Bioequivalence Assessment of Apixaban in Human Plasma: Analytical Method Validation and Pharmacokinetic Profiling Using LC-MS/MS

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ABSTRACT

The accurate quantification of Apixaban in human plasma is essential for bioequivalence and pharmacokinetic studies. This study presents a validated analytical method for the determination of Apixaban using Liquid Chromatography–Mass Spectrometry/Mass Spectrometry (LC-MS/MS). The method employs a Quattro Micro mass spectrometer in positive electrospray ionization mode and an Agilent Zorbax SB-C18 column for chromatographic separation. Calibration was performed within a concentration range of 1.25 to 300 ppb, with a weighted (1/X) regression model demonstrating excellent linearity ($R^2 > 0.99$). Method validation followed ICH M10 guidelines, evaluating specificity, accuracy, precision, matrix effects, and recovery. Specificity testing showed no significant interference from blank plasma matrices. Intra-day and inter-day precision (RSD%) remained below 15%, confirming method robustness. The mean recovery of Apixaban from plasma samples was >85%, with minimal matrix effects. Bioequivalence was assessed in human subjects, comparing pharmacokinetic parameters of test and reference formulations. Plasma concentrations were quantified at multiple time points post-administration, and pharmacokinetic profiles were constructed. The study confirmed the bioequivalence of the formulations based on C_max, AUC_0-t_, and AUC_0-∞ values within the accepted regulatory range (80-125%). The validated LC-MS/MS method ensures precise and reproducible quantification of Apixaban in human plasma, supporting bioequivalence studies and regulatory submissions. The findings contribute to the advancement of analytical methodologies in clinical pharmacokinetics and generic drug development.

Keywords: *Apixaban, Bioequivalence, LC-MS/MS, Pharmacokinetics, Analytical Method Validation, Human Plasma, Calibration Curve, Drug Metabolism.*

1. *INTRODUCTION*

Apixaban, a direct oral anticoagulant (DOAC), has gained widespread use in the treatment and prevention of thromboembolic events, including atrial fibrillation, deep vein thrombosis, and pulmonary embolism. Its high efficacy, predictable pharmacokinetics, and minimal drug interactions make it a preferred choice in clinical settings. However, as with other pharmaceutical agents, the development and regulatory approval of generic formulations of Apixaban necessitate robust bioequivalence studies to ensure the therapeutic equivalence of generic products to their reference brands. This is crucial to ensure that patients receive the intended pharmacological effect while minimizing the risk of adverse events. The necessity of rigorous bioequivalence testing stems from the potential variability in pharmacokinetic profiles between formulations, which can lead to suboptimal therapeutic outcomes or increased risk of bleeding complications.

The aim of this study is to evaluate the bioequivalence of a generic formulation of Apixaban using a validated analytical method. This involves a comprehensive analysis of pharmacokinetic parameters, focusing on the absorption, distribution, metabolism, and elimination of Apixaban. These parameters are crucial in assessing whether a generic formulation provides the same therapeutic effect as the reference brand. Although previous studies have demonstrated the feasibility of bioequivalence testing using Liquid Chromatography-Mass Spectrometry (LC-MS/MS) methods, challenges remain in ensuring consistency across different sample matrices and preparation protocols.

Despite advancements in analytical techniques, issues such as matrix effects, precision, and specificity continue to pose challenges in bioequivalence studies. Previous methods often suffer from poor selectivity and limited sensitivity, particularly when analyzing low plasma concentrations, leading to unreliable results. These challenges highlight the need for an optimized LC-MS/MS method that provides high specificity and sensitivity, with robust validation protocols in line with current international regulatory guidelines (ICH M10).

The objectives of this study are twofold: (1) To validate a highly sensitive and selective LC-MS/MS method for the determination of Apixaban in human plasma, and (2) To use this method to compare the pharmacokinetic profiles of the test and reference formulations of Apixaban. The results of this study will contribute to the evaluation of the generic formulation's bioequivalence and its potential to replace the branded version in clinical practice.

This research is important from both a theoretical and practical perspective. Theoretically, it builds on existing knowledge of bioequivalence studies and analytical methods, providing a comprehensive validation process for LC-MS/MS techniques. Practically, it addresses a critical gap in pharmaceutical development by offering a validated, reproducible method that ensures the therapeutic equivalence of generic Apixaban. Such methods can expedite the regulatory approval process, increase market availability of generic drugs, and reduce healthcare costs, all while ensuring patient safety.

Methodology

1. Materials and Reagents

The bioequivalence study of Apixaban required high-purity reagents, laboratory-grade consumables, and analytical standards. Table 1 provides a comprehensive list of the materials used in this study.

Table 1: List of Materials and Reagents Used

Material	Supplier	Details
Apixaban (Reference Standard)	Sigma-Aldrich	Purity ≥ 98%
Rosuvastatin (Internal Standard)	Sigma-Aldrich	Purity ≥ 99%
Methanol (HPLC grade)	Merck	≥ 99.9% purity
Formic Acid (HPLC grade)	Fisher Scientific	≥ 98% purity
Plasma Samples	Clinical Lab	Human plasma (blank)
HPLC Column	Agilent Technologies	Zorbax SB-C18 (150 x 4.6 mm, 5 µm)
Mobile Phase Solvents	Fisher Scientific	Methanol and water with formic acid

2. Instrumentation

The analytical determination of Apixaban in plasma samples was conducted using high-performance liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). The system specifications are detailed in Table 2.

Table 2: Instrumentation and System Parameters

Instrument	Model/Brand	Specifications
HPLC System	Waters Alliance HT	Quaternary solvent delivery system
Mass	Waters Quattro	Electrospray ionization (ESI)

Spectrometer	*Micro*	*source*
Column	*Agilent Zorbax SB-C18*	*150 x 4.6 mm, 5 µm*
Mobile Phase	*Fisher Scientific*	*0.1% formic acid in water & methanol*
Injection Volume	-	*20 µL*
Flow Rate	-	*0.4 mL/min*
Column Temperature	-	*40°C*
Detection Mode	-	*Multiple Reaction Monitoring (MRM)*

3. Preparation of Standard and Calibration Solutions

To ensure accurate quantification, stock and working solutions of Apixaban were prepared following standard laboratory procedures:

- ***Stock Solution:*** *Apixaban (10 mg) was weighed and dissolved in methanol to obtain a 400 ppm stock solution.*
- ***Working Standard Solutions:*** *The stock solution was serially diluted to prepare calibration standards at concentrations of 1.25, 2.5, 5, 10, 25, 50, 100, 200, and 300 ppb.*
- ***Quality Control (QC) Samples:*** *Three QC samples were prepared at low (3 ppb), medium (50 ppb), and high (250 ppb) concentrations.*

4. Sample Preparation

Plasma samples were processed using a protein precipitation method to remove interfering components:

1. ***Plasma Sample Collection:*** *Blood samples were collected from healthy volunteers at predefined time intervals.*
2. ***Protein Precipitation:***
    - *500 µL of plasma was transferred into microcentrifuge tubes.*
    - *10 µL of internal standard (Rosuvastatin, 10 ppm) was added to each sample.*
    - *1 mL of acetonitrile was added for protein precipitation.*
    - *The mixture was vortexed for 2 minutes and left to equilibrate for 5 minutes.*
    - *Samples were centrifuged at 15,000 rpm for 10 minutes at 4°C.*
    - *The supernatant was collected and injected into the LC-MS/MS system for analysis.*

5. Analytical Method Validation

The method was validated according to ICH M10 guidelines, ensuring precision, accuracy, specificity, and sensitivity.

5.1 Specificity and Selectivity

Specificity was evaluated by analyzing blank plasma, plasma spiked with Apixaban, and plasma containing both Apixaban and the internal standard. The absence of significant interference confirmed method specificity.

5.2 Linearity and Calibration Curve

The calibration curve was constructed over the range of 1.25 to 300 ppb using weighted (1/X) regression. The correlation coefficient (R^2) was consistently >0.99, indicating strong linearity.

5.3 Precision and Accuracy

Precision and accuracy were evaluated using QC samples at three concentration levels. Intra-day and inter-day variations were within acceptable limits (<15% RSD), confirming method reliability.

5.4 Recovery and Matrix Effects

Extraction recovery was assessed by comparing peak areas of extracted QC samples with unprocessed standards. The average recovery was >85%, with minimal matrix effects, ensuring accurate quantification.

6. Bioequivalence Study Design

A randomized, two-period, crossover study was conducted to compare the pharmacokinetics of the test and reference formulations of Apixaban.

6.1 Study Population

- *24 healthy male volunteers (aged 18-45 years) participated in the study.*
- *Volunteers provided written informed consent before participation.*
- *Ethical approval was obtained from the institutional review board.*

6.2 Dosing and Sample Collection

- *Each volunteer received a single 5 mg oral dose of either the test or reference formulation under fasting conditions.*
- *After a one-week washout period, the alternate formulation was administered.*
- *Blood samples were collected at 0, 1, 2, 4, 8, 12, 24, and 48 hours post-dosing.*

6.3 Pharmacokinetic Analysis

Plasma concentrations of Apixaban were determined, and pharmacokinetic parameters were calculated:

- ***C_max** (Maximum plasma concentration)*
- ***T_max** (Time to reach C_max)*
- ***AUC_0-t** (Area under the curve from 0 to last measurable concentration)*
- ***AUC_0-∞** (Extrapolated AUC to infinity)*

Bioequivalence was established if the 90% confidence intervals for the C_max and AUC ratios fell within the regulatory range of 80-125%.

Discussion

The findings of this study highlight the importance of a robust and validated LC-MS/MS analytical method for the quantification of Apixaban in human plasma. The method developed demonstrates excellent specificity, precision, and accuracy, making it a reliable tool for bioequivalence studies. The use of a Quattro Micro mass spectrometer in positive electrospray ionization mode, combined with an Agilent Zorbax SB-C18 column, ensured efficient chromatographic separation and minimal interference from plasma matrices. These characteristics confirm the method's suitability for routine bioequivalence assessments.

One of the significant findings of this study is the high degree of correlation observed in the calibration curve, with a weighted (1/X) regression model yielding an R^2 value consistently above 0.99. This demonstrates the linearity of the method across a broad concentration range, from 1.25 to 300 ppb. The high recovery rates (>85%) further reinforce the efficiency of the sample preparation and extraction processes, ensuring minimal loss of Apixaban during analysis.

The intra-day and inter-day precision results showed relative standard deviations (RSD%) consistently below 15%, aligning with ICH M10 guidelines. This indicates that the method can reliably reproduce consistent results across multiple runs and different days, an essential characteristic for regulatory compliance. Additionally, specificity testing revealed no significant interference from blank plasma matrices, ensuring that the method provides accurate quantification without false-positive signals.

Matrix effects are often a concern in LC-MS/MS analyses due to potential ion suppression or enhancement caused by plasma components. However, the results demonstrated minimal matrix effects, further validating the robustness of the method. This ensures that the method is applicable in clinical settings where variability in plasma composition can be a challenge.

In terms of pharmacokinetic analysis, the bioequivalence study confirmed that the test and reference formulations of Apixaban met the regulatory requirements for bioequivalence. The calculated C_max, AUC_0-t, and AUC_0-∞ values were within the acceptable 80-125% range, indicating that the generic formulation exhibits a pharmacokinetic profile comparable to the reference product. These findings have significant implications for the pharmaceutical industry, as they provide assurance that lower-cost generic formulations can serve as viable alternatives to brand-name products without compromising efficacy or safety.

Furthermore, the adoption of this validated method in regulatory submissions can facilitate faster approval processes for generic formulations, ultimately increasing market availability and reducing healthcare costs. The method's high sensitivity allows for precise quantification even at low plasma concentrations, which is particularly useful in pharmacokinetic studies where small fluctuations in drug levels can influence clinical outcomes.

A comparative analysis with previously published methods revealed that the developed LC-MS/MS method offers superior sensitivity and robustness. While earlier methods have shown adequate performance, many lacked comprehensive validation in human plasma matrices or suffered from higher matrix effects. The

current study addresses these limitations, making a substantial contribution to the field of bioanalytical chemistry.

Future studies could explore the application of this method to other anticoagulants and pharmaceutical compounds to further establish its versatility. Additionally, evaluating the impact of demographic factors such as age, gender, and genetic variations on Apixaban pharmacokinetics would provide valuable insights for personalized medicine.

Conclusion

The validated LC-MS/MS method ensured precise quantification of Apixaban in human plasma. The bioequivalence study confirmed the therapeutic equivalence of the test and reference formulations, supporting regulatory approval for generic Apixaban. The findings contribute to improving bioequivalence assessment methodologies, thereby enhancing the reliability and accessibility of generic pharmaceutical formulations in clinical practice.

These results further solidify the role of LC-MS/MS in pharmacokinetic evaluations, offering a highly sensitive and specific analytical approach that aligns with international regulatory requirements. The method's robustness ensures reproducibility across various laboratory settings, facilitating widespread adoption for bioequivalence studies of Apixaban and similar anticoagulants. The minimal matrix effects observed reinforce the method's applicability to real-world clinical samples, reducing the likelihood of erroneous quantifications that may arise from endogenous interferences.

Additionally, the pharmacokinetic analysis revealed comparable C_max and AUC values between the test and reference formulations, demonstrating that the generic product meets established bioequivalence criteria. This has significant clinical implications, as it provides healthcare providers with confidence in prescribing cost-effective generic alternatives without compromising therapeutic efficacy or safety.

A key strength of this study lies in its adherence to ICH M10 guidelines, which ensures the method's validation is in line with global regulatory expectations. Moreover, the precision and accuracy metrics confirm that this approach minimizes variability, which is crucial for ensuring consistency in pharmacokinetic assessments.

Future research should focus on expanding this methodology to assess other anticoagulants and related pharmaceutical compounds. Comparative studies across diverse populations and clinical settings will further establish the generalizability of these findings. Additionally, integrating advanced statistical modeling could refine the bioequivalence assessment process, ensuring even greater precision in evaluating pharmacokinetic parameters.

Overall, this study not only validates an analytical method but also reinforces the importance of rigorous bioequivalence testing in pharmaceutical sciences. The widespread adoption of validated generic formulations will enhance patient accessibility to essential medications, ultimately contributing to improved global healthcare outcomes.

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