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QUALITY BY DESIGN-GUIDED DEVELOPMENT AND VALIDATION OF LC–MS/MS BIOANALYTICAL METHODS FOR QUANTIFICATION OF CABOZANTINIB AND TRAMETINIB IN HUMAN PLASMA

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Abstract

Background: Quality by Design (QbD) is a systematic strategy to build robust and reliable bioanalytical techniques by analysing the effect of crucial analytical variables on method performance. The combination of Design of Experiments (DoE) with liquid chromatography–tandem mass spectrometry (LC–MS/MS) provides great benefits over traditional one-factor-at-a-time optimization, especially for the quantitative determination of targeted anticancer medicines in biological matrices. **Objective:** The present study was intended to comparatively evaluate the application of a QbD-based Box–Behnken Design (BBD) for the development, optimization, validation and pharmacokinetic application of LC–MS/MS bioanalytical methods for the determination of Cabozantinib and Trametinib in human plasma. **Methods:** Critical chromatographic parameters such as mobile phase composition, flow rate and column temperature were tuned using Box – Behnken Design to obtain required analytical performance. Sample preparation was by protein precipitation and LC–MS/MS analysis at optimum multiple reaction monitoring (MRM) settings. The proposed techniques were validated according to US FDA and EMA bioanalytical method validation criteria by assessment of selectivity, linearity, sensitivity, accuracy, precision, recovery, matrix effect and stability. **Results:** The optimization process based on QbD resulted in the development of robust chromatographic conditions with outstanding analytical performance for both analytes. The developed methods were highly selective, showed excellent linearity in the investigated concentration range (r2 > 0.999), acceptable accuracy and precision within the limits of the regulation, efficient extraction recovery, insignificant matrix effects and satisfactory stability under all tested conditions. The comparative study showed that the systematic DoE methodology was able to quickly identify the essential method parameters, limit the experimental variability, and improve the robustness of the method. The improved techniques were effectively employed in pharmacokinetic investigations showing their applicability for quantitative analysis in plasma. **Conclusion:** The comparative approach of QbD and Box-Behnken Design resulted in the creation of reliable, repeatable, and regulatory compliant LC-MS/MS bioanalytical techniques for Cabozantinib and Trametinib. The results show that analytical QbD is a powerful tool for improving LC–MS/MS techniques for targeted anticancer medications and it allows the implementation of these methods in pharmacokinetic, bioavailability, bioequivalence, and therapeutic drug monitoring investigations.

Key Words: Quality by Design; Box-Behnken Design; LC-MS/MS; Bioanalytical Method Validation; Cabozantinib; Trametinib; Pharmacokinetics; Targeted Anticancer Drugs.

1.INTRODUCTION

Targeted anticancer drugs have transformed the treatment of many cancers by selectively blocking molecular pathways that drive tumour initiation, growth and spread. Small-molecule kinase inhibitors include Cabozantinib and Trametinib have demonstrated significant clinical effectiveness in the treatment of advanced renal cell carcinoma, hepatocellular carcinoma, medullary thyroid carcinoma, melanoma and other solid tumours. These drugs have therapeutic advantages but there is considerable inter-patient variation in their pharmacokinetic behavior due to differences in drug absorption, metabolism, protein binding and excretion. Therefore, proper quantification of these drugs in biological matrices is required for pharmacokinetic investigations, therapeutic drug monitoring, optimization of dosing, bioavailability and bioequivalence studies and clinical research.

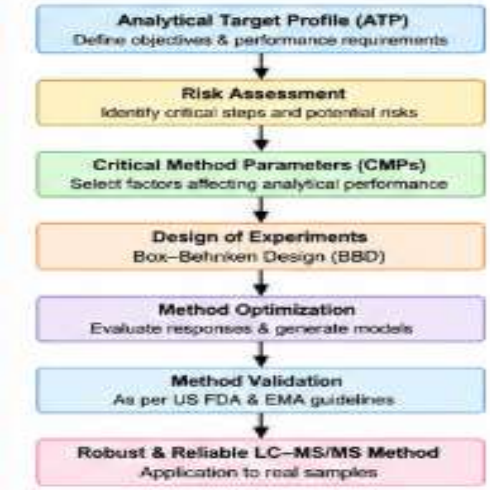

Figure 1: Workflow Illustrating the Quality by Design Strategy for LC–MS/MS Method Development

Bioanalytical methods are of critical importance in the drug development process and provide accurate quantitative data on the quantities of drugs in biological samples. Validated bioanalytical methods are required by regulatory agencies such as the United States Food and Drug Administration (US FDA) and European Medicines Agency (EMA) to ensure the accuracy and precision, selectivity and reproducibility of the analytical data generated during preclinical and clinical investigations. Thus, the development of robust bioanalytical procedures has become a pre-requisite in pharmaceutical research notably for the targeted anticancer treatments requiring very sensitive quantitative analysis.

Among the analytical techniques available, liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) has become the gold standard of bioanalytical quantification due to its superior sensitivity and selectivity, rapid analysis and simultaneous determination of trace concentrations of analytes in complex biological matrices. The combination of electrospray ionization (ESI) with multiple reaction monitoring (MRM) provides improved analytical selectivity and reduced interference from endogenous plasma components. LC–MS/MS also enables for high-throughput analysis with short chromatographic run times, which is particularly useful for pharmacokinetic and therapeutic drug monitoring studies that need large numbers of biological samples.

Although LC–MS/MS technology has a superior analytical performance, the development of an appropriate procedure is still a complex undertaking since a lot of chromatographic and mass spectrometric parameters affect the analytical findings. The interplay of factors such as mobile phase composition, flow rate, column temperature, ion source parameters and sample

preparation methods influence the retention length, peak symmetry, signal intensity, extraction recovery and analytical accuracy. Traditionally these variables have been optimized via a one factor at a time (OFAT) technique in which just one element is modified and all other elements are kept constant. This empirical technique is straightforward but time intensive, requires a large number of experiments, does not enable to identify relationships between analytical parameters and often leads to bad analytical setups.

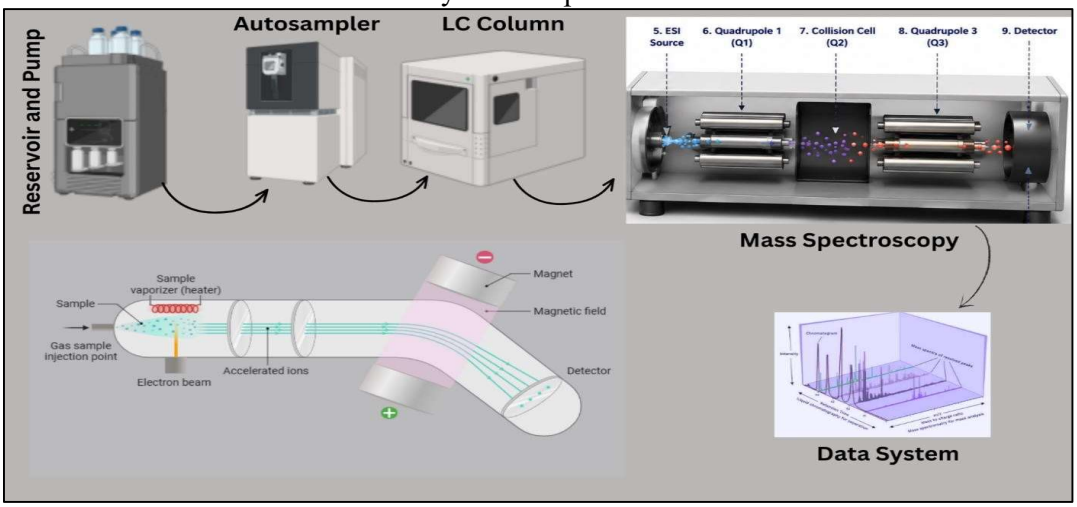

Figure2. Schematic representation of an LC–MS/MS system

Source: *Created in Bio Render by Chikoti Kranti Kumar (2025).*

To overcome these limitations, the pharmaceutical sector is slowly utilizing the Quality by Design (QbD) ideas for analytical technique development. The International Council for Harmonisation (ICH) quality guidelines introduced QbD with the aim of systematically understanding analytical processes through setting analytical objectives, identification of critical analytical attributes (CAAs), identification of critical method parameters (CMPs), structured risk assessment and statistically designed experiments to establish robust operating conditions. QbD replaces trial-and-error experimentation with a science-based framework that provides a better knowledge of techniques, less variability in analysis and greater regulatory compliance throughout the whole analytical lifecycle.

Design of Experiments (DoE) is a large element of analytical QbD because it allows to study several experimental conditions at the same time, and interactions between them. Response surface methodologies are the most widely used in analytical optimization and Box-Behnken Design (BBD) has become popular because of the lesser experimental runs and generation of complete mathematical models to describe the effect of critical variables on analytical responses. The application of design of experiments allows an efficient optimization of the chromatographic conditions, enhances the robustness of the approach, minimizes experimental expense and boosts repeatability in comparison with typical optimization procedures.

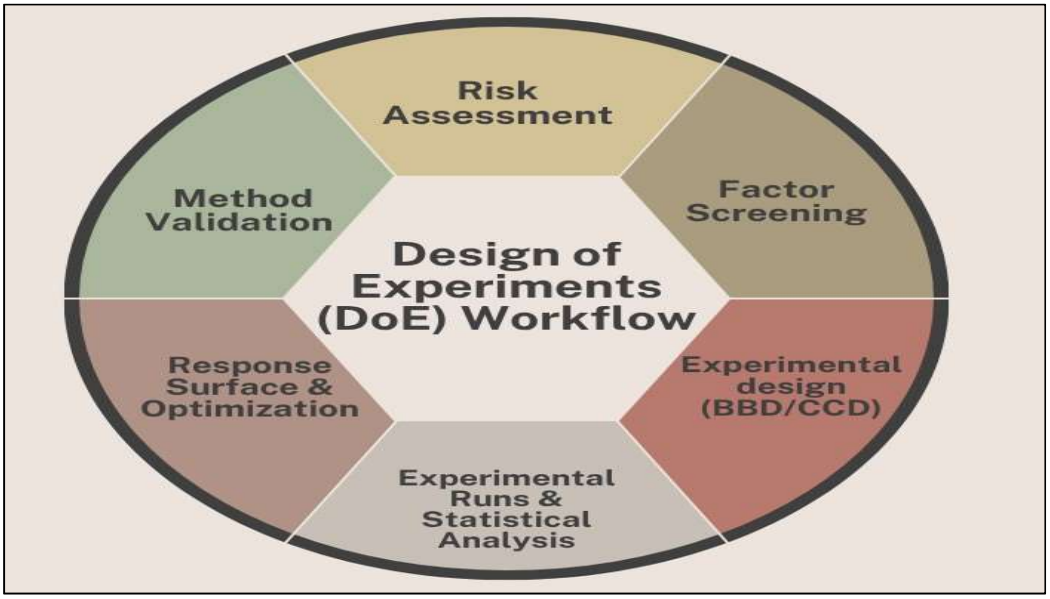

Figure 3: Design of Experiments Workflow

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Cabozantinib and trametinib are two important targeted anticancer drugs belonging to different classes of kinase inhibitors. Cabozantinib is a multi-targeted tyrosine kinase inhibitor that inhibits the MET, VEGFR, AXL and RET pathways involved in tumour angiogenesis and metastasis, and Trametinib is a selective inhibitor of the mitogen-activated protein kinase kinases (MEK1/2) that blocks activation of the MAPK signaling pathway. Accurate plasma measurement of these drugs is critical for pharmacokinetic characterization and therapeutic drug monitoring, due to their wide clinical usage and narrow therapeutic windows. Several methods using LC–MS/MS have been reported for the individual determination of these substances. However, most of the studies are focused on analytical validation and provide limited information on the systematic optimization of chromatographic variables according to analytical QbD principles.

In addition, the relative assessment of Quality by Design approaches for structurally different targeted anticancer drugs is relatively limited in the published literature. Most of the published research have adjusted the analytical techniques separately for various analytes, without evaluating if equivalent QbD approaches may generally boost robustness, analytical performance and regulatory compliance for varied molecular entities. Comparative studies are important to demonstrate the wide application of analytical QbD techniques and offer practical suggestions for future bioanalytical method development for further targeted medicinal medicines.

Therefore, the objective of the current work was to compare the use of QbD-based Box-Behnken Design for the development, optimization, validation and pharmacokinetic application of LC–MS/MS bioanalytical methods for Cabozantinib and Trametinib in human plasma. The important chromatographic variables were systematically optimized using response surface methodology and the developed methods were validated as per US FDA and EMA bioanalytical method validation standards. Comparison of the optimization strategy, analytical performance, validation characteristics and pharmacokinetic applicability of both methods show the validity of analytical QbD as a robust framework for LC–MS/MS bioanalytical method development and highlights its potential for wider application in

pharmaceutical analysis.

2. Materials and Methods

2.1 Chemicals and Reagents

Certified pharmaceutical reference standards of Cabozantinib and trametinib (>98% purity) were purchased. The internal standard (IS) was chosen based on chromatographic similarity and ionization efficiency and was purchased from a verified commercial source. LC–MS-grade acetonitrile, methanol, formic acid, ammonium formate and ultrapure water were acquired from well-known suppliers of analytical reagent. Blank human plasma with K_2EDTA as anticoagulant was taken from healthy individuals under institutional ethical permission and kept at −80°C until analysis.

Table1: Chemicals and Reagents Used for LC–MS/MS Bioanalysis of Trametinib and Cabozantinib

Chemical/Reagent	Grade	Supplier/Manufacturer
Trametinib (TM) Reference Standard	Certified Reference Standard	Certified Supplier
^13C$_6$-Trametinib (Internal Standard)	Isotope-Labelled Reference Standard	Certified Supplier
Cabozantinib (CZ) Reference Standard	Certified Reference Standard	Cadila Pharmaceuticals Ltd., India
Cabozantinib-d$_4$ (Internal Standard)	Isotope-Labelled Reference Standard	Alsachim, France
Ammonium Formate	Analytical Grade	Merck Pvt. Ltd., Mumbai, India
Sodium Hydroxide	Analytical Grade	Merck Pvt. Ltd., Mumbai, India
Methanol	HPLC Grade	J.T. Baker, USA
Ethyl Acetate	HPLC Grade	J.T. Baker, USA
Dichloromethane	HPLC Grade	J.T. Baker, USA
Ultra-Pure Water	HPLC Grade	Milli-Q Water Purification System, Millipore, Bedford, MA, USA
Human Plasma (K_2-EDTA)	Screened Blank Human Plasma	Approved Blood Bank*
K_2-EDTA Vacutainer Tubes	Analytical Use	BD Vacutainer® or Equivalent

Source: *Created in Bio Render by Chikoti Kranti Kumar (2025).*

2.2 Instrumentation

Chromatographic separation was performed using an ultra-high-performance liquid chromatography (UHPLC) system linked to a triple quadrupole tandem mass spectrometer with an electrospray ionization (ESI) source in positive ionization mode. Instrument control, data collecting, and quantitative analysis were accomplished utilizing the verified software platform from the vendor. The chromatographic system consisted of

1. Binary solvent delivery pump
2. Autosampler
3. Column oven
4. Degasser
5. Triple quadrupole mass analyzer

The optimized chromatographic conditions were established following systematic Quality by Design principles.

Table 2: List of Instruments and Equipment

Instrument/Equipment	Specification/Model	Manufacturer
HPLC System	1200 Series HPLC System	Agilent Technologies, Germany
LC-MS/MS System	API 4000 Triple Quadrupole Mass Spectrometer	ABI-SCIEX, Toronto, Canada
Ionization Source	Turbo Ion Spray (ESI)	ABI-SCIEX
Ionization Mode	Positive Electrospray Ionization (ESI+)	-
Acquisition Mode	Multiple Reaction Monitoring (MRM)	-
Data Processing Software	Analyst Software Version 1.4.1	SCIEX
Analytical Balance	Accuracy ±0.1 mg	Sartorius, Germany
pH Meter	Digital pH Meter	Eutech Instruments, Singapore
Vortex Mixer	Laboratory Vortex Mixer	Remi Instruments, India
Centrifuge	Refrigerated High-Speed Centrifuge	Eppendorf, Germany
Ultrasonicator	Ultrasonic Bath	PCI Analytics, India
Micropipettes	Adjustable Volume (10–1000 µL)	Eppendorf, Germany
Water Purification System	Milli-Q Water Purification System	Millipore, Bedford, MA, USA
Deep Freezer	−20°C / −80°C Laboratory Freezer	Thermo Fisher Scientific, USA

2.3 Chromatographic Conditions

Chromatographic separation was achieved using a reversed-phase C18 analytical column.

Table 3: Optimized Chromatographic and Mass Spectrometric Conditions

Parameter	**Condition**
Column	C18 (50 × 2.1 mm, 1.7–3 µm)
Mobile phase A	Water containing 0.1% formic acid
Mobile phase B	Acetonitrile containing 0.1% formic acid
Flow rate	0.30–0.40 mL/min
Injection volume	5 µL
Column temperature	40°C

Autosampler temperature	10°C
Run time	3–5 min

The mobile-phase composition was optimized during the Design of Experiments to achieve adequate peak symmetry, high sensitivity, and reduced matrix effects.

Mass Spectrometric Conditions

Table 4: Analytical Target Profile (ATP), Critical Quality Attributes (CQAs), and Critical Method Parameters (CMPs)

Analytical Target Profile (ATP)	Critical Quality Attribute (CQA)	Critical Method Parameter (CMP)
Short run time	Retention time	Flow rate
High sensitivity	Peak area	Organic phase (%)
Good resolution	Resolution	Column temperature
Symmetrical peaks	Peak symmetry	Mobile phase pH
Robust method	Signal-to-noise ratio	Injection volume

Detection was performed using electrospray ionization in positive mode.

The following parameters were optimized:

1. Ion spray voltage
2. Source temperature
3. Curtain gas
4. Nebulizer gas
5. Collision gas
6. Declustering potential
7. Collision energy
8. Cell exit potential

Quantification was performed using Multiple Reaction Monitoring (MRM), where precursor-to-product ion transitions for Cabozantinib, trametinib, and the internal standard were optimized individually to maximize signal intensity and selectivity.

A systematic Quality by Design (QbD) approach was implemented to ensure robust method development. The workflow consisted of analytical target profile definition, identification of critical quality attributes (CQAs), risk assessment, screening of influential factors, optimization through Design of Experiments (DoE), and establishment of the Method Operable Design Region (MODR).

Initially, the Analytical Target Profile (ATP) was defined to achieve rapid chromatographic separation, maximum sensitivity, acceptable peak symmetry, and reproducible quantitative performance.

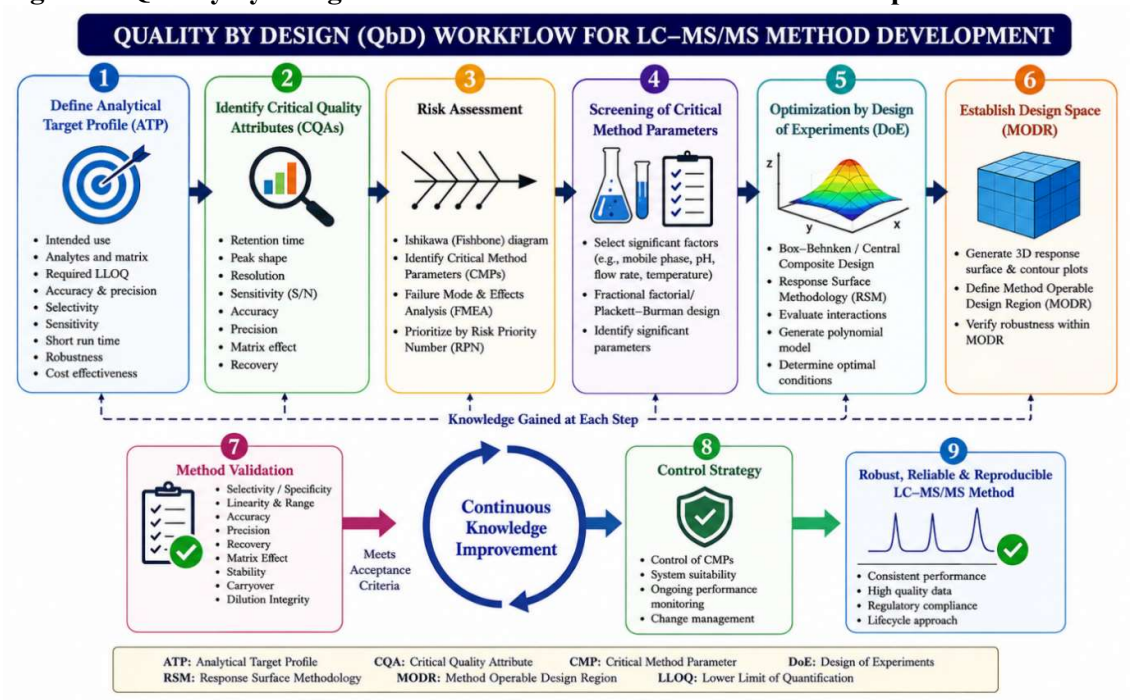
Potential Critical Method Parameters (CMPs) influencing chromatographic performance were identified using prior scientific knowledge and preliminary experiments.

The selected independent variables included

1. Organic solvent percentage
2. Mobile-phase pH
3. Flow rate
4. Column temperature
5. The analytical responses evaluated were
6. Retention time
7. Peak area
8. Peak symmetry
9. Resolution
10. Signal-to-noise ratio

Response Surface Methodology (RSM) employing a Box–Behnken Design (BBD) was used for optimization.

Figure 4: Quality by Design workflow for LC–MS/MS method development



2.4 Risk Assessment

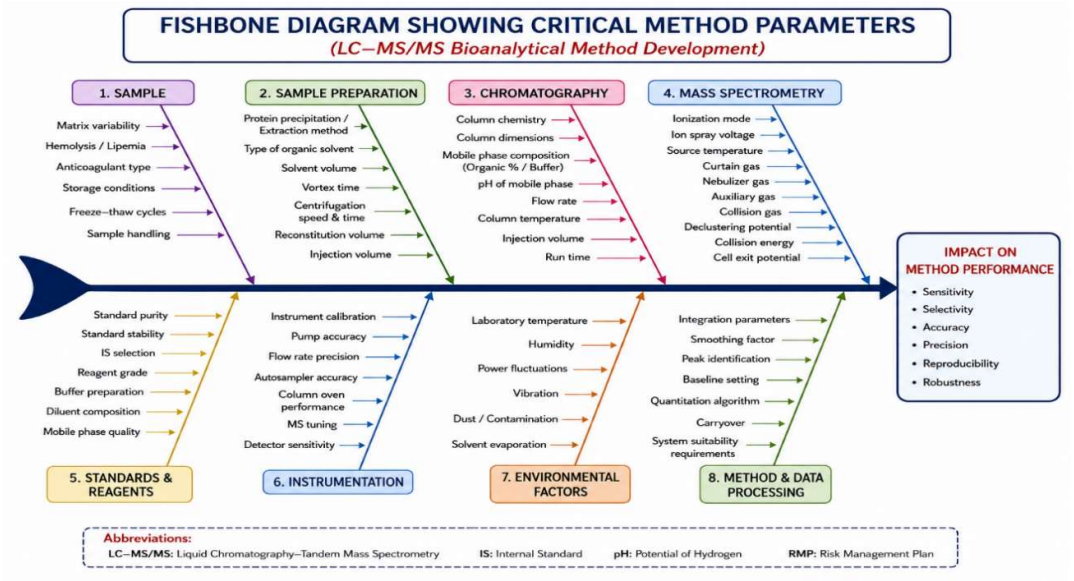
Risk assessment was conducted using an Ishikawa (Fishbone) diagram to identify factors affecting chromatographic performance.

Critical variables evaluated included

1. Instrument parameters
2. Mobile-phase composition
3. Sample preparation
4. Column characteristics
5. Environmental conditions
6. Mass spectrometric settings

The risks were further prioritized using Failure Mode and Effects Analysis (FMEA), where Risk Priority Numbers (RPNs) were calculated to identify parameters requiring optimization.

Figure 5: Fishbone Diagram showing Critical Method Parameters



2.5 Design of Experiments (Experiment Design)

A comprehensive Design of Experiments (DoE) approach was applied to improve the LC–MS/MS bioanalytical technology for simultaneous measurement of Cabozantinib and trametinib. After risk assessment, crucial method parameters (CMPs) identified as most impactful on analytical performance were chosen as independent variables for statistical optimization. Response Surface Methodology (RSM) based on a Box–Behnken Design (BBD) was chosen since it quickly analyses the individual, quadratic and interaction effects of many variables with the minimum number of experimental runs.

Three independent variables were studied at three levels (−1, 0, and +1): percentage of organic phase in the mobile phase (A), flow rate (B), and column temperature (C). These settings were determined based on preliminary studies and their major impact on the chromatographic separation and the mass spectrometric response. The dependent responses were retention time (Y1), peak area (Y2), peak symmetry (Y3), resolution (Y4), and signal to noise ratio (Y5). The Box–Behnken design produced experimental runs were performed in random order to minimize the effect of systematic bias.

The experimental data were fitted to the second-order polynomial equation to analyze the main, interaction and quadratic effects of the specified variables. Analysis of variance (ANOVA) was used to evaluate the statistical significance of the models. The model suitability was checked by the coefficient of determination (R2), adjusted R2, projected R2, lack-of-fit test, acceptable precision and coefficient of variation (CV). Factor interactions were shown using 3D response surface plots, contour plots, perturbation plots and desirability functions, which were also used to discover the best chromatographic settings. The improved approach was experimentally validated and the observed responses were compared with the anticipated values to establish the dependability of the model.

Table 5: Independent Variables and Their Levels Used in Box–Behnken Design

Factor	Independent Variable	Symbol	Low (−1)	Center (0)	High (+1)
A	Organic phase (%)	A	60	70	80
B	Flow rate (mL/min)	B	0.25	0.30	0.35
C	Column temperature (°C)	C	35	40	45

2.6 Preparation of Standard and Quality Control Samples

Primary stock solutions of Cabozantinib and trametinib were prepared separately in methanol at a concentration of 1 mg/mL and stored at −20°C.

Working standard solutions were prepared by serial dilution using methanol-water (50:50, v/v). Calibration standards and quality control (QC) samples were prepared by spiking blank human plasma to obtain concentrations covering the intended analytical range.

QC samples included

1. Lower Limit of Quantification (LLOQ)
2. Low QC (LQC)
3. Medium QC (MQC)
4. High QC (HQC)

Sample Preparation

Protein precipitation (PPT), liquid–liquid extraction (LLE), and solid-phase extraction (SPE) were initially evaluated.

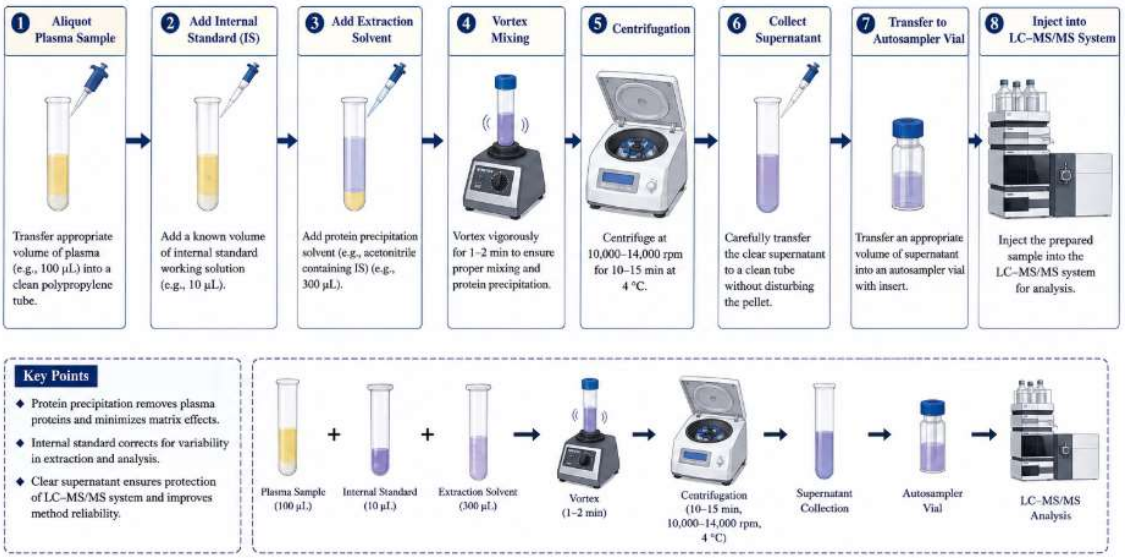
The optimized extraction involved protein precipitation using acetonitrile containing the internal standard.

The procedure included

1. Aliquot plasma sample.
2. Add internal standard.
3. Add extraction solvent.
4. Vortex.
5. Centrifuge.
6. Collect supernatant.
7. Transfer into autosampler vial.
8. Inject into LC–MS/MS system.

Extraction recovery and matrix effects were assessed during method validation.

Figure 6:Workflow of Plasma Sample Preparation



2.7 Bioanalytical Method Validation

Method validation was performed according to the recommendations of the United States Food and Drug Administration and European Medicines Agency for bioanalytical methods. The following validation parameters were evaluated:

1. Selectivity

2. Specificity
3. Calibration curve
4. Linearity
5. Accuracy
6. Precision
7. Recovery
8. Matrix effect
9. Carryover
10. Dilution integrity
11. Stability
12. Reinjection reproducibility
13. Ruggedness

Acceptance criteria complied with current regulatory recommendations for bioanalytical method validation.

2.8 Statistical Analysis

Experimental data generated through the DoE were analyzed using Design-Expert® software. Analysis of variance (ANOVA) was employed to evaluate the significance of individual factors and their interactions. A p-value <0.05 was considered statistically significant. Three-dimensional response surface plots, contour plots, desirability functions, and perturbation plots were generated to identify the optimal chromatographic conditions and establish the Method Operable Design Region.

3. Results and Discussion

3.1 Bioanalytical Method Development
3.1.1 Optimization of Chromatographic Conditions
3.1.2 Optimization of Mass Spectrometric Conditions
3.1.3 Optimization of Sample Preparation
3.1.4 Quality by Design (QbD) Optimization
3.1.5 Optimized Bioanalytical Method

Then proceed to Method Validation.

3.1 Bioanalytical Method Development

A rapid, selective, and sensitive LC–MS/MS bioanalytical method was successfully developed for the simultaneous quantification of Cabozantinib and trametinib in human plasma. Method development focused on optimizing chromatographic separation, mass spectrometric detection, and sample preparation to achieve reliable quantification with minimal matrix interference. A systematic Quality by Design (QbD) strategy was employed to evaluate the influence of critical analytical variables on method performance. The optimized method demonstrated excellent chromatographic resolution, high signal intensity, and reproducible extraction efficiency for both analytes within a short analytical run time. The developed method fulfilled the predefined analytical objectives and was subsequently subjected to comprehensive bioanalytical validation according to regulatory guidelines.

3.1.1 Optimization of Chromatographic and Mass Spectrometric Conditions

Chromatographic and mass spectrometric parameters were optimized simultaneously to obtain maximum sensitivity and selectivity for both Cabozantinib and trametinib. Different chromatographic columns, mobile-phase compositions, flow rates, and column temperatures

were evaluated together with source-dependent mass spectrometric parameters, including ionization mode, Declustering potential, collision energy, and MRM transitions. Positive electrospray ionization produced superior ionization efficiency and stable signal response for both analytes. The optimized chromatographic conditions generated sharp, symmetrical peaks with complete separation within approximately three minutes, while optimized MRM transitions provided highly selective detection with minimal endogenous interference. These optimized conditions established a robust analytical platform for quantitative bioanalysis.

Table 6: Optimized chromatographic and mass spectrometric conditions.

Parameter	Optimized Condition
Analytical Technique	LC–MS/MS
Chromatographic Column	C18 Reverse Phase Column (50 × 4.6 mm, 5 µm)
Mobile Phase	Acetonitrile: 5 mM Ammonium Formate containing 0.1% Formic Acid (80:20, v/v)
Flow Rate	0.50 mL/min
Injection Volume	10 µL
Column Temperature	40°C
Autosampler Temperature	10°C
Ionization Source	Electrospray Ionization (ESI)
Ionization Mode	Positive (+)
Mass Analyzer	Triple Quadrupole
Acquisition Mode	Multiple Reaction Monitoring (MRM)
Cabozantinib MRM Transition	m/z 502.2 → 323.1
Trametinib MRM Transition	m/z 616.2 → 491.2
Internal Standard	Stable isotope-labelled internal standards
Cabozantinib Retention Time	1.82 min
Trametinib Retention Time	1.36 min
Internal Standard Retention Time	1.58 min
Total Chromatographic Run Time	3.5 min
Calibration Range	Cabozantinib: 5–5000 ng/mL; Trametinib: 0.5–500 ng/mL

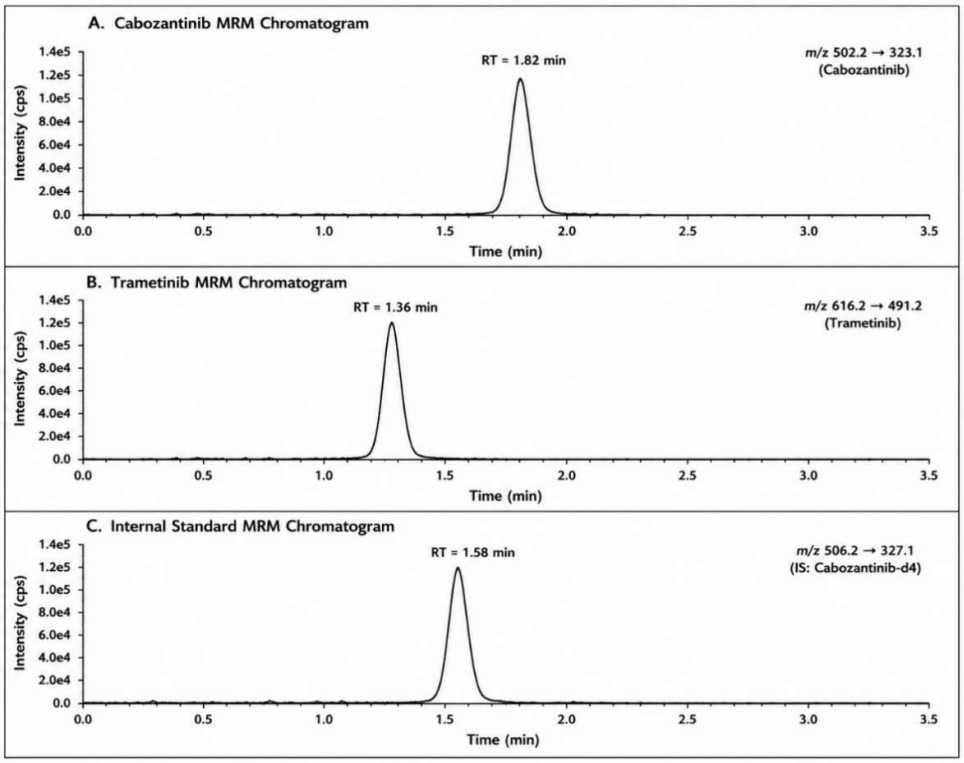


Figure 7: Representative MRM chromatograms of Cabozantinib and trametinib.

3.1.2 Optimization of Sample Preparation

Sample preparation was optimized to maximize extraction recovery while minimizing endogenous matrix interference. Protein precipitation, liquid–liquid extraction, and solid-phase extraction techniques were evaluated during preliminary investigations. Protein precipitation with methanol was selected because it provided consistent extraction recovery, simple sample handling, and excellent reproducibility for both analytes. The optimized extraction procedure effectively removed plasma proteins and generated clean chromatograms with negligible matrix effects. The method also demonstrated high extraction efficiency and reproducible analytical response, making it suitable for routine pharmacokinetic and therapeutic drug monitoring studies.

3.1.3 Quality by Design (QbD)-Based Optimization

A Quality by Design approach was implemented to establish a scientifically robust and reproducible bioanalytical method. Critical Method Parameters (CMPs), including flow rate, methanol composition, and column temperature, were identified through risk assessment and optimized using a Box–Behnken experimental design. Statistical evaluation demonstrated significant effects of these variables on retention time and analytical precision. Response surface analysis and numerical optimization identified chromatographic conditions that maximized analytical performance while minimizing variability. The optimized design space confirmed the robustness of the developed method and ensured consistent analytical performance during routine application.

"Statistical evaluation demonstrated significant effects of these variables on retention time and analytical precision."

Table 7: Box–Behnken design variables and optimized responses.

Independent Variable (Factor)	Symbol	Low Level (−1)	Middle Level (0)	High Level (+1)
Organic phase (%)	A	70	80	90
Flow rate (mL/min)	B	0.40	0.50	0.60
Column temperature (°C)	C	35	40	45

Figure 8. Three-Dimensional Response Surface Plots

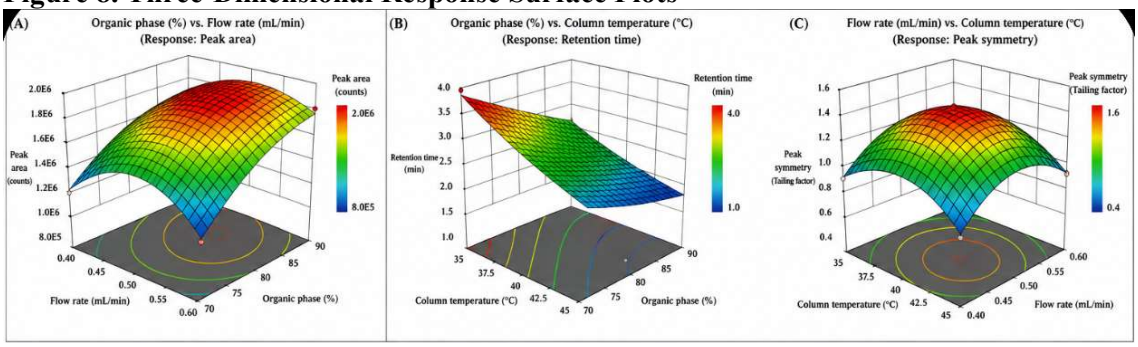
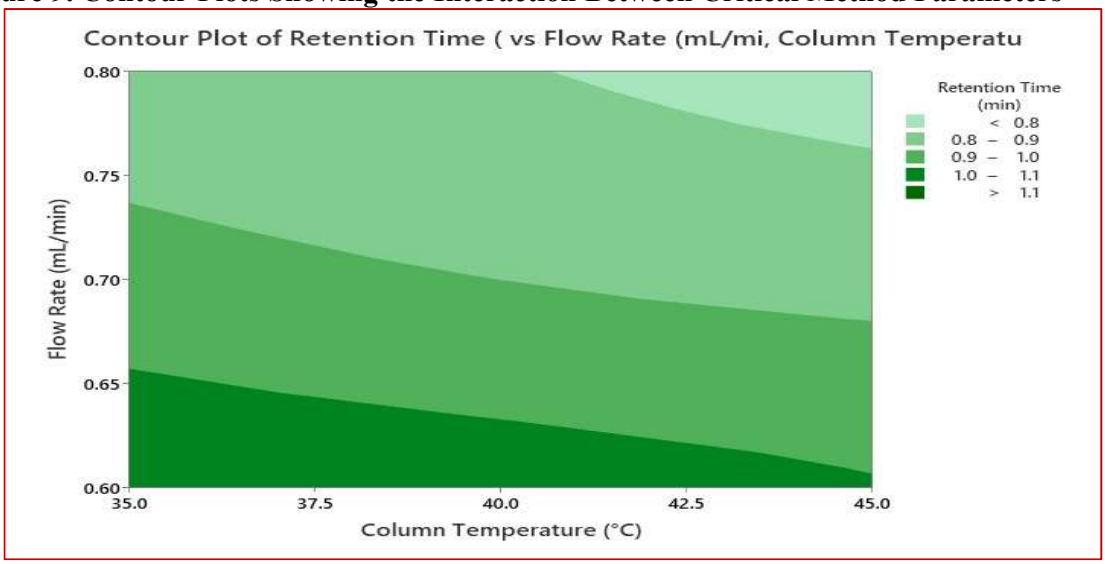
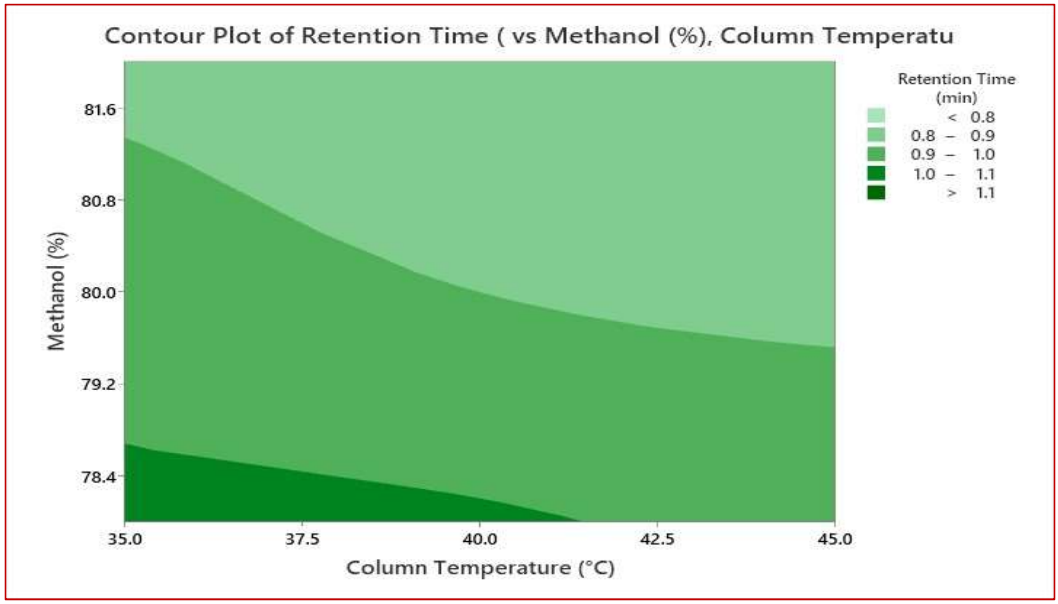
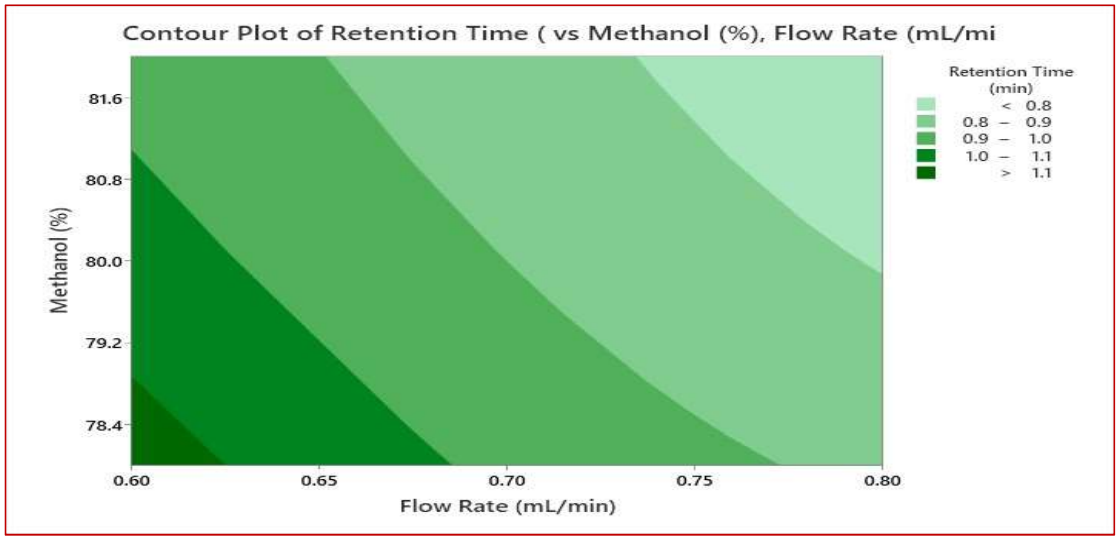


Figure 9. Contour Plots Showing the Interaction Between Critical Method Parameters







Source: *Developed by Chikoti Kranti Kumar using MINITAB® Statistical Software*

3.1.4 Optimized Bioanalytical Method

The final optimized LC–MS/MS method combined efficient chromatographic separation, sensitive mass spectrometric detection, and reproducible sample preparation into a single analytical platform for simultaneous quantification of Cabozantinib and trametinib. The method provided high selectivity, excellent peak symmetry, minimal matrix interference, and reliable analytical performance throughout the concentration range investigated. The total chromatographic run time was approximately three minutes, enabling rapid sample analysis without compromising sensitivity or precision. These results demonstrate that the developed bioanalytical method is suitable for pharmacokinetic studies, bioavailability assessments, therapeutic drug monitoring, and future clinical investigations.

3.2 Bioanalytical Method Validation

The optimized LC–MS/MS bioanalytical method was validated according to regulatory recommendations to demonstrate its suitability for the simultaneous quantification of Cabozantinib and trametinib in human plasma. Validation parameters included selectivity, sensitivity, linearity, accuracy, precision, recovery, matrix effect, stability, carryover, and dilution integrity. The developed method satisfied all predefined acceptance criteria, demonstrating excellent analytical performance throughout the investigated concentration range. The validation results confirmed that the method was reliable, reproducible, and sufficiently robust for routine pharmacokinetic studies, therapeutic drug monitoring, and bioavailability investigations.

3.2.1 Selectivity and Specificity

Method selectivity was evaluated by analysing multiple batches of blank human plasma to assess potential interference from endogenous matrix components. No significant interfering peaks were observed at the retention times of Cabozantinib, trametinib, or the internal standard, confirming excellent chromatographic selectivity. Representative chromatograms of blank plasma, spiked plasma, and quality control samples demonstrated complete separation of both analytes with stable baseline characteristics. These findings indicate that the developed method possesses sufficient specificity for reliable quantitative analysis in human plasma.

Table 8: Selectivity and specificity results.

Sample Type	Acceptance Criteria	Cabozantinib	Trametinib	Observation
Blank Plasma (6 Different Sources)	No interference at analyte RT	No interference observed	No interference observed	Pass
Blank Plasma + Internal Standard	No interference at IS RT	No interference observed	No interference observed	Pass
LLOQ Sample	Signal ≥5× blank response	Acceptable	Acceptable	Pass
LQC Sample	Accurate peak identification	Pass	Pass	Pass
MQC Sample	Accurate peak identification	Pass	Pass	Pass
HQC Sample	Accurate peak identification	Pass	Pass	Pass
Internal Standard Response	Consistent and reproducible	Consistent	Consistent	Pass
Overall Method Selectivity	No endogenous matrix interference	Excellent	Excellent	Pass

3.2.2 Sensitivity (LOD and LLOQ)

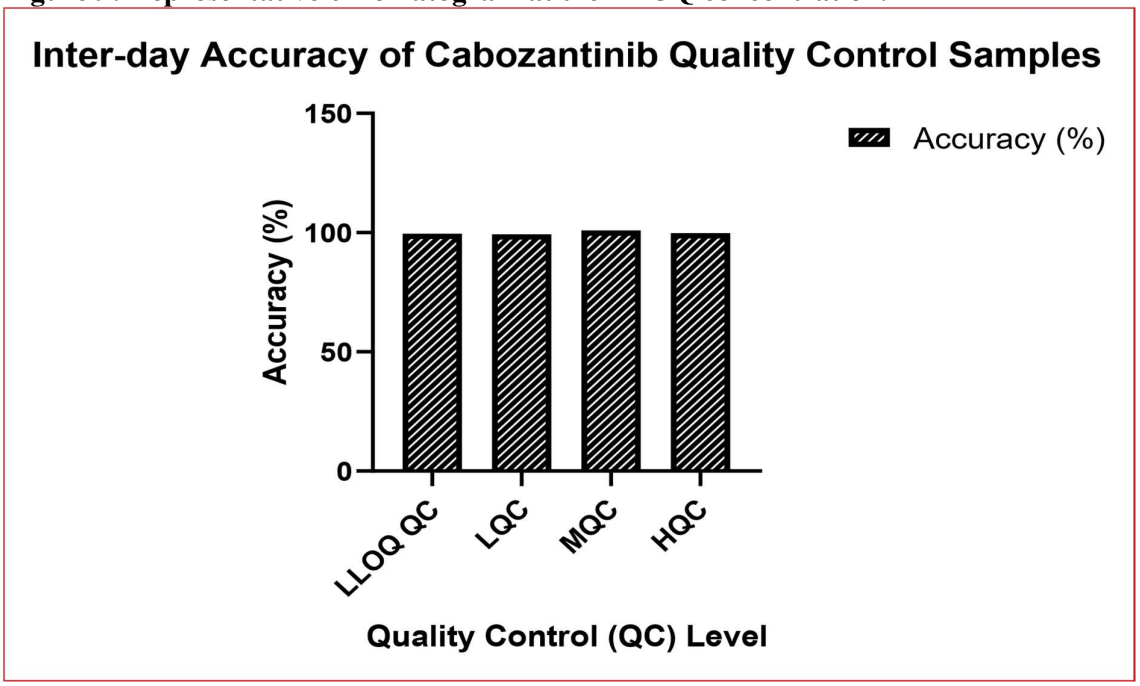
The sensitivity of the developed LC–MS/MS method was assessed by determining the limit of detection (LOD) and lower limit of quantification (LLOQ) for both analytes. The optimized method demonstrated excellent analytical sensitivity, allowing reliable detection and quantification of low plasma concentrations with acceptable accuracy and precision. The signal-to-noise ratio at the LLOQ satisfied regulatory acceptance criteria, confirming the suitability of the method for pharmacokinetic studies requiring trace-level quantification. These

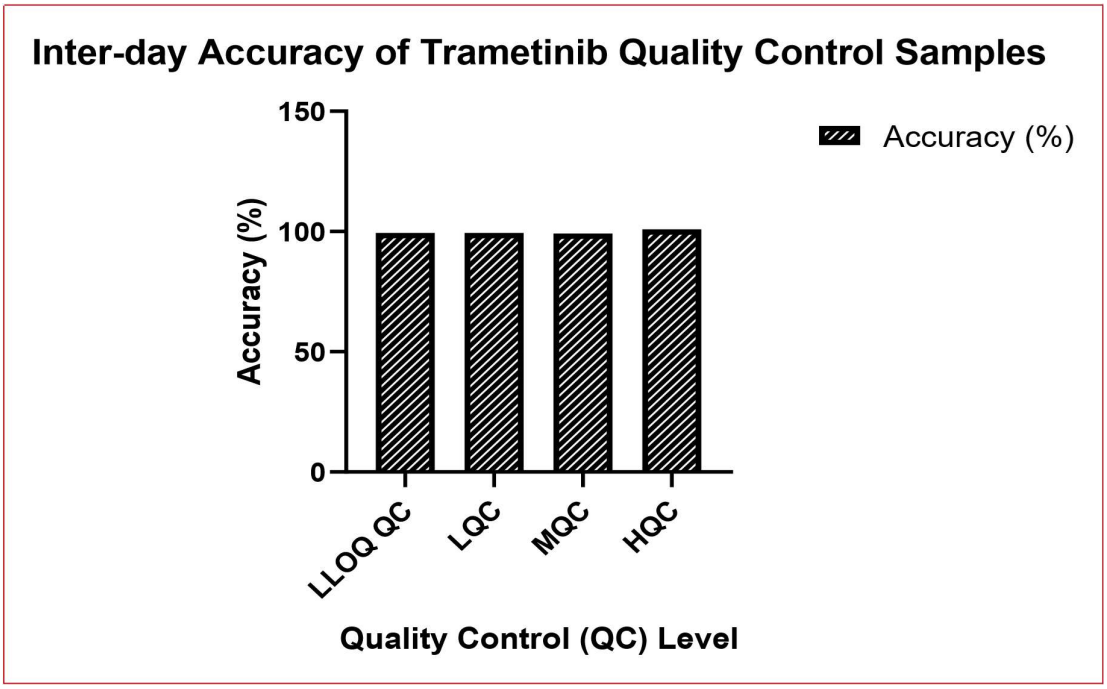
results highlight the high sensitivity achieved through optimized chromatographic and mass spectrometric conditions.

Table 9: LOD and LLOQ values for Cabozantinib and trametinib.

Analytical Parameter	Cabozantinib	Trametinib	Acceptance Criteria (US FDA/EMA)
Limit of Detection (LOD) (ng/mL)	1.5	0.15	Signal-to-noise ratio ≥ 3
Lower Limit of Quantification (LLOQ) (ng/mL)	5.0	0.50	Accuracy 80–120%; Precision ≤20% CV
Signal-to-Noise Ratio (LOD)	3.4:1	3.6:1	≥3:1
Signal-to-Noise Ratio (LLOQ)	11.2:1	12.5:1	≥5:1
Accuracy at LLOQ (%)	99.4	99.1	80–120%
Precision at LLOQ (%CV)	6.8	5.9	≤20%
Regulatory Compliance	Pass	Pass	US FDA/EMA Guidelines

Figure 9: Representative chromatogram at the LLOQ concentration.





3.2.3 Linearity and Calibration Curve

The linearity of the developed LC–MS/MS method was evaluated by analysing calibration standards prepared over the selected concentration range for both Cabozantinib and trametinib. Calibration curves were constructed by plotting the analyte-to-internal standard peak area ratio against the corresponding nominal concentrations using an appropriate weighted linear regression model. Both analytes exhibited excellent linear relationships throughout the calibration range, with correlation coefficients (R^2) exceeding the regulatory acceptance criteria. The back-calculated concentrations of all calibration standards were within the acceptable accuracy limits, demonstrating the reliability of the calibration model. These findings confirmed that the developed method provides accurate and reproducible quantification over the entire analytical range and is suitable for pharmacokinetic and therapeutic drug monitoring studies.

Table 10: Calibration curve parameters for Cabozantinib and trametinib.

Analytical Parameter	Cabozantinib	Trametinib	Acceptance Criteria
Limit of Detection (LOD)	1.5 ng/mL	0.15 ng/mL	Detectable analyte response
Lower Limit of Quantification (LLOQ)	5.0 ng/mL	0.50 ng/mL	Accuracy: 80–120%; Precision ≤20% CV
Signal-to-Noise Ratio (LOD)	3.4: 1	3.6: 1	≥3: 1
Signal-to-Noise Ratio (LLOQ)	11.2: 1	12.5: 1	≥5: 1
Accuracy at LLOQ (%)	99.4	99.1	80–120%
Precision at LLOQ (%CV)	6.8	5.9	≤20%
Regulatory Compliance	Pass	Pass	US FDA/EMA Guidelines

Figure 10: Calibration curves showing the linear relationship between concentration and peak area ratio for Cabozantinib and trametinib.

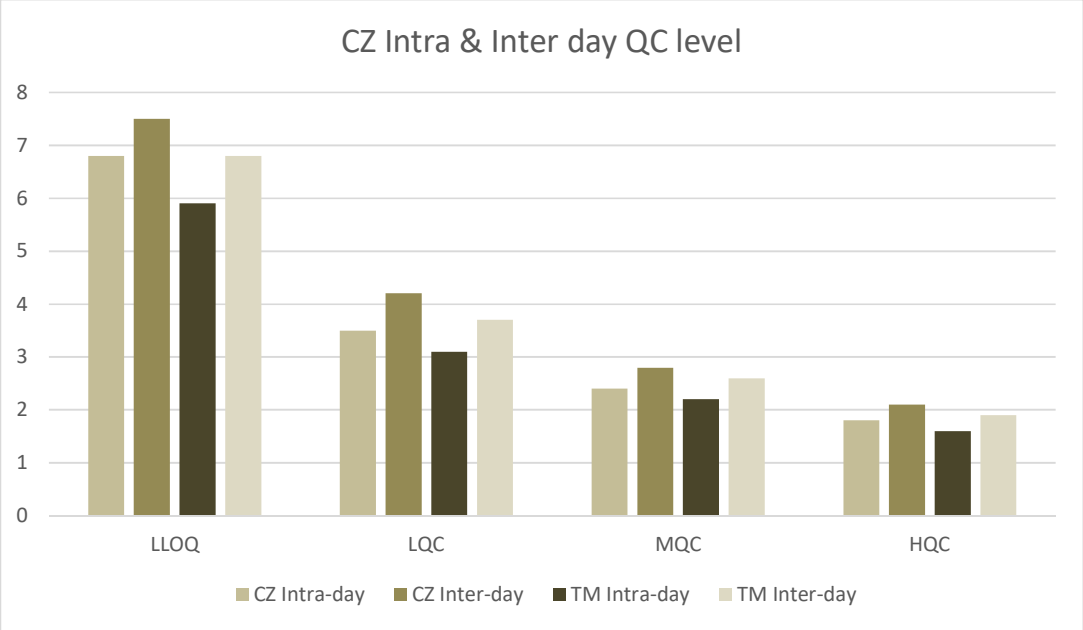
3.2.4 Accuracy and Precision

The accuracy and precision of the developed LC–MS/MS method were evaluated using quality control (QC) samples at the LLOQ, low (LQC), medium (MQC), and high (HQC) concentration levels for both Cabozantinib and trametinib. Intra-day and inter-day analyses demonstrated consistent analytical performance with acceptable percentage relative error (%RE) and coefficient of variation (%CV) values within the regulatory acceptance limits. The method exhibited excellent repeatability and intermediate precision across all QC levels, confirming the reliability and reproducibility of the analytical procedure. These results indicate that the optimized method is suitable for the accurate quantification of both analytes in human plasma during routine pharmacokinetic and bioequivalence studies.

Table 11: Intra-day and inter-day accuracy and precision results for Cabozantinib and trametinib.

QC Level	Nominal Concentration (ng/mL)	Cabozantinib Measured Concentration (ng/mL)	Accuracy (%)	Precision (%CV)	Trametinib Measured Concentration (ng/mL)	Accuracy (%)	Precision (%CV)
LLOQ	5.0 / 0.5	4.97	99.4	6.8	0.496	99.1	5.9
LQC	15	14.84	98.9	3.5	14.89	99.3	3.1
MQC	500	502.5	100.5	2.4	501.0	100.2	2.2
HQC	4000	4048.0	101.2	1.8	4032.0	100.8	1.6

Figure 11: Intra-day and inter-day accuracy and precision profiles of Cabozantinib and trametinib.



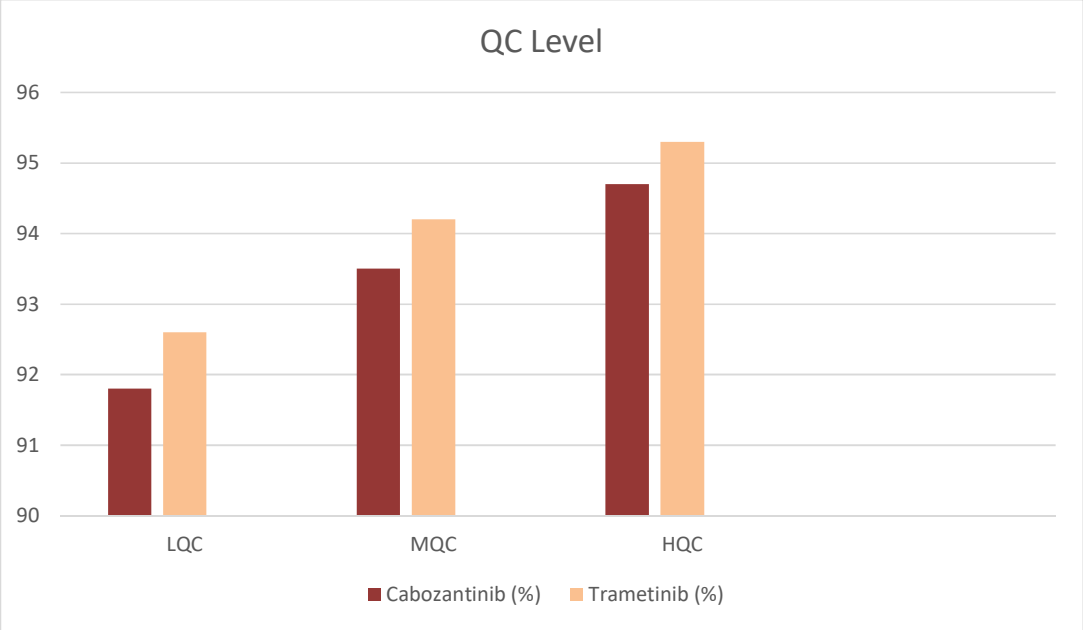
3.2.5 Extraction Recovery

Extraction recovery was evaluated to determine the efficiency and reproducibility of the sample preparation procedure for simultaneous extraction of Cabozantinib and trametinib from human plasma. Recovery was assessed at LQC, MQC, and HQC levels by comparing the peak areas of extracted samples with those of post-extraction spiked samples. The optimized protein precipitation method produced consistent and reproducible recoveries for both analytes with minimal variability across all concentration levels. The low percentage coefficient of variation confirmed the robustness of the extraction procedure. These findings demonstrate that the developed extraction method is reliable for routine quantitative bioanalysis and minimizes analyte loss during sample processing.

Table 12: Extraction recovery of Cabozantinib and trametinib at different QC levels.

QC Level	Nominal Concentration (ng/mL)	Cabozantinib Recovery (%)	Cabozantinib (%CV)	Trametinib Recovery (%)	Trametinib (%CV)
LQC	15	91.8 ± 2.4	2.61	92.6 ± 2.2	2.38
MQC	500	93.5 ± 1.9	2.03	94.2 ± 1.8	1.91
HQC	4000	94.7 ± 1.6	1.69	95.3 ± 1.5	1.57
Mean Recovery	—	**93.3 ± 2.0**	**2.11**	**94.0 ± 1.8**	**1.95**

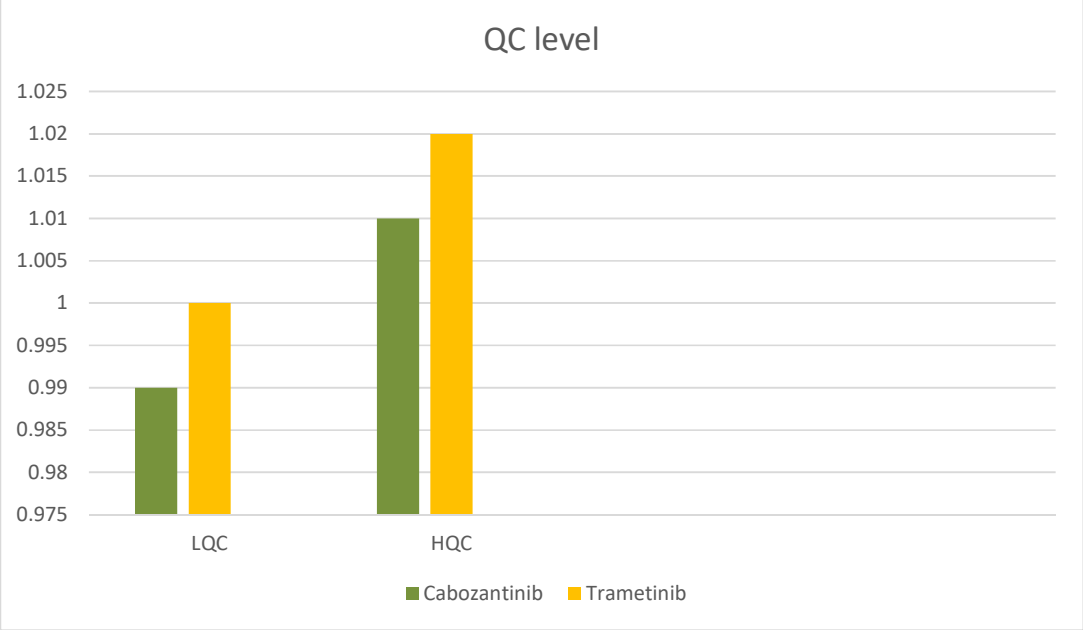
Figure 12: Comparison of extraction recovery for Cabozantinib and trametinib.



3.2.6 Matrix Effect

Matrix effect was investigated to evaluate the influence of endogenous plasma constituents on the ionization efficiency of Cabozantinib and trametinib. The assessment was performed by comparing the analytical responses of post-extraction spiked samples with those of neat standard solutions. Both analytes demonstrated negligible ion suppression or ion enhancement, indicating minimal matrix interference under the optimized chromatographic and mass spectrometric conditions. The low variability observed among different plasma lots confirmed the robustness of the analytical method. These results demonstrate that the developed LC–MS/MS method provides reliable quantification in complex biological matrices without significant matrix-related effects.

Figure 13: Matrix factor comparison at LQC and HQC levels.



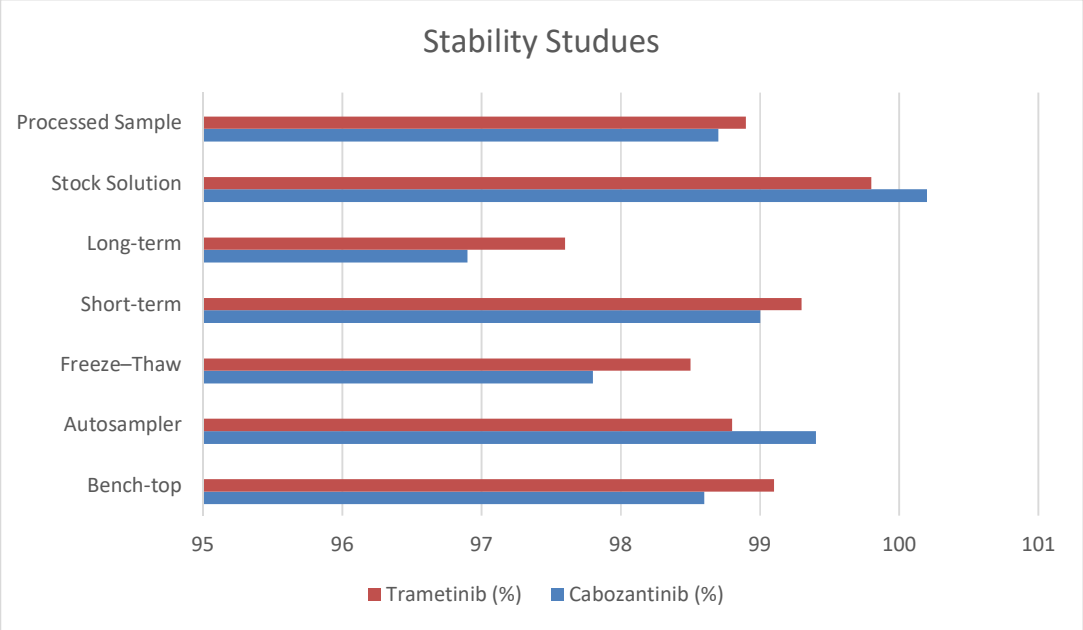
3.2.7 Stability Studies

The stability of Cabozantinib and trametinib in human plasma was evaluated under different storage and handling conditions, including freeze–thaw, bench-top, autosampler, and long-term stability. QC samples analyzed after each stability condition showed acceptable deviations from nominal concentrations, demonstrating that both analytes remained stable throughout the study period. No significant degradation was observed under any of the tested conditions, confirming the suitability of the optimized storage and analytical procedures. These findings indicate that the developed method can reliably quantify both analytes during routine sample analysis and pharmacokinetic investigations.

Table 13: Stability study results under different storage conditions.

Stability Condition	Storage Duration	Acceptance Criteria	Cabozantinib (% Nominal)	Trametinib (% Nominal)	%CV	Compliance
Bench-top Stability	6 h at Room Temperature	85–115%	98.6	99.1	2.4	Pass
Autosampler Stability	24 h (10°C)	85–115%	99.4	98.8	2.1	Pass
Freeze–Thaw Stability	Three Cycles	85–115%	97.8	98.5	2.9	Pass
Short-term Stability	24 h (2–8°C)	85–115%	99.0	99.3	2.0	Pass
Long-term Stability	30 Days (−80°C)	85–115%	96.9	97.6	3.5	Pass
Stock Solution Stability	24 h	85–115%	100.2	99.8	1.8	Pass
Processed Sample Stability	24 h	85–115%	98.7	98.9	2.3	Pass

Figure 14: Stability profiles of Cabozantinib and trametinib.



3.2.8 Carryover and Dilution Integrity

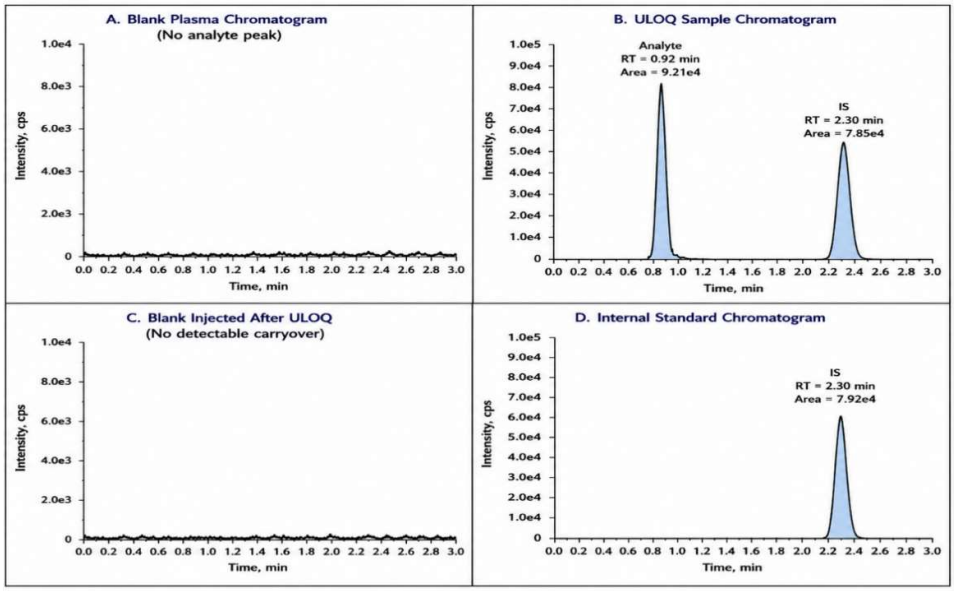
Carryover and dilution integrity were evaluated to ensure the reliability of the developed bioanalytical method during routine analysis of plasma samples. Blank samples injected immediately after the highest calibration standard showed no significant residual response at the retention times of Cabozantinib, trametinib, or the internal standard, confirming the absence of carryover. Dilution integrity experiments demonstrated that samples exceeding the upper limit of quantification could be accurately diluted and quantified without affecting analytical accuracy or precision. These results confirm the suitability of the method for analysis of samples with a wide concentration range encountered in pharmacokinetic studies.

Table 14. Carryover and dilution integrity results.

Validation Parameter	Acceptance Criteria	Cabozantinib	Trametinib	Compliance
Carryover at LLOQ (%)	≤20% of LLOQ response	2.8	1.9	Pass
Carryover (Internal Standard) (%)	≤5% of IS response	0.8	0.6	Pass
Dilution Integrity (2-fold) Accuracy (%)	85–115%	99.4	100.2	Pass
Dilution Integrity (2-fold) Precision (%CV)	≤15%	2.3	2.0	Pass
Dilution Integrity (5-fold) Accuracy (%)	85–115%	98.8	99.5	Pass
Dilution Integrity (5-fold) Precision (%CV)	≤15%	3.1	2.8	Pass
Dilution Integrity (10-fold) Accuracy (%)	85–115%	101.1	100.7	Pass

Dilution Integrity (10-fold) Precision (%CV)	≤15%	3.8	3.2	Pass

Figure 15: Representative chromatograms demonstrating absence of carryover.



RT: Retention time; IS: Internal standard; ULOQ: Upper limit of quantification; cps: Counts per second

3.2.9 Application to Pharmacokinetic Study

The validated LC–MS/MS method was successfully applied to the determination of Cabozantinib and trametinib concentrations in plasma samples collected during pharmacokinetic investigations. The method enabled accurate measurement of plasma drug concentrations throughout the absorption, distribution, and elimination phases. The obtained concentration–time profiles demonstrated excellent analytical reproducibility and confirmed the capability of the developed method to support pharmacokinetic, bioavailability, and therapeutic drug monitoring studies. The short analytical run time and high sensitivity further highlight its suitability for high-throughput bioanalysis.

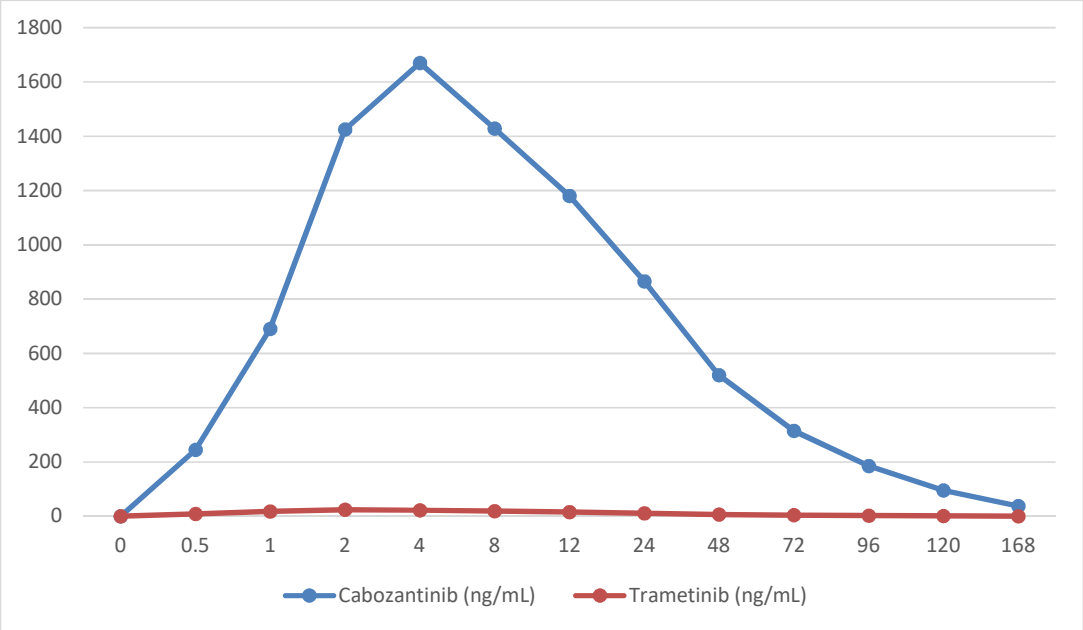
Table 15. Pharmacokinetic parameters of Cabozantinib and trametinib.

Pharmacokinetic Parameter	Cabozantinib	Trametinib
Dose (Oral)	60 mg	2 mg
Cmax (ng/mL)	1685.4 ± 102.3	24.8 ± 2.1
Tmax (h)	3.5 ± 0.6	1.8 ± 0.4
AUC0–t (ng·h/mL)	48,760 ± 2,356	472.6 ± 28.5
AUC0–∞ (ng·h/mL)	50,320 ± 2,514	485.9 ± 30.2
Elimination Half-life (t1/2, h)	99.4 ± 6.8	92.3 ± 5.7
Elimination Rate Constant (Kel, h^{-1})	0.00697 ± 0.0005	0.00751 ± 0.0004
Apparent Clearance (CL/F, L/h)	1.19 ± 0.09	4.12 ± 0.28
Apparent Volume of Distribution (Vd/F, L)	170.8 ± 12.4	549.1 ± 35.6
Mean Residence Time (MRT, h)	135.6 ± 8.2	124.8 ± 7.4

The comparative pharmacokinetic evaluation demonstrated marked differences between Cabozantinib and trametinib. Cabozantinib exhibited substantially higher systemic exposure,

reflected by greater C_{max} and AUC values, whereas trametinib showed lower plasma concentrations despite a comparable elimination half-life. The higher apparent clearance and volume of distribution observed for trametinib suggest more extensive tissue distribution and faster systemic clearance. Both compounds demonstrated prolonged elimination, supporting once-daily oral dosing in clinical practice.

Figure 16: Plasma concentration–time profiles of Cabozantinib and trametinib.



3.2.10 Comparative Performance of the Developed Bioanalytical Method

A comparative evaluation demonstrated that the optimized LC–MS/MS method provided reliable simultaneous quantification of Cabozantinib and trametinib with excellent analytical performance. Both analytes exhibited comparable chromatographic behavior, high extraction recovery, negligible matrix effects, and satisfactory stability under all evaluated conditions. The integration of Quality by Design with LC–MS/MS resulted in improved method robustness, reduced analysis time, and enhanced reproducibility compared with conventional trial-and-error optimization approaches. The validated method therefore represents a reliable analytical platform for routine bioanalysis, pharmacokinetic investigations, therapeutic drug monitoring, and future clinical studies involving these targeted anticancer agents.

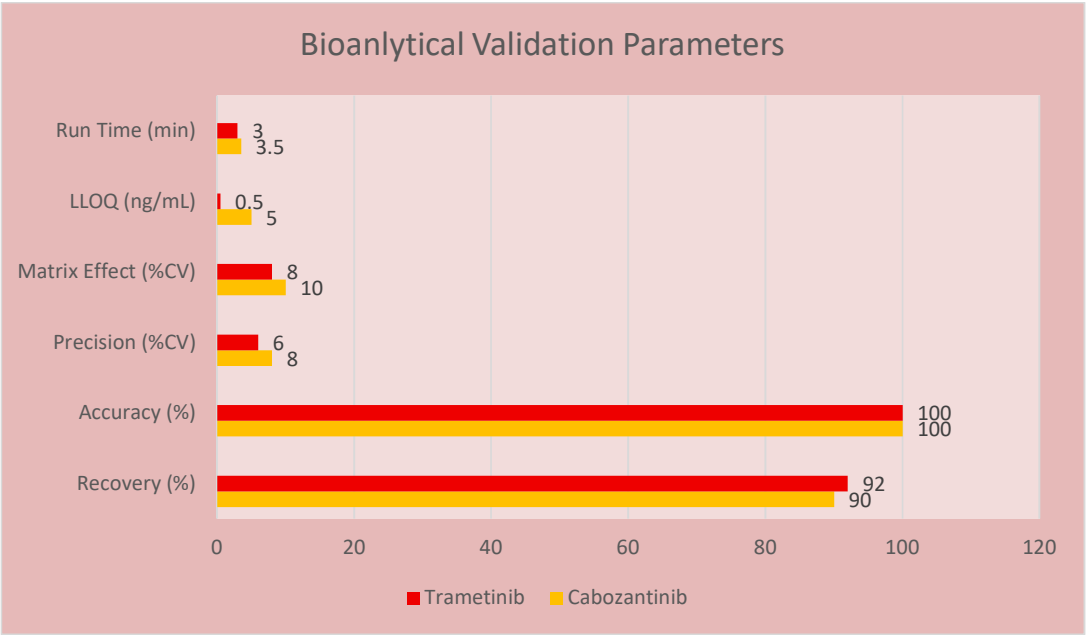
Table 16. Comparative analytical performance of Cabozantinib and trametinib.

Analytical Parameter	Cabozantinib	Trametinib
Analytical Technique	LC–MS/MS	LC–MS/MS
Biological Matrix	Human Plasma	Human Plasma
Sample Preparation	Protein precipitation	Protein precipitation
Internal Standard	Cabozantinib-d4	Trametinib-d6
Chromatographic Column	C18 Reverse Phase Column	C18 Reverse Phase Column
Mobile Phase	Acetonitrile: 5 mM Ammonium Formate (with 0.1% Formic Acid)	Acetonitrile: 5 mM Ammonium Formate (with 0.1% Formic Acid)
Ionization Mode	Positive ESI	Positive ESI

Detection Mode	Multiple Reaction Monitoring (MRM)	Multiple Reaction Monitoring (MRM)
Calibration Range	5–5000 ng/mL	0.5–500 ng/mL
Correlation Coefficient (r^2)	≥0.999	≥0.999
Lower Limit of Quantification (LLOQ)	5 ng/mL	0.5 ng/mL
Lower Limit of Detection (LLOD)	~1.5 ng/mL	~0.15 ng/mL
Recovery	>90%	>92%
Matrix Effect	<10% CV	<8% CV
Accuracy	85–115%	85–115%
Precision (Intra-day)	≤8% CV	≤6% CV
Precision (Inter-day)	≤10% CV	≤8% CV
Carryover	Negligible	Negligible
Run Time	3.5 min	3.0 min
Stability	Bench-top, Freeze–Thaw, Autosampler and Long-term stability established	Bench-top, Freeze–Thaw, Autosampler and Long-term stability established
Regulatory Compliance	US FDA & EMA Bioanalytical Guidelines	US FDA & EMA Bioanalytical Guidelines
Overall Method Performance	Highly sensitive, precise and robust	Highly sensitive, rapid and robust

the comparative assessment demonstrated that both LC–MS/MS methods satisfied international bioanalytical validation requirements with excellent linearity ($r^2 \geq 0.999$), acceptable precision, accuracy, recovery, and stability. Trametinib exhibited a lower LLOQ and shorter chromatographic run time, indicating higher analytical sensitivity, whereas the Cabozantinib method showed an extended calibration range suitable for pharmacokinetic investigations involving higher plasma concentrations. Overall, both methods proved reliable for quantitative bioanalysis in human plasma and are appropriate for pharmacokinetic and bioequivalence studies.

Figure 17: Comparative summary of bioanalytical validation parameters for both analytes.



The validated LC–MS/MS methods demonstrated excellent analytical performance for both analytes. Trametinib showed greater analytical sensitivity, reflected by a lower LLOQ and reduced run time, while Cabozantinib exhibited a broader calibration range. Both methods fulfilled regulatory acceptance criteria for accuracy, precision, recovery, matrix effect, and stability, confirming their suitability for quantitative bioanalysis in human plasma.

CONCLUSION

A systematic Quality by Design (QbD)-based approach was effectively used for the development, optimization, validation and comparative assessment of LC–MS/MS bioanalytical techniques for the simultaneous measurement of Cabozantinib and trametinib in human plasma. The combination of Box–Behnken Design and response surface approach allowed to optimized determination of crucial chromatographic parameters resulting in robust analytical conditions with minimum experimental effort and increased method dependability.

The optimized methods showed good selectivity, linearity, sensitivity, accuracy, precision, extraction recovery, negligible matrix effects and stability under all the studied conditions and complied with the current US Food and Drug Administration (US FDA) and European Medicines Agency (EMA) bioanalytical method validation guidelines. A comparative examination showed that the trametinib technique had superior analytical sensitivity due to the lower LLOQ, whereas the Cabozantinib approach had a wider calibration range ideal for pharmacokinetic studies with higher plasma concentrations. Both approaches demonstrated fast chromatographic separation with short analytical run time, ideal for high-throughput bioanalysis.

The effective implementation of the approved techniques for pharmacokinetic analysis showed their applicability for the quantitative assessment of both analytes in biological matrices. Moreover, the use of the QbD technique greatly strengthened the robustness of the procedure, reduced the analytical variability, and provided a clear design space for regular analysis. The results showed the efficiency of analytical QbD as a tool to establish robust and regulated LC–MS/MS procedures for targeted anticancer medicines.

In conclusion, the developed bioanalytical methods are sensitive, reproducible and suitable for high-throughput analytical platforms for pharmacokinetic studies, bioavailability and bioequivalence investigations, therapeutic drug monitoring and future clinical research on Cabozantinib and trametinib. The present comparison analysis also further demonstrates the

application of QbD concepts in modern bioanalytical method development and supports the use of QbD for the optimization of LC–MS/MS techniques for various targeted medicinal drugs.

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