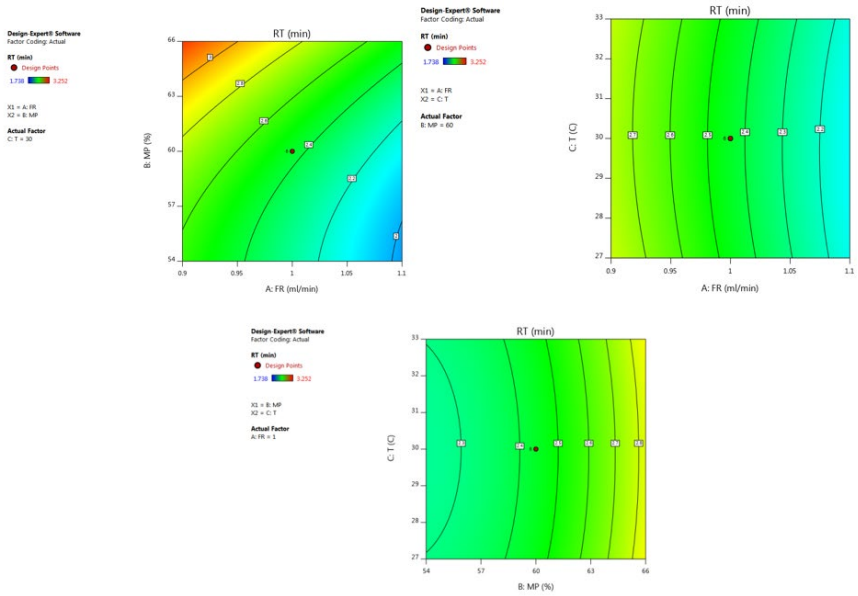

Figure 2. Contour plots showing the effect of Flow Rate, Organic Ratio, and Temperature on Retention Time

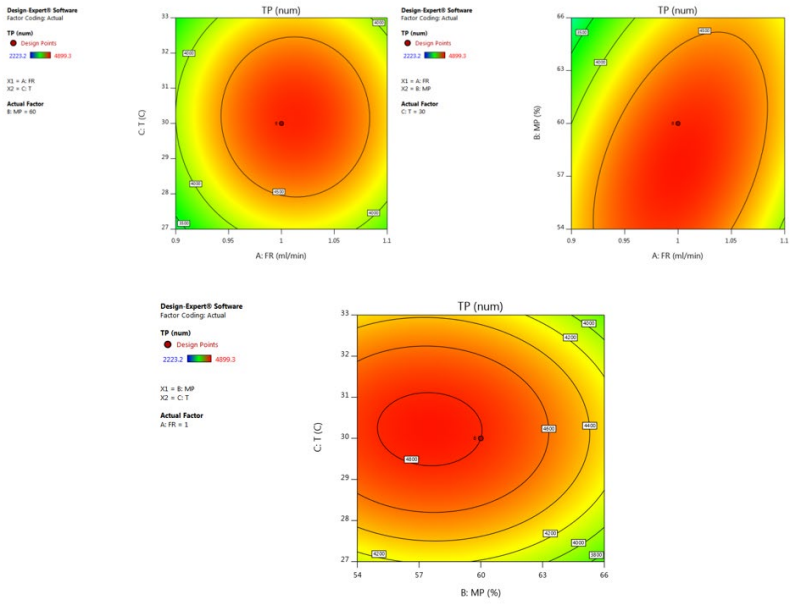

Figure 3. Contour plots showing the effect of Flow Rate, Organic Ratio, and Temperature on Theoretical Plates

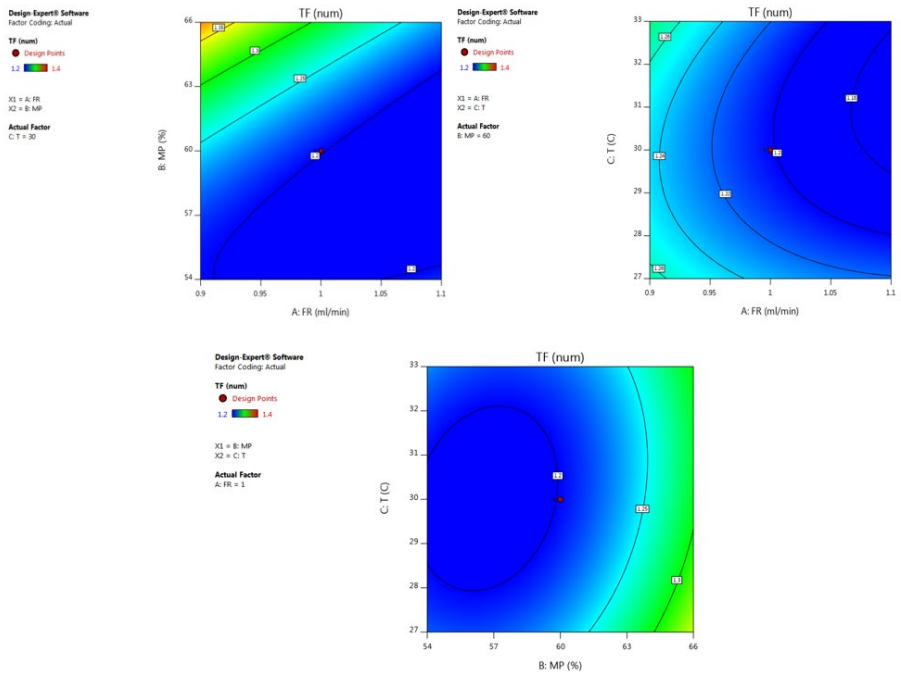

Figure 4. Contour plots showing the effect of Flow Rate, Organic Ratio, and Temperature on Peak Asymmetry

Using a multi-criteria desirability function approach, a composite desirability (D) of 1.00 was achieved.

The optimized chromatographic parameters derived from this mathematical optimization were: a mobile phase composition of 0.01 M monobasic potassium dihydrogen phosphate buffer (adjusted to pH 3.0) and acetonitrile in a 59:41 v/v ratio, a flow rate of 0.93 mL/min, and a column temperature maintained at 27°C. This optimized method yielded a standard peak elution at 2.66 min, resolving gilteritinib with a symmetrical peak shape (T_f = 1.21) and high column efficiency (N = 4850), which was subsequently used for method validation and forced degradation studies.

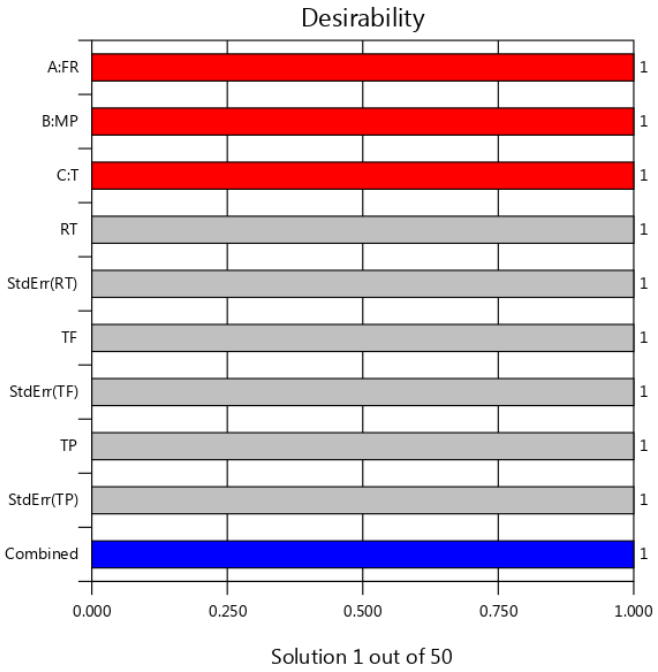

Figure 5. Desirability bar chart for the multi-objective optimization

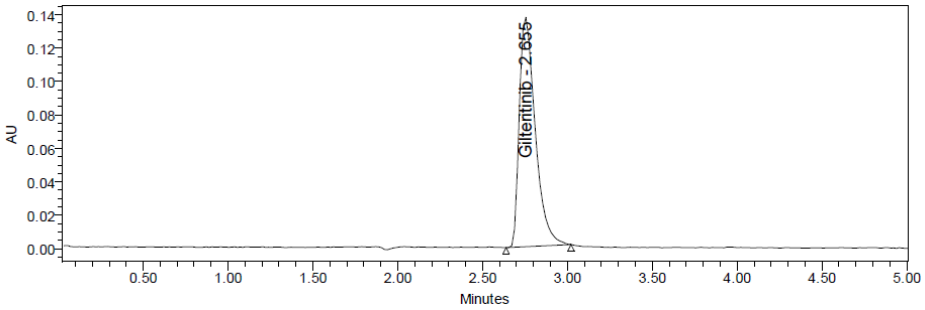

Figure 6. Chromatogram of gilteritinib standard solution under optimized chromatographic conditions

3.3. Validation of the Optimized Method

3.3.1. System Suitability

The system suitability parameters were determined from six replicate injections of the standard drug solution (40 µg/mL). The relative standard deviation (% RSD) of the peak areas was found to be 0.3%. The average retention time was 2.66 min (% RSD = 0.2%). The column theoretical plate count exceeded 4500, and the tailing factor was maintained below 1.3. These results met the regulatory acceptance specifications of N > 2000 and $T_f \leq 2.0$, confirming the readiness of the system for quantitative evaluation.

3.3.2. Specificity

Specificity was verified by injecting blank diluent, placebo mixtures, gilteritinib standard, and stressed solutions into the HPLC system. The blank and placebo chromatograms did not display any peaks at the retention time of gilteritinib.

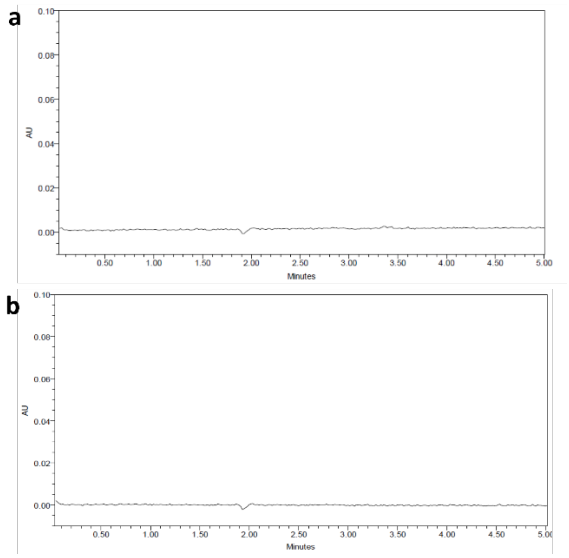

Figure 7. Chromatograms of blank diluent and placebo solution showing absence of peak interferences

Under all forced stress conditions, the degradation products were resolved from the parent drug peak, confirming that the developed method is selective and stability-indicating.

3.3.3. Linearity

The calibration curve for gilteritinib showed excellent linearity over the evaluated concentration range of 10 to 60 µg/mL. The linear regression equation was determined to be: y = 23398 x + 11401 The correlation coefficient (r^2) was calculated to be 0.999, indicating a strong linear relationship between the analyte concentration and the detector response.

3.3.4. Precision

The reproducibility of the method was evaluated by performing repeatability (intra-day) and intermediate (inter-day) precision studies, as summarized in the tables below.

Table 2. Results of Repeatability (Intra-day Precision)

Injection	Peak Area	Amount Found (µg/mL)	% Nominal Assay
1	937798	39.59	98.98
2	932539	39.37	98.42
3	930848	39.29	98.24
4	935096	39.48	98.69
5	936856	39.55	98.88
6	934695	39.46	98.65
Mean	934639	39.46	98.64
SD	2603.4	0.11	0.27
% RSD	0.28	0.28	0.27

Table 3. Results of Intermediate (Inter-day Precision)

Injection	Peak Area	Amount Found (µg/mL)	% Nominal Assay
1	932491	39.37	98.41
2	923317	38.97	97.44
3	937308	39.57	98.93
4	921659	38.90	97.26
5	927166	39.14	97.85
6	927960	39.17	97.93
Mean	928317	39.19	97.97
SD	5814.5	0.25	0.62
% RSD	0.63	0.63	0.63

The standard deviations and percentage relative standard deviations for both intra-day and inter-day precision studies were well below the regulatory threshold of 2.0%, confirming the reliability and precision of the analytical method.

3.3.5. Accuracy

The recovery of the standard addition experiments at three concentration levels (50%, 100%, and 150%) is summarized in the table below.

Table 4. Results of Accuracy

Spiked Level (%)	Nominal Amount (µg/mL)	Spiked Amount (µg/mL)	Recovered Amount (µg/mL)	% Recovery	Mean % Recovery
50	40.0	20.0	19.91	99.56	100.25
50	40.0	20.0	19.92	99.61	
50	40.0	20.0	20.96	104.82	
100	40.0	40.0	39.69	99.22	
100	40.0	40.0	39.69	99.22	
100	40.0	40.0	39.79	99.48	
150	40.0	60.0	59.99	99.98	
150	40.0	60.0	60.07	100.11	
150	40.0	60.0	60.15	100.25	

The recovery values ranged from 99.22% to 104.82%, with an overall mean recovery of 100.25%, indicating that the method is accurate and free from systematic matrix interferences.

3.3.6. LOD and LOQ

The Limit of Detection and Limit of Quantitation were calculated from the regression parameters of the calibration curve, yielding an LOD of 0.17 µg/mL and an LOQ of 0.53 µg/mL. These values confirm that the method is sensitive enough for the quantitative determination of gilteritinib in pharmaceutical dosage forms and the monitoring of trace degradation products.

3.3.7. Robustness

The robustness of the method was evaluated by introducing small, deliberate changes to the optimized flow rate, column temperature, and mobile phase ratio. The relative standard deviation (% RSD) of the target responses remained below 1.0% across all modified runs, as detailed in the table below.

Table 5. Results of Robustness

Modified Parameter	Applied Condition	Response % RSD
Flow Rate (-10%)	0.837 mL/min	0.90
Flow Rate (+10%)	1.023 mL/min	0.80
Organic Ratio (-10%)	36.9% Acetonitrile	0.60
Organic Ratio (+10%)	45.1% Acetonitrile	0.80
Column Temp (-10%)	24.3°C	0.10
Column Temp (+10%)	29.7°C	0.60

These results confirm that the method is robust and insensitive to minor variations in operational parameters.

3.4. Forced Degradation and Stability-Indicating Studies

The chromatograms obtained from the forced degradation of gilteritinib under various stress conditions are shown in the following figures.

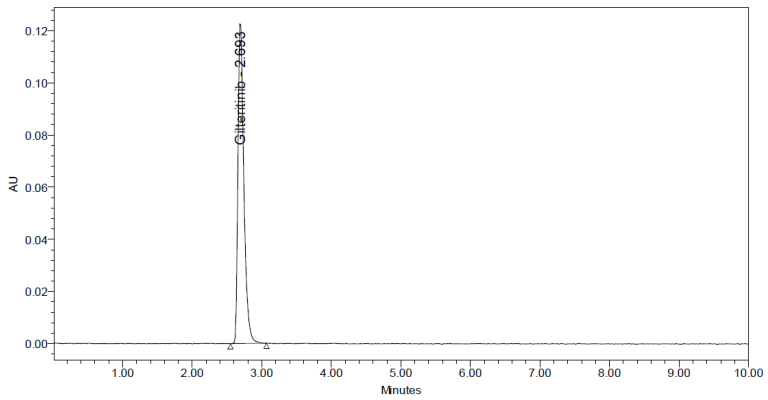

Figure 8. Chromatogram of acid degradation in 2N HCl at 70°C for 8 hours

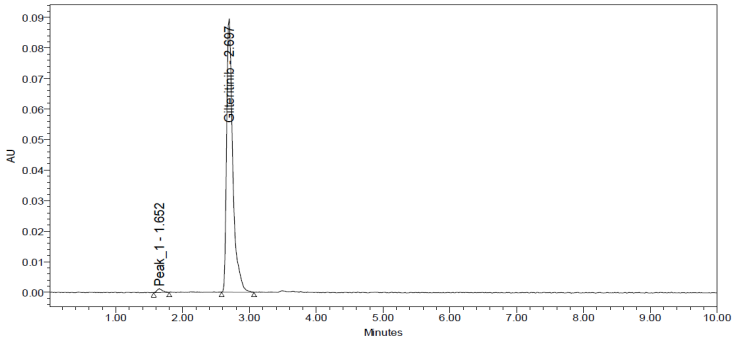

Figure 9. Chromatogram of alkaline degradation in 2N NaOH at 70°C for 8 hours

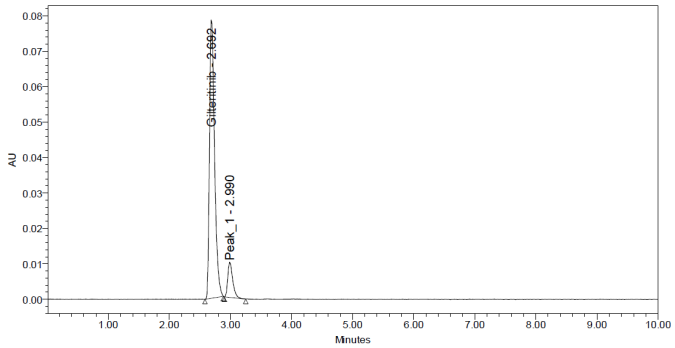

Figure 10. Chromatogram of oxidative degradation in 20% H_2O_2 at room temperature for 24 hours

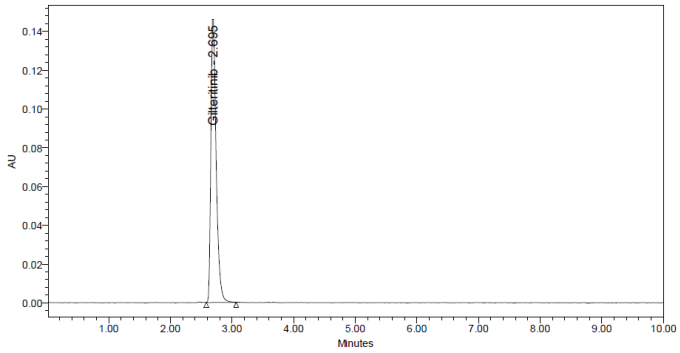

Figure 11. Chromatogram of thermal degradation at 70˚C for 24 hours

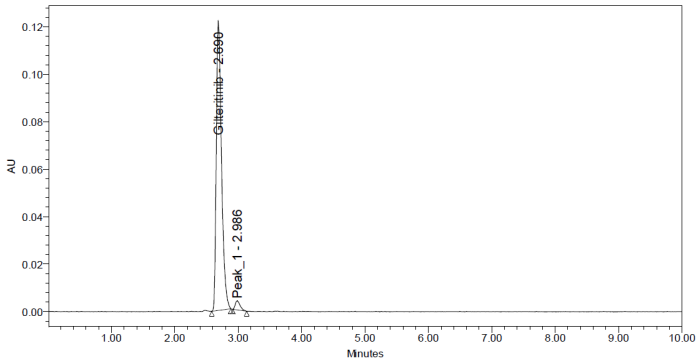

Figure 12. Chromatogram of photolytic degradation under sunlight for 24 hours

The percentage degradation and retention times of the degradation products under the applied stress conditions is presented in the table below.

Table 6. Percentage degradation and retention times of the degradation products under the applied stress conditions

Stress Condition	Applied Reagent / Environment	Exposure Duration	% Degradation	Retention Time of Degradants (t_R, min)
Acid Hydrolysis	2N HCl at 70°C	8 hours	6.82	No UV-absorbing peak resolved
Base Hydrolysis	2N NaOH at 70°C	8 hours	4.21	1.65
Neutral Water	Purified water at 70 °C	24 hours	0.28	None detected
Oxidative Stress	20% H_2O_2 at room temp	24 hours	5.45	2.99
Thermal Stress	Solid state at 70 °C	24 hours	3.48	No UV-absorbing peak resolved
Photolytic Stress	Natural direct sunlight	24 hours	1.68	2.99

Under base hydrolysis, gilteritinib underwent 4.21% degradation, yielding a polar degradant peak at 1.65 min. In the oxidative stress study, 5.45% degradation of the drug occurred, with a distinct degradant peak eluting at 2.99 min. This peak at 2.99 min was also observed under photolytic stress (1.68% degradation), suggesting a shared degradation pathway.

Under acid hydrolysis (6.82% degradation) and thermal stress (3.48% degradation), no distinct UV-absorbing degradant peaks were detected. This suggests that the degradation products under these conditions do not absorb at the target wavelength (225 nm) or elute within the dead volume. Gilteritinib remained stable under neutral hydrolytic conditions, showing only 0.28% degradation after 24 hours. These results indicate that the method can resolve gilteritinib from its degradation products, confirming its stability-indicating capability.

3.5. Assay of Marketed Formulation

The validated method was applied for the quantitative determination of gilteritinib in commercially available tablet formulations. The standard and sample solutions were injected separately in replicate preparations, and the percentage assay was calculated.

Table 7. Results of Commercial Formulation Assay

Replicate Sample	Calculated % Assay
Preparation 1	100.26
Preparation 2	99.70
Preparation 3	99.52
Preparation 4	99.97
Preparation 5	100.16
Preparation 6	99.93
Mean Assay	99.92
SD	0.28
% RSD	0.28

The average assay of the marketed formulation was found to be 99.92% with a statistical standard deviation of 0.28%, indicating that the method is accurate and suitable for routine quality control of commercial formulations.

4. Conclusion

A robust, stability-indicating reversed-phase high-performance liquid chromatography method was developed and validated for the determination of gilteritinib using a Quality by Design approach. System optimization was achieved using a Central Composite Design, which established a robust design space. The optimized mobile phase consisted of a 0.01 M potassium dihydrogen phosphate buffer (pH 3.0) and acetonitrile (59:41 v/v) at a flow rate of 0.93 mL/min and a column temperature of 27°C, yielding a symmetrical peak with a retention time of 2.66 min. The method was validated in compliance with the ICH guidelines, showing acceptable sensitivity, linearity, precision, accuracy, and robustness. Forced degradation studies under hydrolytic, oxidative, photolytic, and thermal stress conditions showed that the degradation products were resolved from the parent drug peak, confirming the stability-indicating capability of the method. The assay results of the commercial formulation were accurate and precise, indicating that the developed method is suitable for routine quality control and stability testing of gilteritinib in pharmaceutical dosage forms.

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