Comprehensive Validation of Rosuvastatin and Apixaban Quantification in Plasma Using LC-MS/MS: A Novel Analytical Method for Bioequivalence Studies

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ABSTRACT

This study presents the development and comprehensive validation of a highly sensitive and selective LC-MS/MS method for the simultaneous quantification of rosuvastatin and apixaban in human plasma, aimed at bioequivalence and pharmacokinetic studies. Analytical method validation was conducted following EMEA and ICH M10 guidelines. The method employed a Zorbax SB-C18 column with a mobile phase comprising 0.2% formic acid in water (Phase A) and methanol (Phase B) under gradient elution. Rosuvastatin and apixaban were quantified using multiple reaction monitoring (MRM) in positive electrospray ionization mode. The lower limit of quantification (LLOQ) was established at 0.625 ppb for rosuvastatin, ensuring a signal-to-noise ratio exceeding 10.

Validation parameters included specificity, carry-over, calibration curve linearity, accuracy, precision (within-run and between-run), matrix effects, and stability under various conditions. Specificity tests revealed no interference from blank plasma, with placebo interference below 1%. Calibration curves exhibited excellent linearity ($R^2 > 0.999$) over the concentration range of 0.625–60 ppb. Accuracy and precision were within acceptable limits, with relative standard deviation (RSD) values below 10% for all quality control levels. Matrix effects were minimal, with matrix effect factors (MEF) consistently meeting validation criteria. Stability assessments, including short-term, freeze-thaw, and long-term evaluations, demonstrated negligible degradation under simulated bioanalytical conditions.

Application of the validated method to plasma samples from study volunteers demonstrated its robustness and reproducibility in real-world scenarios. These results confirm the method's suitability for bioequivalence and pharmacokinetic analyses of rosuvastatin and apixaban, contributing to enhanced therapeutic monitoring and optimization.

Keywords: *LC-MS/MS, rosuvastatin, apixaban, method validation, bioequivalence, plasma quantification, pharmacokinetics, ICH M10 guidelines, matrix effect, stability.*

1. *INTRODUCTION*

Rosuvastatin is a widely prescribed statin used to manage hypercholesterolemia by inhibiting HMG-CoA reductase, thereby reducing low-density lipoprotein cholesterol levels. Apixaban, an oral anticoagulant, functions as a direct factor Xa inhibitor and is commonly used for preventing thromboembolic events. Accurate quantification of these drugs in human plasma is essential for pharmacokinetic studies, therapeutic drug monitoring, and bioequivalence assessments.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) has emerged as a preferred analytical technique due to its high sensitivity, specificity, and robustness. Several studies have reported validated LC-MS/MS methods for the determination of rosuvastatin and apixaban in human plasma. For instance, a study developed and validated a bioanalytical method using LC-MS/MS to measure rosuvastatin in 10 µL of blood

collected by volumetric absorptive microsampling (VAMS), highlighting the method's precision and accuracy ([TC Pharm](https://www.tcpharm.org/pdf/10.12793/tcp.2021.29.e13?utm_source=chatgpt.com)). Another study introduced a fast, simple, and sensitive LC-MS/MS technique for determining apixaban in human plasma, emphasizing its application in clinical therapeutic drug monitoring and bioequivalence studies ([Akjournals](https://akjournals.com/view/journals/1326/34/3/article-p332.xml?utm_source=chatgpt.com)).

Despite these advancements, there remains a need for standardized methodologies that can simultaneously quantify both rosuvastatin and apixaban in human plasma with high precision and accuracy. This study aims to develop and validate an LC-MS/MS method for the simultaneous determination of rosuvastatin and apixaban in human plasma, providing a reliable tool for clinical and pharmacokinetic applications.

Methodology

Chemicals and Reagents

Rosuvastatin calcium (purity ≥99%) and apixaban (purity ≥99%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The internal standard (IS), atorvastatin (purity ≥99%), was obtained from Cayman Chemicals (Ann Arbor, MI, USA). Acetonitrile (HPLC grade) and methanol (HPLC grade) were sourced from Merck (Darmstadt, Germany). Formic acid (analytical grade) and ammonium acetate (analytical grade) were supplied by Sigma-Aldrich. Deionized water was prepared using a Milli-Q purification system (Millipore, Bedford, MA, USA).

Instrumentation and Chromatographic Conditions

The analysis was conducted on an LC-MS/MS system comprising a Waters Acquity UPLC coupled with a triple quadrupole mass spectrometer (Xevo TQ-S, Waters, Milford, MA, USA) equipped with an electrospray ionization (ESI) source. Chromatographic separation was achieved on a Zorbax SB-C18 column (150 mm × 4.6 mm, 5 µm; Agilent Technologies, Santa Clara, CA, USA) maintained at 30°C. The mobile phase consisted of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile), delivered in a gradient mode. The flow rate was set at 0.4 mL/min, and the injection volume was 20 µL.

Gradient Program

Time (min)	*Solvent A (%)*	*Solvent B (%)*
0.0	*90*	*10*
2.0	*50*	*50*
5.0	*10*	*90*
7.0	*90*	*10*

Mass spectrometric detection was performed in positive ionization mode using multiple reaction monitoring (MRM) transitions. The transitions were as follows:

- *Rosuvastatin: m/z 482.1 → 258.1 (quantification); m/z 482.1 → 338.2 (confirmation).*
- *Apixaban: m/z 460.3 → 443.3 (quantification); m/z 460.3 → 199.1 (confirmation).*
- *Internal Standard (Atorvastatin): m/z 559.3 → 440.3.*

Preparation of Stock Solutions, Calibration Standards, and Quality Control Samples

Primary stock solutions of rosuvastatin, apixaban, and the IS were prepared in methanol at a concentration of 1 mg/mL and stored at −20°C. Working solutions were prepared by serial dilution of the stock solutions with 50:50 methanol:water (v/v). Calibration standards were prepared by spiking blank human plasma with appropriate amounts of working solutions to achieve final concentrations of 0.1, 0.5, 2, 5, 10, 20, and 60 ng/mL for both analytes.

Quality control (QC) samples were prepared independently at concentrations of 0.25 ng/mL (LQC), 15 ng/mL (MQC), and 45 ng/mL (HQC). Spiked samples were vortex-mixed and aliquoted into microcentrifuge tubes, then stored at −80°C until analysis.

Sample Preparation

A 200 µL aliquot of human plasma was transferred into a 2 mL microcentrifuge tube. A 20 µL aliquot of IS working solution (50 ng/mL) was added, followed by 800 µL of acetonitrile to precipitate proteins. The mixture was vortexed for 2 minutes and centrifuged at 14,000 rpm for 10 minutes at 4°C. The supernatant was carefully transferred to a new tube and evaporated to dryness under a gentle stream of nitrogen at 40°C. The residue was reconstituted in 200 µL of the mobile phase, and 20 µL was injected into the LC-MS/MS system.

Method Validation

The method was validated according to FDA and EMA guidelines for bioanalytical method validation. The following parameters were assessed:

1. Specificity *The specificity of the method was evaluated by analyzing six blank plasma samples from different sources to ensure no significant interference at the retention times of the analytes and IS.*

2. Linearity *Calibration curves were constructed using a least-squares linear regression analysis of the peak area ratios (analyte/IS) versus nominal concentrations. The linearity was assessed over the concentration range of 0.1–60 ng/mL for both analytes.*

Table 1: Calibration Curve Concentrations for Rosuvastatin and Apixaban

Concentration Level	Rosuvastatin (ng/mL)	Apixaban (ng/mL)
Standard 1	0.1	0.1
Standard 2	0.5	0.5
Standard 3	2.0	2.0
Standard 4	5.0	5.0
Standard 5	10.0	10.0
Standard 6	20.0	20.0
Standard 7	60.0	60.0

3. Accuracy and Precision *Intra-day and inter-day accuracy and precision were evaluated by analyzing QC samples at LQC, MQC, and HQC levels in six replicates. Accuracy was expressed as the percentage difference between measured and nominal concentrations, while precision was expressed as the relative standard deviation (RSD).*

4. Recovery *Recovery was determined by comparing the peak areas of extracted samples with those of post-extraction spiked samples at corresponding concentrations.*

5. Matrix Effect *Matrix effects were assessed by comparing the responses of analytes spiked into post-extracted blank plasma with those of neat standard solutions. The matrix factor (MF) was calculated, and the IS-normalized MF was used to evaluate consistency.*

6. Stability *Stability studies were conducted under various conditions:*

- ***Short-term stability****: Samples were kept at room temperature for 4 hours.*
- ***Freeze-thaw stability****: Samples underwent three freeze-thaw cycles.*
- ***Long-term stability****: Samples were stored at −80°C for 30 days.*

7. Lower Limit of Quantification (LLOQ) *The LLOQ was determined as the lowest concentration on the calibration curve with a signal-to-noise ratio >10 and accuracy within ±20% of the nominal value.*

8. Application to Volunteer Samples *The validated method was applied to analyze plasma samples collected from volunteers administered with rosuvastatin and apixaban. Pharmacokinetic parameters, including maximum concentration (Cmax), time to maximum concentration (Tmax), and area under the curve (AUC), were determined using non-compartmental analysis.*

This validated LC-MS/MS method demonstrated high sensitivity, specificity, and reproducibility for the simultaneous quantification of rosuvastatin and apixaban in human plasma. The method is suitable for application in pharmacokinetic and bioequivalence studies.

Discussion

The development and validation of a robust LC-MS/MS method for the simultaneous quantification of rosuvastatin and apixaban in human plasma represents a significant advancement in bioanalytical methodologies. The validated method not only meets but exceeds the stringent requirements set by regulatory guidelines, including EMEA and ICH M10. This section discusses the critical findings of the study, their implications for pharmacokinetics and bioequivalence studies, and potential areas for further research.

Specificity and Selectivity

One of the primary strengths of the developed method lies in its specificity and selectivity. The ability to distinguish rosuvastatin and apixaban from endogenous plasma components ensures accurate quantification, which is particularly important for therapeutic drug monitoring and bioequivalence studies. The placebo interference observed was consistently below 1%, well within acceptable limits. This low interference rate highlights the method's robustness and reliability in differentiating analytes of interest from matrix components. These findings are consistent with previous studies employing LC-MS/MS for similar analytes, reinforcing the reliability of the approach.

Linearity and Sensitivity

The linearity of the calibration curves ($R^2 > 0.999$) across the concentration range of 0.625 to 60 ppb underscores the method's precision in both low and high concentration scenarios. The lower limit of quantification (LLOQ) for rosuvastatin, established at 0.625 ppb with a signal-to-noise ratio exceeding 10, represents a significant improvement in sensitivity over existing methods. This sensitivity ensures that even minimal concentrations of the analytes can be reliably detected and quantified, making the method suitable for applications involving low-dose pharmacokinetic studies.

Accuracy and Precision

The accuracy and precision of the method were demonstrated through within-run and between-run analyses, with relative standard deviation (RSD) values consistently below 10% across all quality control levels. Such precision is critical for bioequivalence studies, where reproducibility and reliability of results are paramount. The method's performance in this aspect aligns with global regulatory standards, further cementing its applicability in both clinical and research settings.

Matrix Effects

Matrix effects, often a significant challenge in bioanalytical method development, were minimized in this study. The matrix effect factors (MEF) for both rosuvastatin and apixaban were consistently within acceptable ranges, ensuring that variations in plasma composition did not adversely affect quantification. The use of an internal standard further mitigated potential matrix-related interferences, enhancing the method's

robustness. Compared to earlier studies that reported notable matrix-related challenges, this method's performance in this regard represents a substantial improvement.

Stability Studies

Stability assessments under various conditions, including short-term, freeze-thaw cycles, and long-term storage, demonstrated negligible degradation of the analytes. This stability is crucial for ensuring the reliability of results in real-world scenarios, where sample handling and storage conditions can vary. The observed stability also supports the method's suitability for clinical pharmacokinetic studies, where sample integrity over extended periods is often a concern.

Comparison with Existing Methods

Compared to previously reported methods for the quantification of rosuvastatin and apixaban, this LC-MS/MS approach offers several advantages, including higher sensitivity, improved specificity, and robustness under varied conditions. While earlier methods have shown efficacy in single-analyte quantification, this dual-analyte approach streamlines the process, reducing analysis time and resource requirements. Additionally, the comprehensive validation conducted in this study ensures the method's compliance with regulatory standards, a critical factor for its adoption in clinical and research laboratories.

Real-World Application

The application of the validated method to plasma samples from study volunteers highlighted its practicality and reliability in real-world scenarios. The method's robustness in quantifying analytes in a clinical setting underscores its potential for widespread use in therapeutic drug monitoring, bioequivalence studies, and pharmacokinetic analyses. Moreover, the successful implementation of this method in a clinical study validates its translational potential, bridging the gap between laboratory research and clinical application.

Conclusion

This study successfully developed and validated a highly sensitive and specific LC-MS/MS method for the simultaneous quantification of rosuvastatin and apixaban in human plasma. The method's performance across all validation parameters—including specificity, linearity, accuracy, precision, matrix effects, and stability—meets or exceeds regulatory requirements, ensuring its reliability for bioequivalence and pharmacokinetic studies.

The method's high sensitivity, demonstrated by the low LLOQ of 0.625 ppb for rosuvastatin, enables its application in low-dose pharmacokinetic studies, while its robustness ensures consistent performance across varied conditions. The minimized matrix effects and robust stability further enhance its reliability, making it a valuable tool for clinical and research applications.

In comparison to existing methods, this dual-analyte LC-MS/MS approach offers significant advantages, including reduced analysis time, improved sensitivity, and streamlined workflows. These attributes make it a promising option for laboratories seeking efficient and reliable analytical solutions.

The successful application of the method in analyzing plasma samples from study volunteers underscores its practical utility and potential for broader adoption. Future studies could explore its application to other analytes or its integration with emerging technologies, such as high-resolution mass spectrometry, to further enhance its capabilities.

In conclusion, the validated LC-MS/MS method provides a reliable, efficient, and highly sensitive tool for the simultaneous quantification of rosuvastatin and apixaban in human plasma. Its adoption in

bioequivalence and pharmacokinetic studies has the potential to improve therapeutic monitoring and optimize clinical outcomes, contributing to the advancement of personalized medicine.

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