QbD-Oriented Bioanalytical Method Development for Erlotinib: Achieving Enhanced Accuracy and Robustness via LC–MS/MS

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Abstract

An LC–MS/MS analytical method guided by the Quality by Design (QbD) approach was carefully optimized and validated for the estimation of Erlotinib in human plasma, using Erlotinib-D_6 as the internal standard (IS). Excellent linearity was demonstrated by the approach over the concentration range of 2.01–2219.09 ng/mL, and it was backed by an easy and affordable liquid–liquid extraction process. A Box–Behnken experimental design was implemented to optimize critical chromatographic factors flow rate, buffer pH, and mobile phase ratio with retention time and peak arae defined as the dependent responses. Optimization results demonstrated a significant reduction in variability across analytical parameters, enhancing the robustness and reliability of the method. Validation outcomes confirmed that the assay met all acceptance criteria for linearity, accuracy, precision, selectivity, and sensitivity. Stability studies, including bench-top, autosampler, short-term, long-term, and freeze–thaw evaluations, confirmed that Erlotinib remained stable under all tested conditions. Overall, the developed LC–MS/MS method offers a precise, robust, and reproducible approach for quantifying Erlotinib in plasma, rendering it highly suitable for pharmacokinetic profiling and bioequivalence investigations in clinical research.

Keywords: Bioanalytical Validation, Analytical Quality by Design, Stability, Robustness

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Introduction

Erlotinib, a strong tyrosine kinase activity inhibitor that specifically targets the epidermal growth factor receptor (EGFR), is frequently recommended to treat pancreatic cancers and non-small cell lung cancers. Its pharmacological action primarily involves the blockade of EGFR-mediated intracellular signaling cascades that are responsible for promoting uncontrolled cellular proliferation, angiogenesis, and inhibition of apoptosis in cancerous tissues (Bardin C et al., 2014, Ling J et al 2008, Clark GM et al., 2006, Samuel JR et al., 2019). Chemically, Erlotinib belongs to the anilinoquinazoline class, with a molecular weight of 393.44 g/mol and a molecular formula of $C_{22}H_{23}N_3O_4$ (National Center for Biotechnology Information 2023). The substance has a moderate lipophilicity and is extensively metabolized by the cytochrome P450 isoenzyme in the liver, producing active and inactive metabolites that can influence therapeutic response and interindividual variability.

Analytical methods such as liquid chromatography–mass spectrometry (LC–MS) have become indispensable tools for the quantification of Erlotinib in biological matrices due to their superior sensitivity, specificity, precision, and reproducibility, enabling accurate pharmacokinetic profiling and therapeutic drug monitoring. Compared with conventional spectrophotometric or HPLC–UV assays, LC–MS/MS offers better selectivity and significantly lower limits of detection, which is crucial for trace-level quantification in plasma or serum samples collected during pharmacokinetic studies**.** As per the USFDA product-specific guidance, a two-treatment, two-period, fasting bioequivalence study design is recommended to assess comparative bioavailability, using the 150 mg tablet strength as the reference dose (U.S. Food and Drug Administration 2023).

For the determination of tyrosine kinase inhibitors, such as sunitinib, dasatinib, imatinib, and erlotinib, several analytical techniques have been developed, such as mass spectrometry (MS), gas chromatography (GC), liquid chromatography–mass spectrometry (LC–MS), and high-performance liquid chromatography coupled with ultraviolet detection (HPLC–UV) (Ohgami M et al., 2016, Yuta Y et al., 2022, Faivre L et al., 2011, Jing Z et al., 2017, Bouchet S et al., 2011, Honeywell R et al., 2010, Götze L et al., 2012). In more recent reports, significant attention has been directed toward optimizing sample preparation strategies—such as solid-phase extraction, protein precipitation, and liquid–liquid extraction, to enhance recovery and minimize matrix interference in LC–MS/MS and GC–MS analyses of Erlotinib Chongliang L et al., 2013, Ghasemian E et al., 2023, Herbrink M et al., 2016. However, these conventional analytical methods are often

associated with lengthy and labor-intensive sample preparation steps, high solvent consumption, and the need for stringent control of multiple chromatographic parameters. Consequently, they may exhibit substantial variability and reduced reproducibility, which can adversely impact the accuracy of quantification.

To address these limitations, the utilization of a Quality by Design (QbD)-based framework has emerged as an effective approach for the development of bioanalytical methods in complex biological matrices. Within the QbD paradigm, the Design of Experiments (DoE) tool provides a statistically robust and systematic strategy to identify critical method variables, evaluate their interactions, and optimize them to achieve the desired analytical performance. This scientific approach facilitates method robustness and transferability, ensures regulatory compliance, and minimizes trial-and-error experimentation, thereby reducing both development time and cost. Additionally, the QbD framework supports risk assessment and establishes a design space that guarantees method reliability throughout its life cycle Jaiprakash NS et al., 2017, Hasnain MS et al., 2013, Khan S et al., 2023, Khan S et al., 2025.

In the present investigation, a rapid, highly sensitive, and cost-effective LC–MS/MS method was developed and optimized for the quantitative determination of Erlotinib in human plasma. Key chromatographic parameters influencing retention time, peak shape, and overall separation efficiency were optimized using the Box–Behnken design (BBD) under the DoE framework. The developed method demonstrated excellent linearity, precision, and recovery, with negligible matrix effects and improved signal-to-noise ratio compared with previously reported procedures. In compliance with ICH M10 and USFDA requirements for bioanalytical technique validation, the optimized approach underwent rigorous validation. This included evaluations of Erlotinib's stability, matrix effect, recovery, accuracy, precision, and selectivity under various processing and storage settings. The validated LC–MS/MS method thus offers a robust and reproducible analytical platform suitable for pharmacokinetic, bioequivalence, and therapeutic drug monitoring applications in clinical and preclinical settings.

Materials and methods

Analyte and Internal Standard (IS)

Erlotinib and its deuterated internal standard, Erlotinib-D6, were procured from Artis Biotech (Mumbai, India).

Biological Matrix

The biological matrix employed in the study was sourced from Sycon Clinical Research Pvt. Ltd., Gandhinagar, Gujarat. Human plasma containing K_2-EDTA served as the matrix for preparing calibration curve (CC) standards and quality control (QC) samples. The analyte was isolated from the plasma to eliminate possible interference from endogenous substances. Additionally, blank plasma samples were analyzed with each batch to confirm the absence of any interfering peaks at the retention times of Erlotinib and its internal standard.

Reagents

HPLC-grade methanol, ammonium acetate, acetonitrile, ammonia, ethyl acetate, and water were acquired from Merck (Mumbai, India) and used as received for all experimental procedures.

Preparation of Solutions Used in the LC–MS/MS Method

Ammonium Acetate Buffer (Buffer-1)

Approximately 0.385 g of ammonium acetate was precisely weighed and transferred into a 1,000 mL reagent bottle. About 1,000 mL of HPLC-grade water was then added, and the contents were thoroughly mixed. The solution was sonicated in an ultrasonic bath until all solids were completely dissolved. The freshly prepared buffer was used within five days, with necessary dilutions made whenever required.

Mobile Phase Preparation

A clean 1,000 mL reagent container was filled with 200 mL of Buffer-1, 800 mL of HPLC-grade acetonitrile, and 0.01% liquid ammonia to create the mobile phase. After properly mixing the solution, any trapped air was removed by sonicating and degassing it in an ultrasonic bath. After being produced, the mobile phase was used within three days and kept at room temperature.

Ammonia Solution

A 10 mL portion of ammonia solution was accurately measured and transferred to a 1,000 mL volumetric flask. The solution was diluted to the mark using HPLC-grade water, mixed thoroughly, and then sonicated and degassed in an ultrasonic bath. After being prepared, the solution was used within three days and kept at room temperature.

Diluent Solution

An accurately measured 500 mL of HPLC-grade methanol was transferred to a 1,000 mL reagent flask, and the volume was adjusted to the mark with HPLC-grade water. The resulting mixture was well mixed, sonicated, and degassed to ensure homogeneity. After being prepared, the solution was used within three days and kept at room temperature.

Reconstitution Solution

A total of 700 mL of HPLC-grade acetonitrile was measured and transferred into a 1,000 mL reagent flask. The final volume was made up with HPLC-grade water. The solution was mixed thoroughly and sonicated in an ultrasonic bath to eliminate any dissolved gases. After being prepared, the Reconstitution solvent was used within three days and kept at room temperature.

Rinsing Solution

HPLC-grade acetonitrile was used as the rinsing solvent. Before use, it was sonicated for about 15 minutes to remove any dissolved air bubbles and ensure optical clarity of the solvent.

Preparation of Stock Solutions

Erlotinib and Erlotinib-D$_6$ Stock Solutions

To reach a concentration of 1 mg/mL, a stock solution of Erlotinib was made in the chosen diluent using the spiking technique. This stock was serially diluted to yield a 50 ng/mL solution, which was subsequently utilized to create quality control (QC) and calibration curve (CC) samples. These samples were essential for evaluating accuracy, precision, and determining the lower limit of quantification (LLOQ) of the developed method. The internal standard (IS), Erlotinib-D$_6$, was similarly prepared and analyzed by LC–MS/MS to confirm the absence of any interfering peaks or impurities.

Instrumentation and LC–MS/MS Conditions

A Shimadzu HPLC system with an LC-20 pump and a SIL-HTc autosampler connected to an AB SCIEX API 4000 triple quadrupole mass spectrometer was used to quantitatively determine the amount of erlotinib in human plasma. An XTerra MS C18 column (75 × 4.6 mm, 5 µm) kept at room temperature was used to produce chromatographic separation. Analyst® software version 1.6.3 was used for data processing and acquisition. The mobile phase consisted of ammonium acetate buffer and acetonitrile in a 20:80 (v/v) ratio, delivered at a flow rate of 0.8 mL/min. A 5 µL injection volume was used for all runs, and the total chromatographic run time was 1.24 minutes. Under these optimized conditions, Erlotinib and the internal standard (Erlotinib-D$_6$) showed well-resolved and distinct retention times.

A Turbo Ion Spray source was used to run the mass spectrometer in the positive ionization mode. Through the continuous infusion of a standard solution at a rate of 10 µL/min, instrumental parameters were improved. The optimized source conditions included an ion source temperature of 550°C, curtain gas at 50, collision gas (CAD) at 12, and an ion spray voltage of 4000 V. The de-clustering potential (DP), entrance potential (EP), and focusing potential (FP) were set to 60 V, 45 V, and 10 V, respectively. Quantitation was performed using multiple reaction monitoring (MRM) transitions at m/z 394.1 → 278.0 for Erlotinib and m/z 400.3 → 278.0 for Erlotinib-D$_6$ (internal standard).

Calibration Standards and Quality Control Samples

The calibration curve for Erlotinib was generated using a series of standard solutions spanning concentrations from 2.01 ng/mL to 2219.08 ng/mL. To ensure the reliability of the analytical method, quality control (QC) samples were prepared at four distinct levels: 2.01 ng/mL (LLOQ QC), 5.89 ng/mL (LQC), 776.67 ng/mL (MQC), and 1664.29 ng/mL (HQC). All QC samples were stored at temperatures below −50°C until analysis to preserve sample integrity. For assessing long-term stability, a set of 36 LQC and HQC samples was maintained at temperatures below −20°C and analyzed periodically to confirm consistency and stability of the analyte under storage conditions.

Method validation studies

The developed LC–MS/MS method was validated through three analytical runs, each comprising eight non-zero calibration curve (CC) standards, two standard zero samples (containing the internal standard, IS), and one blank plasma sample (without IS). A minimum of six out of the eight calibration standards (≥75%) were required to meet the acceptance limits of ±15% deviation from the nominal concentration, except for the LLOQ, where a deviation of ±20% was permitted. Both the LLOQ and ULOQ were required to comply with these criteria, and the calibration curve had to exhibit a correlation coefficient (r^2) of not less than 0.98.

Accuracy and precision were assessed at four QC levels across intra- and inter-run analyses. The mean accuracy for QC samples was expected to be within ±15% of the nominal values, and precision, expressed as the coefficient of variation (%CV), was not to exceed 15% at any QC level.

Method selectivity was evaluated by analyzing 12 different lots of blank human plasma to confirm the absence of significant interference at the retention times of Erlotinib and IS. The observed response at the analyte's retention time was required to be below 20% of the LLOQ response, and interference at the IS retention time below 5% of its mean response.

Stability studies were conducted using LQC and HQC samples (n = 6) under various storage conditions to assess short-term, long-term, and freeze–thaw stability. Acceptance criteria required at least four of the six replicates to fall within ±15% of the nominal value, with mean accuracy and %RSD also within ±15%. The stability of stock and working standard solutions was evaluated by comparing freshly prepared solutions with those stored under defined conditions, allowing for a maximum deviation of 10%.

Short-term stability was assessed by keeping spiked plasma samples at room temperature for 8 hours, followed by analysis. Reinjection reproducibility was verified by reanalyzing previously injected samples and comparing results with initial data.

Dilution integrity was confirmed by diluting plasma samples with concentrations above the ULOQ (~1.8×) using blank plasma (2× and 4×

dilutions). At least two-thirds of the diluted samples were required to meet the accuracy acceptance criteria of ±15% for the method to be considered valid.

Robustness evaluation using experimental design.

Method robustness was assessed using *Design-Expert® version 13* software. A Box–Behnken design (BBD) was employed to evaluate the robustness of the developed LC–MS/MS method by examining three independent variables at three levels each. The selected factors included the mobile phase composition (ratio of ammonium acetate solution to acetonitrile, 30:70–10:90, v/v), pH (6.5–6.7), and flow rate (0.6–1.0 mL/min). The experimental design generated a total of 17 runs (Table 1), each representing a unique combination of these factors to assess their collective and individual effects on method performance.

The response parameters evaluated were peak area and retention time (Rt). Response surface methodology (RSM) was applied to interpret the influence of each factor and their interactions on the measured responses. Numerical optimization using a desirability function was then performed to determine the optimal chromatographic conditions that achieved a balanced compromise between peak area and retention time (Table 1).

Table 1: Run list as per box Behnken design with there responses.

Run	Mobile Phase Composition % (v/v)	Flow rate ml/min	pH	Retention Time (Min)	Area
1	70	6.6	0.6	2.3	131988
2	80	6.5	0.6	1.7	135852
3	80	6.6	0.8	1.24	161988
4	90	6.7	0.8	2.45	158852
5	80	6.6	0.8	1.24	161988
6	80	6.5	1	2.3	159835
7	90	6.6	0.6	2.21	158842
8	90	6.6	1	2.34	158769
9	80	6.7	1	1.89	130890
10	80	6.6	0.8	1.24	161988
11	90	6.5	0.8	2.2	158918
12	70	6.5	0.8	1.98	139004
13	80	6.6	0.8	1.24	161988
14	70	6.7	0.8	2.42	138843
15	80	6.7	0.6	1.96	141004
16	80	6.6	0.8	1.24	161988
17	70	6.6	1	2.81	132576

Extraction Procedure

Frozen spiked plasma samples were removed from storage and allowed to thaw naturally at room temperature. After complete thawing, the samples were vortex-mixed to ensure homogeneity. Except for the blank samples, which received 50 µL of the dilution solution, each labeled polypropylene tube was spiked with 50 µL of the internal standard (IS) solution containing approximately 4000.00 ng/mL of Erlotinib-D6. Subsequently, 250 µL of the plasma sample was added to each tube and briefly vortexed. To facilitate extraction, 200 µL of ammonia solution was added, followed by additional vortex mixing to ensure thorough blending. Thereafter, 2 mL of ethyl acetate was introduced, and the mixture was vortexed for 2 minutes to promote efficient extraction of the analyte and IS. The samples were centrifuged at 4000 RCF for 2 minutes at 5°C to achieve phase separation. Following centrifugation, 1 mL of the clear organic layer was carefully transferred into pre-labeled glass tubes and evaporated to dryness at 50°C under a gentle stream of nitrogen. The resulting residue was reconstituted in 1 mL of the reconstitution solution, transferred to autosampler vials, and analyzed by LC–MS/MS.

Results

Sample Extraction

Solid-phase extraction (SPE) was utilized as the primary sample preparation technique to minimize matrix interference, particularly at the lower limit of quantification (LLOQ) of 2.01 ng/mL. Several extraction approaches were initially tested; however, SPE demonstrated superior cleanliness, reproducibility, and recovery, making it the preferred method for the study. The analytes were eluted with an optimized solvent system that provided mean recoveries of 80.1% for Erlotinib and 77.23% for the internal standard, Erlotinib-D_6. The resulting chromatographic peaks were sharp and symmetrical, and recovery values were consistent across all tested levels, confirming the robustness and reliability of the extraction process.

Tandem Mass Spectrometry

Mass spectrometric detection was carried out in positive ionization mode for both Erlotinib and Erlotinib-D_6. The precursor ion scans revealed intense protonated molecular ions at m/z 394.1 for Erlotinib and m/z 400.3 for Erlotinib-D_6. Product ion spectra were subsequently obtained to verify

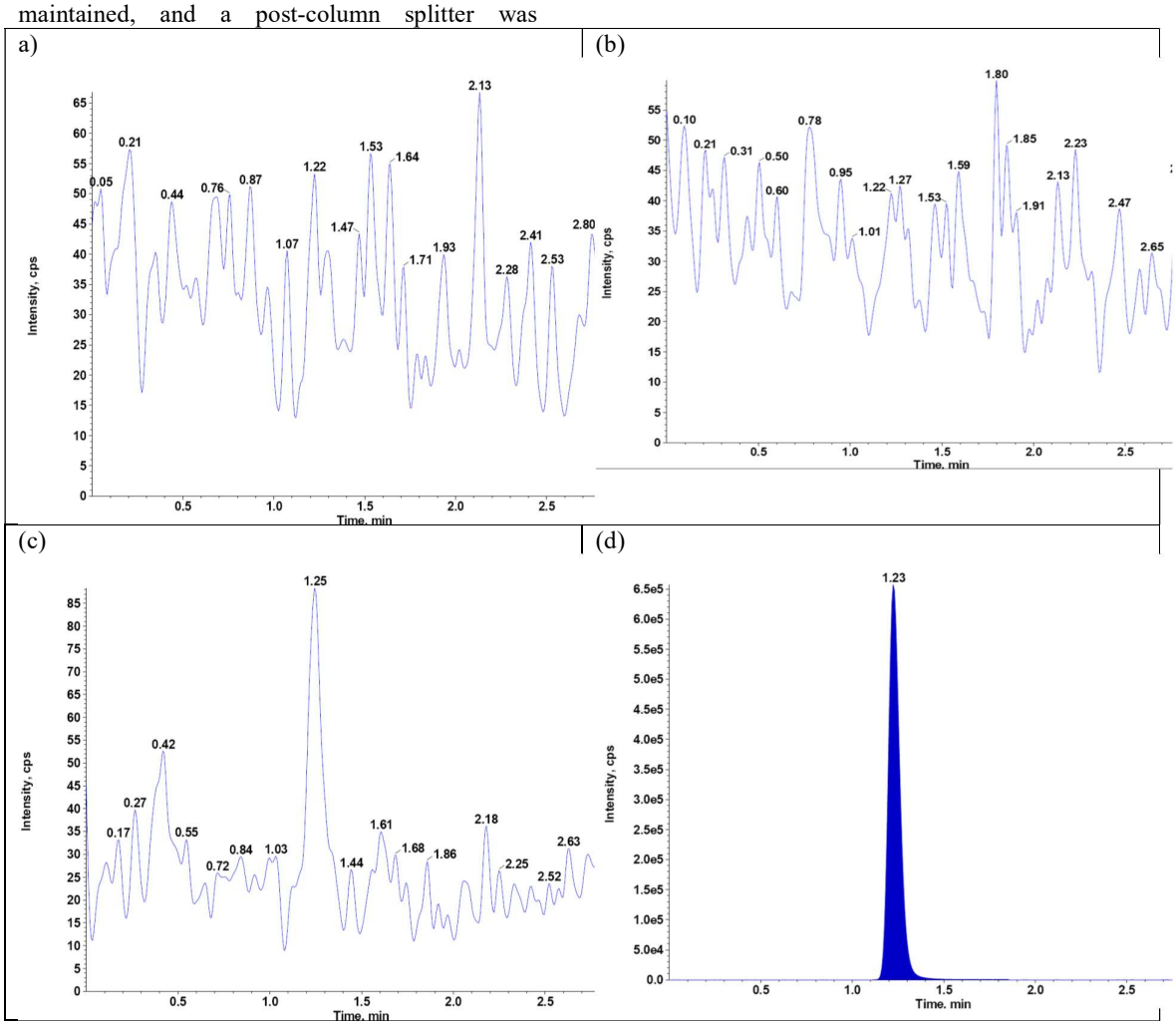
the fragmentation behavior of each compound. For quantitative analysis, the transitions m/z 394.1 → 278.0 for Erlotinib and m/z 400.3 → 278.0 for Erlotinib-D_6 were monitored in Multiple Reaction Monitoring (MRM) mode, ensuring high specificity and accurate detection of the analyte and internal standard.

Separation

During preclinical analysis, multiple metabolites of Erlotinib were detected in volunteer samples, which posed potential challenges for chromatographic separation. To overcome this issue, an XTerra MS C18 column (75 × 4.6 mm, 5 µm) was employed, providing excellent selectivity and peak resolution. A flow rate of 0.8 mL/min was maintained, and a post-column splitter was integrated into the system to ensure efficient isolation of Erlotinib from its metabolites, resulting in clean and well-defined chromatographic peaks.

Sensitivity

Method sensitivity was assessed as part of the validation procedure at the lower limit of quantification (LLOQ) of 2.01 ng/mL using six replicate plasma samples spiked with Erlotinib. The precision and accuracy, calculated based on the analyte-to-internal standard response ratio, were found to be 2.42% and 103.30%, respectively, indicating excellent reproducibility and reliability of detection at trace levels. Representative chromatograms of Erlotinib and Erlotinib-D_6 at the LLOQ concentration are presented in Figure 1.



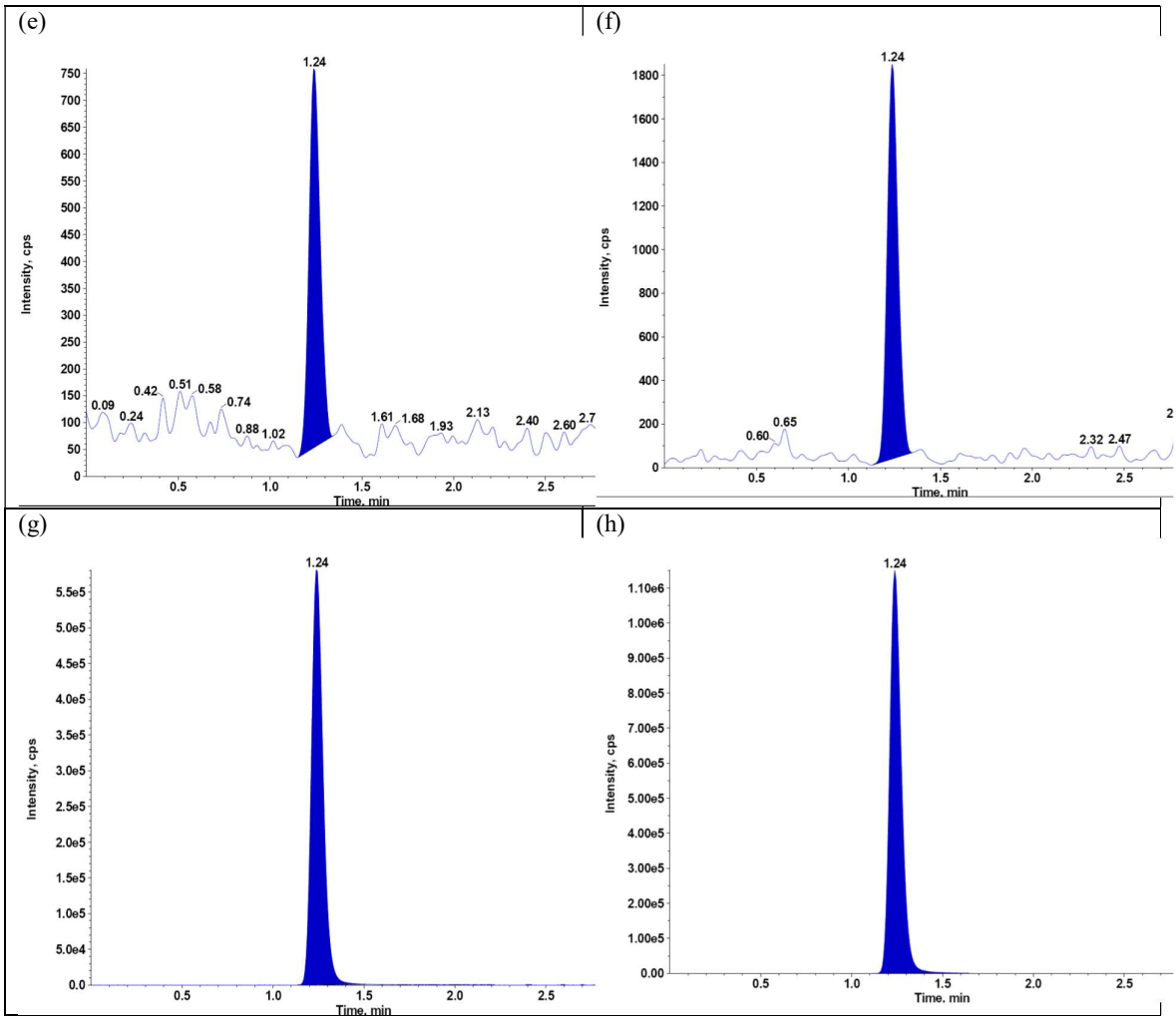


Figure 1: Representative chromatogram of Blank Erlotinib (a) Erlotinib D6 IS in blank(b) Erlotinib blank sample with IS (c) Erlotinib D6 (d) LLOQ-QC (e) LQC (f) MQC (g) HQC (h).

Matrix Effect

Matrix effect refers to the suppression or enhancement of analyte ionization due to components present in the biological matrix. The method was designed to minimize any such effects. welve plasma lots, including lipemic and hemolysed samples, were tested for matrix effect at LQC, MQC, and HQC levels. No substantial ion suppression or amplification was found.

Selectivity

Selectivity was assessed using twenty different plasma lots containing K2-EDTA as the anticoagulant. The representative chromatogram (Figure 1) demonstrated the absence of any significant interference from endogenous components at the retention times of Erlotinib and its internal standard in all plasma batches analyzed.

Calibration Curve Regression

Erlotinib responses were analyzed using quadratic regression with a 1/concentration weighting, which produced the best correlation, exhibiting a coefficient of determination (r^2) greater than 0.997 demonstrated excellent linearity across the concentration range of 2.01–2219.08 ng/mL (Table 2).

Table 2. Erlotinib concentrations obtained from calibration standards.

CC STD ID	Nominal Conc.	Average	Standard Deviation	Precision (%)	Accuracy (%)
STD 1	2.01	2.013	0.0058	0.29	100.15
STD 2	4.43	4.343	0.0416	0.96	98.04
STD 3	12.76	12.847	0.1823	1.42	100.68
STD 4	52.91	55.003	1.7312	3.15	103.96
ST	229.2	224.8	3.1114	1.38	98.09

D 5	4	6			
STD 6	835.44	841.627	1.9324	0.23	100.74
STD 7	1606.62	1559.397	4.9784	0.32	97.06
STD 8	2219.08	2141.987	3.4225	0.16	96.53

Accuracy and Precision

Table 3 summarizes the accuracy (% nominal) and precision for Erlotinib within and between batches. Accuracy ranged from 96.26 % to 103.88%, while the intraday and interday precision, demonstrated excellent reproducibility.

Table 3. Within batch, within-day, and different-day precision and accuracy (% average and % RSD).

Within batch (n=6)					
Nominal Concentration		2.01	5.90	776.91	1663.62
LLOQ QC	Mean	2.088	5.873	747.830	1609.630
LQC	Standard Deviation	0.0979	0.2137	19.2766	32.3696
MQC	% RSD (Precision)	4.69	3.64	2.58	2.01
HQC	% Accuracy	103.88	99.54	96.26	96.75
Within day (n=12)					
LLOQ QC	Mean	2.010	5.794	741.268	1608.576
LQC	Standard Deviation	0.1311	0.2286	19.5518	43.4063
MQC	% RSD (Precision)	6.52	3.95	2.64	2.70
HQC	% Accuracy	100.00	98.20	95.41	96.69
Different days (n=18)					
LLOQ QC	Mean	1.993	5.737	745.404	1620.124
LQC	Standard Deviation	0.1280	0.2139	18.1469	43.0892
MQC	% RSD (Precision)	6.42	3.73	2.43	2.66
HQC	% Accuracy	99.15	97.24	95.94	97.39

Recovery

Recovery was evaluated by comparing the mean peak areas of QC samples before and after extraction. Mean extraction recovery for Erlotinib was 82.23% with 3.26% precision, while for Erlotinib-D6 it was 89.71%, with precision ranging from 1.32% to 4.52%. indicating efficient extraction of both the analyte and internal standard.

Integrity of Dilution

Dilution integrity was tested using QC samples diluted two- and four-fold with blank matrix. Samples diluted 2- and 4-fold showed precision of 1.57% and 2.38%, and accuracy of 95.49% and 98.14% respectively, confirming the method's integrity above the ULOQ.

Stability Studies

Stability evaluations were performed to confirm the integrity of Erlotinib and its internal standard (IS) under various handling and storage conditions that may occur during sample collection, processing, transport, and analysis. Quality control (QC) samples (n = 6) at low, medium, and high concentration levels were compared against freshly prepared pooled QCs.

Erlotinib demonstrated excellent stability in human plasma across all tested conditions, including stock solution stability, refrigerated stock stability, freeze–thaw stability, autosampler stability, short-term stability, and long-term stability. All stability parameters met the acceptance criteria for bioanalytical validation, confirming that Erlotinib and its internal standard are stable under various laboratory and storage conditions. These results demonstrate the robustness and reliability of the developed method for routine quantitative analysis.

Table 4. Summary of Stability Results.

Stability Test	**Conditions**	**Duration / Cycles**	**Accuracy (%)**	**Precision (% CV)**
Stock Solution Stability	Standard and dilution stock solutions	26 h / 23 h	96.74 – 102.48	–

Refrigerated Stock Stability	Erlotinib and Erlotinib-D6 stock solutions at 2–8 °C	8 days	96.74	–
Freeze–Thaw Stability	Plasma samples at LQC and HQC	3 cycles	97.21 – 101.92	2.71 – 6.51
Autosampler Stability	QC samples at autosampler temperature (~10 °C)	67 h	96.79 – 99.04	3.32 – 5.19
Short-Term Stability	Spiked plasma at room temperature	19 h	93.59 – 98.45	1.95 – 4.48
Long-Term Stability (−50 °C)	Plasma samples stored at −50 °C	98 days	97.74 – 99.32	0.78 – 3.50
Long-Term Stability (−20 °C)	Plasma samples stored at −20 °C	98 days	96.21 – 97.34	1.64 – 4.65

Ruggedness

Ruggedness was evaluated by analyzing precision and accuracy batches performed by different analysts using the same and/or different instruments. The results confirmed that the method is highly rugged and reliable for routine application, with precision and accuracy ranging from 0.90–8.36% and 96.45–100.75%, respectively.

Robustness Testing Using Experimental Design

Robustness describes the ability of an analytical method to maintain its performance despite minor, intentional variations in chromatographic parameters such as mobile phase composition, flow rate, or column temperature. In this study, method robustness was systematically assessed using a Box–Behnken Design (BBD), which considered three independent variables: mobile phase composition (% v/v of ammonium acetate buffer to acetonitrile), flow rate (mL/min), and pH. The effects of these factors were examined on two key responses — retention time (Rt) and peak area — to understand the stability and reliability of the developed LC–MS/MS method.

A total of 17 experimental runs were performed according to the BBD matrix, and each experimental condition was executed in a randomized sequence to minimize bias caused by uncontrolled variables. The optimized chromatographic settings determined from the experimental outcomes were a mobile phase ratio of 19.32:80.68 (ammonium acetate buffer:acetonitrile), a flow rate of 0.7 mL/min, and a pH of 6.5. Under these optimized conditions, the method produced a peak area of 163,080 and a retention time of 1.24 minutes (as presented in Table 5).

Table 5. Chromatographic Conditions and Parameters Evaluated During Robustness Assessment.

Factor	Range	Low level	High level	Optimized Value
Composition of Mobile phase	30:70 – 10:90	10	30	19.32:80.68
Flow Rate	0.6-1	0.6	1	0.7
pH	6.5-6.7	6.5	6.7	6.5

The data obtained from the design were best described by a second-order quadratic model, which was selected for both response variables based on the statistical fit. The coefficients of determination (r^2) were 0.9602 for retention time and 0.9572 for peak area, indicating excellent correlation between predicted and experimental data. The lack-of-fit test was statistically insignificant, confirming the suitability of the model for representing the experimental data.

Further evaluation through Analysis of Variance (ANOVA) demonstrated that the developed models were statistically significant. For retention time, the model showed an F-value of 18.77 (P = 0.0001), indicating a highly reliable model with only a 0.04% chance of the result being due to random variation. Similarly, for peak area, the F-value was 17.40, corresponding to a 0.05% probability of being influenced by noise. The close agreement between predicted and experimental responses confirmed the accuracy, precision, and predictive reliability of the quadratic model.

Mathematical modeling was conducted using Design Expert® software, employing a quadratic polynomial equation to describe the effect of the independent variables on both response factors. The final equations developed for retention time and peak area are presented below, illustrating the influence and interaction of the selected parameters on chromatographic performance.

Retention time = 1.24-0.0387A+0.0675B+0.1463C-0.0475AB-0.0950AC-0.1675BC+

$0.7375A^2+0.2850B^2+0.4375C^2$

Peak Area = 1.620E+05+11621.25A-3002.50B+1798.00C+23.75AB-165.25AC-8524.25BC-4717.63A^2-8366.13B^2-11726.62C^2.

Evaluation of the polynomial equations demonstrated that the experimental data were well-described by the chosen model, confirming its suitability and accuracy in representing the observed responses.

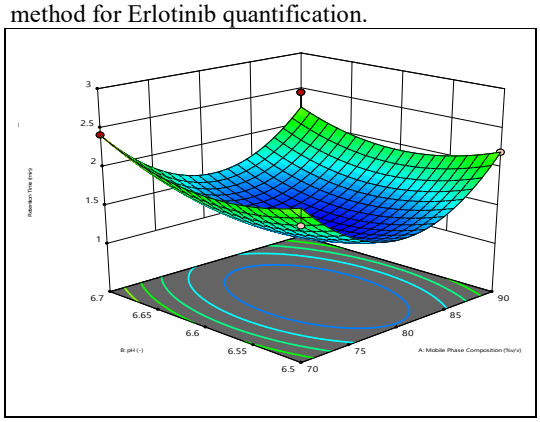
Response Surface Analysis

The response surface methodology was employed to explore and visualize the interactive effects of pH and flow rate on the chromatographic performance of Erlotinib. The 3D surface plots (Figures 2) clearly demonstrate how variations in these parameters influence the retention time and peak area.

For retention time, the surface plot exhibited a concave (bowl-shaped) curvature, indicating that an initial increase in both pH and flow rate led to a decrease in Rt, followed by a gradual increase at higher levels of the factors. This pattern suggests a nonlinear interaction between the two parameters, where both exert significant combined effects on the analyte's elution behavior.

Conversely, the peak area response surface showed a convex (dome-shaped) profile, revealing that increasing pH and flow rate initially enhanced the peak area, after which a decline was observed beyond the optimal range. This indicates that an intermediate pH and moderate flow rate provide the most favorable ionization and signal intensity conditions for Erlotinib detection.

Overall, the curvature and interaction trends in the response surfaces confirm that pH and flow rate play crucial, interdependent roles in determining method robustness. The use of the Box–Behnken Design effectively predicted these relationships, enabling precise optimization of chromatographic conditions to achieve maximum response, reproducibility, and robustness in the LC–MS/MS method for Erlotinib quantification.



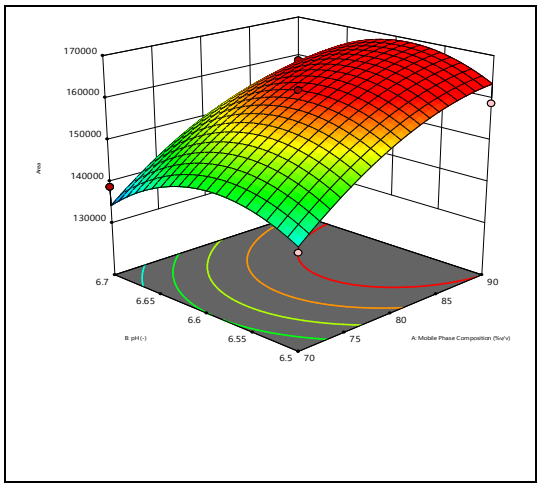


Figure 2. Three-dimensional response surface plots illustrating the combined effects of mobile phase composition, flow rate, and pH on the retention time and peak area.

Table 6. Numerical Optimization Parameters.

Constraints						
Name	Goal	Lower Limit	Upper Limit	Lower weight	Upper Weight	Importance
Composition of Mobile phase	is in range	10	30	1	1	3
Flow Rate	is in range	0.6	1	1	1	3
pH	is in range	6.5	6.7	1	1	3
Run	Mobile Phase Composition	pH	Flow rate (ml/min)	Retention Time	Area	Desirability
1	19.32:80.68	6.5	0.7	1.223	162124.185	1.000

Discussion

This study demonstrates the application of Quality by Design principles in developing a robust LC–MS/MS method for the quantification of Erlotinib in human plasma. A systematic approach using the Box–Behnken Design was employed to enhance method reliability and performance. Key analytical factors including buffer pH, mobile phase composition, and flow rate were optimized to assess their impact on peak area and retention time, as presented in

Table 6. The use of response surface methodology provided comprehensive insights into the interaction and combined effects of these parameters. The validated method demonstrated outstanding sensitivity, selectivity, and specificity. Furthermore, extensive stability assessments under various conditions such as freeze–thaw cycles, short-term, long-term, and bench-top storage confirmed that the analyte remained stable throughout the analytical process. In summary, the developed LC–MS/MS method is accurate, precise, and robust, making it well-suited for routine bioanalytical evaluation of Erlotinib in human plasma samples.

Conclusion

The newly developed LC–MS/MS method for the quantification of Erlotinib in human plasma exhibited excellent linearity across the concentration range of 2.01–2219.08 ng/mL, along with superior precision, accuracy, and simplicity. Validation results confirmed the method's reliability and suitability for pharmacokinetic and bioequivalence applications, supported by its rapid and straightforward sample preparation procedure. The method met all regulatory requirements, demonstrating compliance with essential validation parameters such as sensitivity, selectivity, accuracy, precision, stability, and ruggedness. Moreover, the implementation of the Box–Behnken Design for robustness optimization effectively predicted the impact of subtle variations in chromatographic parameters—such as flow rate and mobile phase composition—on critical analytical responses, including peak area and retention time.

Ethics approval and consent to participate

As this study does not involve animal and patient experiments, the ethical approval and consent to participate are not applicable.

Consent for publication

All authors agreed with the content and that all gave explicit consent to submit and publish.

Conflicts of interest There are no conflicts to declare.

Author contribution

All authors have carefully reviewed and given their approval for final version of the manuscript. The all authors contributed equally.

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Declaration of Generative AI

This manuscript has utilized OpenAI's ChatGPT (version 4) to enhance the clarity and coherence of the language. The tool was employed exclusively for language improvement purposes with ethical and academic standards. The authors take full responsibility for the content and integrity of the manuscript.

References

1. Bardin C, Veal G, Paci A, Chatelut E, Astier A, Levêque D, Widmer N, Beijnen J (2014) Therapeutic drug monitoring in cancer – Are we missing a trick? *Eur J Cancer* 50:2005–2009.
2. Ling J, Fettner S, Lum BL, Riek M, Rakhit A (2008) Effect of food on the pharmacokinetics of erlotinib, an orally active epidermal growth factor receptor tyrosine-kinase inhibitor, in healthy individuals. *Anticancer Drugs* 19:209–216.
3. Clark GM, Zborowski DM, Santabárbara P, Ding K, Whitehead M, Seymour L, Shepherd FA (2006) Smoking history and epidermal growth factor receptor expression as predictors of survival benefit from erlotinib for patients with non–small-cell lung cancer in the National Cancer Institute of Canada Clinical Trials Group study BR.21. *Clin Lung Cancer* 7:389–394.
4. Samuel JR, Olivia C, Jie H, Arzu O, Alberto B, Amar G, Smith CF (2019) Pharmacokinetics and safety of erlotinib and its metabolite OSI-420 in infants and children with primary brain tumors. *Cancer Chemother Pharmacol* 84:829–838.
5. National Center for Biotechnology Information (2023) PubChem Compound Summary for CID 176870, Erlotinib hydrochloride. https://pubchem.ncbi.nlm.nih.gov/compound/Erlotinib-Hydrochloride.
6. U.S. Food and Drug Administration (2023) Product-specific guidances for generic drug development. https://www.accessdata.fda.gov/drugsatfda_docs/psg/Erlotinib_HCl_tab_21743_RC1-06.pdf
7. Ohgami M, Homma M, Suzuki Y, Naito K, Yamada M, Mitsuhashi S, Fujisawa F, Kojima H, Kaburagi T, Uchiumi K, Yamada Y, Bando H, Hara H, Takei K (2016) A simple high-performance liquid chromatography method for determining lapatinib and erlotinib in human plasma. *Ther Drug Monit* 38:657–662.
8. Yuta Y, Eiji N, Miho M, Imoto E, Matsuda T, Shibata M, Kobayashi T, Matsumoto E, Yamashita R, Kawaguchi K, Jinnouchi A,

Kondo H, Sato S, Nakashima T (2022) Simultaneous quantification of dasatinib, nilotinib, bosutinib, and ponatinib using high-performance liquid chromatography–photodiode array detection. *J Clin Lab Anal* 36:e24598.

9. Faivre L, Gomo C, Mir O, Taieb F, Schoemann TA, Ropert S, Vidal M, Dusser D, Dauphin A, Goldwasser F, Blanchet B (2011) A simple HPLC-UV method for the simultaneous quantification of gefitinib and erlotinib in human plasma. *J Chromatogr B* 879:2345–2350.
10. Jing Z, Hualin C, Zhiping J, Qing W, Yan Z, Ping X, Xielan Z (2017) A validated UPLC–MS/MS method for simultaneous determination of imatinib, dasatinib and nilotinib in human plasma. *J Pharm Anal* 7:374–380.
11. Bouchet S, Chauzit E, Ducint D, Castaing N, Canal-Raffin M, Moore N, Titier K, Molimard M (2011) Simultaneous determination of nine tyrosine kinase inhibitors by 96-well solid-phase extraction and ultra-performance LC/MS-MS. *Clin Chim Acta* 412:1060–1067.
12. Honeywell R, Yarzadah K, Giovannetti E, Losekoot N, Smit EF, Walraven M, Lind JSW, Tibaldi C, Verheul HM, Peters GJ (2010) Simple and selective method for the determination of various tyrosine kinase inhibitors used in the clinical setting by liquid chromatography tandem mass spectrometry. *J Chromatogr B* 878:1059–1068.
13. Götze L, Hegele A, Metzelder SK, Renz R (2012) Development and clinical application of a simultaneous determination of various tyrosine kinase inhibitors in plasma. *Clin Chim Acta* 413:143–149.
14. Chongliang L, Mengchun C, Xiaoqiang Z, Congcong W, Qingwei Z, Jinzhang C (2013) Development and validation of an LC–MS method for determination of erlotinib in rat plasma and application in a pharmacokinetic study. *Lat Am J Pharm* 32:1298–1303.
15. Ghasemian E, Sadrai S, Shokri J, Ilka A (2023) Analytical method validation and bioequivalence study of erlotinib 150 mg tablets in Iranian healthy volunteers under fasting condition. *Int J Pharm Pharm Sci* 15:27–32.
16. Herbrink M, Vries N, Rosing H, Huitema AD, Nuijen B, Schellens JH, Beijnen JH (2016) Quantification of 11 therapeutic kinase inhibitors in human plasma for therapeutic drug monitoring using liquid chromatography coupled with tandem mass spectrometry. *Ther Drug Monit* 38:649–656.
17. Jaiprakash NS, Mrinmayee DA, Zahid Z, Devanand BS, Arote R (2017) Quality by design approach: Regulatory need. *Arab J Chem* 10:S3412–S3425.
18. Hasnain MS, Rao S, Singh MK, Vig N, Gupta A, Ansari A, Sen P, Joshi P, Ansari SA (2013) Development and validation of LC–MS/MS method for the quantitation of lenalidomide in human plasma using Box–Behnken experimental design. *Analyst* 138:1581–1588.
19. Khan S, Khan F (2023) QbD-based approach to RP-HPLC method development and validation of bupivacaine hydrochloride in bulk and in-house developed nanostructured lipid carriers. *J Res Pharm* 27:1936–1950.
20. Khan S, Khan FN (2025) Optimized RP-HPLC method development and validation for quantification of articaine in bulk and nanostructured lipid carriers using a quality-by-design framework. *Accred Qual Assur* 30:167–181.
21. U.S. Food and Drug Administration (2018) Bioanalytical Method Validation: Guidance for Industry. U.S. Department of Health and Human Services, Food and Drug Administration.
22. International Council for Harmonisation (2022) ICH Harmonised Guideline: Bioanalytical Method Validation and Study Sample Analysis (M10).