Bioanalytical Method Validation and Bioequivalence Study of Ibrutinib in Human Plasma Using LC-MS/MS: An Advanced Approach

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ABSTRACT

This study presents a comprehensive bioanalytical method validation and bioequivalence analysis for ibrutinib, a Bruton's tyrosine kinase inhibitor, in human plasma samples. The method employs liquid chromatography-tandem mass spectrometry (LC-MS/MS) for quantification, utilizing apixaban as an internal standard. Method validation adhered to ICH M۱۰ guidelines, assessing parameters such as specificity, carry-over, lower limit of quantitation (LLOQ), calibration curve linearity, accuracy, precision, matrix effects, and stability under various conditions. Calibration curves exhibited strong linearity across a range of ۰.۵–٤۸ ppb (R^2 > ۰.۹۹), with an LLOQ of ۰.۵ ppb demonstrating a signal-to-noise ratio exceeding ۱۰. The method demonstrated robust specificity with minimal interference, as well as high precision and accuracy, with intra- and inter-day deviations within acceptable limits (< ۱۵٪). Stability assessments, including freeze-thaw, short-term, and long-term analyses, confirmed analyte integrity under varied conditions. The bioequivalence study compared test and reference formulations of ibrutinib in ۲٦ healthy volunteers over multiple time points, with pharmacokinetic parameters including C_max and AUC analyzed for equivalence. Results affirmed bioequivalence between formulations, meeting regulatory criteria. This validated method provides a reliable tool for therapeutic drug monitoring and pharmacokinetic studies of ibrutinib, contributing to optimized clinical outcomes and regulatory compliance.

Keywords: *Ibrutinib , Bioequivalence, LC-MS/MS, Analytical Method Validation , Pharmacokinetics*

۱. *INTRODUCTION*

General Background

The field of oncology has seen remarkable advancements over the past decade, with targeted therapies emerging as pivotal strategies in cancer treatment. Among these, ibrutinib, a Bruton's tyrosine kinase (BTK) inhibitor, has become a cornerstone for treating B-cell malignancies such as chronic lymphocytic leukemia (CLL) and mantle cell lymphoma (MCL). Despite its clinical success, challenges persist in optimizing its therapeutic efficacy due to inter-individual variability in pharmacokinetics (PK) and potential bioavailability issues. These factors necessitate rigorous bioequivalence (BE) studies to ensure consistent efficacy and safety across formulations, particularly in generic drug development.

Existing Challenges and Need for the Study

While liquid chromatography-tandem mass spectrometry (LC-MS/MS) has become the gold standard for quantifying drugs like ibrutinib in plasma due to its precision, sensitivity, and specificity, method validation remains critical for regulatory compliance. Issues such as matrix effects, variability in calibration accuracy, and stability of analytes under different conditions need comprehensive evaluation. Prior studies have largely focused on individual aspects of method validation without integrating these into a cohesive framework for BE assessment.

Research Objectives

The primary objective of this study is to develop and validate an LC-MS/MS-based analytical method for quantifying ibrutinib in human plasma according to ICH M١٠ guidelines. Specific objectives include:

١. *Ensuring accuracy, precision, and robustness of the method across a range of concentrations.*
٢. *Assessing the stability of ibrutinib under various conditions to simulate real-world scenarios.*
٣. *Conducting a bioequivalence study to compare the pharmacokinetics of test and reference formulations of ibrutinib.*

Research Problem

The lack of a universally validated, standardized method for evaluating ibrutinib in plasma, coupled with variability in BE study outcomes, underscores the need for a robust analytical approach. This research aims to fill this gap by providing a validated method that meets international regulatory standards.

Importance and Theoretical Framework

This study contributes both theoretically and practically to the field of bioanalysis. Theoretically, it strengthens the framework for pharmacokinetic evaluation using LC-MS/MS. Practically, it offers a validated method that can be adopted for therapeutic drug monitoring and regulatory submissions, enhancing drug accessibility and patient safety.

Hypotheses

١. *The LC-MS/MS method will exhibit high sensitivity and specificity for ibrutinib quantification in plasma.*
٢. *The bioequivalence study will confirm statistical equivalence in pharmacokinetic parameters (C_max, AUC) between the test and reference formulations.*

Methods

Materials

The materials used in the study are detailed in Table ١ below. All reagents and solvents were of analytical grade.

Material	***Quantity/Specification***	***Supplier***
Ibrutinib pure standard	*١٠ mg (٢٠٠ ppm stock solution)*	*Sigma-Aldrich*
Apixaban (Internal Std.)	*١٠ mg (٢٠٠ ppm stock solution)*	*Sigma-Aldrich*
Methanol	*HPLC-grade*	*Merck*
Formic Acid	*٠.٣٪ in water (Mobile Phase A)*	*Fisher Scientific*
Mobile Phase B	*Methanol*	*Fisher Scientific*
Blank Human Plasma	*٥٠٠ µL per sample*	*Biobank*
Acetonitrile	*HPLC-grade*	*Merck*

Method Overview

١. ***Preparation of Stock and Working Solutions****:*
   - *Ibrutinib stock solution (٢٠٠ ppm) was prepared by dissolving ١٠ mg of the drug in methanol and diluting to volume. Serial dilutions created working solutions of ٠.٥–٤٨ ppb.*
   - *Apixaban stock solution (٢٠٠ ppm) was prepared similarly, serving as an internal standard (IS).*
٢. ***Sample Preparation****:*
   - *٥٠٠ µL of blank plasma was spiked with ٥٠ µL of working solutions to achieve calibration levels.*

- *Internal standard (١٠ µL, ٥٠٠ ppb) was added to each sample. Proteins were precipitated with ١ mL acetonitrile, vortexed, and centrifuged. The supernatant was injected into the LC-MS/MS system.*

٣. ***LC-MS/MS Analysis**:*
   - *Chromatographic separation was achieved using an Agilent Zorbax SB-C١٨ column (PN٨٨٣٩٧٥-٩٠٢) with a mobile phase gradient of methanol and ٠.٣٪ formic acid in water.*
   - *The mass spectrometer operated in positive ion mode with multiple reaction monitoring (MRM).*

٤. ***Validation and Statistical Analysis**:*
   - *Parameters assessed included specificity, carry-over, LLOQ, linearity, precision, accuracy, matrix effects, and stability. Pharmacokinetic parameters were analyzed using non-compartmental methods.*

Preparation of Stock and Working Solutions

١. ***Stock Solutions:***
   - ***Ibrutinib Stock Solution:** Prepared by dissolving ١٠ mg of ibrutinib in methanol and diluting it to ٥٠ mL in a volumetric flask to achieve a concentration of ٢٠٠ ppm.*
   - ***Apixaban Stock Solution:** Similarly, ١٠ mg of apixaban was dissolved in methanol and diluted to ٥٠ mL to serve as the internal standard (IS) at a concentration of ٢٠٠ ppm.*

٢. ***Serial Dilutions:***
   - *For ibrutinib, serial dilutions were prepared to achieve concentrations ranging from ٠.٥ ppb to ٤٨ ppb. These dilutions served as calibration standards.*
   - *Apixaban was diluted to a working solution of ٥٠٠ ppb.*

٣. ***Quality Control (QC) Samples:***
   - *Low (LQC, ١ ppb), medium (MQC, ٤ ppb), and high (HQC, ٣٢ ppb) concentration levels were prepared to assess method precision and accuracy.*

Plasma Sample Preparation

١. ***Spiking Plasma Samples:***
   - *٥٠٠ µL of blank human plasma was aliquoted into microcentrifuge tubes.*
   - *Calibration and QC standards were prepared by spiking plasma with ٥٠ µL of the respective working solutions.*
   - *Internal standard (١٠ µL, ٥٠٠ ppb) was added to each sample.*

٢. ***Protein Precipitation:***
   - *To precipitate proteins, ١ mL of acetonitrile was added to each tube, followed by vortex mixing for ٢ minutes.*
   - *Samples were centrifuged at ١٥,٠٠٠ rpm at ٤°C for ١٠ minutes.*

٣. ***Supernatant Collection:***
   - *The clear supernatant was carefully transferred to HPLC vials for injection into the LC-MS/MS system.*

Instrumentation and Analytical Conditions

١. ***Chromatographic Separation:***
   - ***Column:** Agilent Zorbax SB-C١٨ (PN٨٨٣٩٧٥-٩٠٢).*
   - ***Mobile Phase:** A gradient elution of methanol (Phase B) and ٠.٣٪ formic acid in water (Phase A) was employed.*
   - ***Flow Rate:** ٠.٤ mL/min.*
   - ***Column Temperature:** Maintained at ٤٠°C.*
   - ***Injection Volume:** ٢٠ µL.*

٢. ***Mass Spectrometric Detection:***

- ***Instrument:*** *Quadrupole mass spectrometer (Waters Quattro Micro) equipped with an electrospray ionization (ESI) source.*
- ***Ionization Mode:*** *Positive.*
- ***MRM Transitions:***
  - *Ibrutinib: m/z ٤٦٠.٢٠ → ١٩٩.٠٠.*
  - *Apixaban (IS): m/z ٤٤١.٢٠ → ١٣٨.١٠.*
- ***Source Parameters:***
  - *Capillary Voltage: ٤ kV*
  - *Cone Voltage: ٣٥ V (ibrutinib), ٣٠ V (IS)*
  - *Source Temperature: ١٢٠°C*
  - *Desolvation Temperature: ٤٠٠°C*
  - *Desolvation Gas Flow: ١٢٠٠ L/h*

Method Validation

Validation followed ICH M١٠ guidelines, addressing parameters critical to analytical method robustness.

١. ***Specificity:***
   - *Six blank plasma samples from different donors were analyzed to evaluate potential interference at the retention times of ibrutinib and the IS. Spiked samples at LLOQ (٠.٥ ppb) were included to confirm peak identity and resolution.*

٢. ***Carry-Over:***
   - *A high-concentration sample (ULOQ, ٤٨ ppb) was injected, followed by a blank sample to assess any residual analyte carry-over.*

٣. ***Lower Limit of Quantitation (LLOQ):***
   - *The LLOQ was determined as the lowest concentration with a signal-to-noise ratio (S/N) ≥ ١٠ and acceptable precision and accuracy (±٢٠٪).*

٤. ***Calibration Curve Linearity:***
   - *Seven calibration standards (٠.٥–٤٨ ppb) were analyzed in triplicate. A weighted (١/x) linear regression model was used to calculate the slope, intercept, and correlation coefficient (R^2).*

٥. ***Precision and Accuracy:***
   - *Intra- and inter-day variability were assessed by analyzing five replicates each of LQC, MQC, and HQC on three separate days. Results were expressed as percent relative standard deviation (RSD%) and percent deviation (%Dev).*

٦. ***Matrix Effects:***
   - *Matrix effects were evaluated by comparing the analyte response in plasma and aqueous solutions at LQC and HQC levels across six different plasma lots.*

٧. ***Stability Studies:***
   - *Short-term, freeze-thaw, and long-term stability were assessed under various conditions, including room temperature exposure (١ hour), repeated freeze-thaw cycles (٢٤ hours, –٢١°C), and storage at –٨٠°C for up to one month.*

Bioequivalence Study Design

١. ***Study Population:***
   - *Twenty-six healthy volunteers participated in a randomized, two-period crossover study with a ٧-day washout period.*

٢. ***Dosing and Sample Collection:***
   - *Volunteers received single oral doses of test and reference ibrutinib formulations. Blood samples were collected at pre-determined intervals (٠.٥, ١, ١.٥, ٢, ٣, ٤, ٦, ٨, ١٢, ٢٤ hours).*

٣. ***Pharmacokinetic Analysis:***

- *Plasma concentrations of ibrutinib were analyzed to determine C_max, AUC_۰-t, and AUC_۰-∞. BE was assessed using log-transformed PK parameters, with the ۹۰٪ confidence interval for the geometric mean ratios falling within ۸۰–۱۲۵٪.*

Statistical Analysis

Statistical evaluation was performed using ANOVA for crossover designs. All calculations were conducted using validated software compliant with regulatory guidelines.

This detailed methodological approach ensures the reliability and reproducibility of results, laying the groundwork for regulatory acceptance and clinical application of the validated method.

Discussion

Overview of Key Findings

The development and validation of an LC-MS/MS-based method for ibrutinib quantification in human plasma were successfully achieved, adhering to ICH M۱۰ guidelines. The calibration curve exhibited exceptional linearity (R^2 > ۰.۹۹) over the concentration range of ۰.۵–۴۸ ppb, confirming the method's robustness. The LLOQ of ۰.۵ ppb, coupled with a signal-to-noise ratio exceeding ۱۰, highlights the method's sensitivity. These findings are consistent with the standards established for therapeutic drug monitoring of kinase inhibitors, offering enhanced precision and accuracy.

Comparison with Existing Literature

Previous studies on ibrutinib quantification have demonstrated the potential of LC-MS/MS; however, few have reported comprehensive validation covering matrix effects, stability under varied conditions, and inter-day precision. This study bridges these gaps by incorporating rigorous assessments across all critical validation parameters. For example, short-term and freeze-thaw stability tests revealed analyte integrity within ±۲۰٪ deviation, aligning with findings from similar analytical studies on small-molecule kinase inhibitors.

Bioequivalence Insights

The bioequivalence study demonstrated statistical equivalence between test and reference formulations of ibrutinib. Pharmacokinetic parameters, including C_max and AUC, fell within the regulatory limits of ۸۰–۱۲۵٪. These results underscore the method's applicability in generic drug development, ensuring that alternative formulations meet therapeutic standards.

Practical Implications

Validated methods like the one developed here are essential for regulatory submissions and therapeutic drug monitoring. By confirming ibrutinib's stability and bioequivalence, this study supports its clinical use in treating B-cell malignancies while providing a blueprint for future

Conclusion

The validated LC-MS/MS method for ibrutinib quantification in human plasma offers a robust analytical framework that fulfills the stringent criteria of ICH M۱۰ guidelines. The high sensitivity, specificity, and reproducibility demonstrated by the method across a broad concentration range establish its suitability for both clinical and regulatory applications. By ensuring accurate and precise measurements of ibrutinib concentrations, the method significantly contributes to therapeutic drug monitoring and the pharmacokinetic evaluation of novel and generic formulations.

The study's bioequivalence analysis provided compelling evidence that test and reference formulations of ibrutinib meet regulatory requirements. The close alignment of key pharmacokinetic parameters, such as C_max and AUC, supports the therapeutic interchangeability of these formulations, enhancing accessibility and affordability for patients requiring long-term treatment.

Furthermore, the comprehensive stability assessments, encompassing short-term, freeze-thaw, and long-term storage conditions, underscore the reliability of the method under real-world scenarios. This not only reinforces its application in bioequivalence studies but also positions it as a valuable tool for quality control in drug manufacturing processes.

The broader implications of this research extend to the development of analytical methodologies for other small-molecule drugs. By addressing key challenges such as matrix effects and inter-day variability, the study sets a benchmark for future bioanalytical investigations. Its findings pave the way for more consistent and reliable drug evaluations, ultimately contributing to improved patient outcomes and regulatory compliance on a global scale.

In conclusion, the outcomes of this study underscore the importance of rigorous method validation in ensuring the efficacy and safety of pharmaceutical products. The adoption of such validated methods can streamline drug development, foster innovation, and ensure that critical medications like ibrutinib remain accessible to patients worldwide. Future research should aim to extend these findings by exploring the applicability of the validated method in special populations and in assessing potential drug-drug interactions, thereby broadening its clinical relevance and impact.

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