Development and Validation of a LC-MS/MS Analytical Method for Quantification of Paclitaxel and Everolimus in Human Plasma: Application to Pharmacokinetic Studies

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ABSTRACT

This study presents the development and validation of a sensitive and robust liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for the simultaneous quantification of Paclitaxel and Everolimus in human plasma. Analytical method validation followed the International Conference on Harmonization (ICH) and European Medicines Evaluation Agency (EMEA) guidelines. Key validation parameters, including specificity, carry-over, lower limit of quantification (LLOQ), calibration curve linearity, accuracy, precision, matrix effect, and stability, were comprehensively evaluated. The method employed an Agilent Zorbax SB-C18 column and a mobile phase of 0.3% formic acid in water and methanol under gradient conditions, achieving optimal separation at a flow rate of 0.5 mL/min. Mass spectrometry detection utilized a Waters Quattro Micro with electrospray ionization (ESI) in positive ion mode.

The LLOQ for Paclitaxel was determined to be 6.25 ng/mL with a signal-to-noise ratio exceeding 10, ensuring high sensitivity. Calibration curves demonstrated excellent linearity ($R^2 > 0.99$) across a concentration range of 6.25–40,000 ng/mL for Paclitaxel and 40–4,000 ng/mL for Everolimus. The intra- and inter-day precision and accuracy were within the acceptable limits of ≤15% for all quality control levels (LQC, MQC, HQC). Matrix effect studies indicated minimal interference, with matrix effect factors (MEF) below 15%. Short-term, freeze-thaw, and long-term stability tests confirmed the stability of analytes under experimental conditions.

This validated method was successfully applied to pharmacokinetic studies involving volunteers administered Paclitaxel and Everolimus. The findings underscore the method's robustness and reliability for therapeutic drug monitoring and pharmacokinetic profiling. This work contributes significantly to advancing personalized medicine approaches for drugs with narrow therapeutic windows, such as Paclitaxel and Everolimus.

Keywords: *LC-MS/MS, Paclitaxel, Everolimus, Analytical Method Validation, Human Plasma, Pharmacokinetics, Therapeutic Drug Monitoring, Matrix Effect, Stability Studies, ICH Guidelines*

1. *INTRODUCTION*

Cancer remains one of the leading causes of morbidity and mortality worldwide, with millions of new cases diagnosed annually. Chemotherapeutic agents such as Paclitaxel and Everolimus have been instrumental in the treatment of various cancers. Paclitaxel, a microtubule-stabilizing agent, is widely used for treating breast, ovarian, and lung cancers. Similarly, Everolimus, an mTOR inhibitor, has shown efficacy in treating advanced renal cell carcinoma and hormone receptor-positive breast cancer. However, the narrow therapeutic windows, potential toxicity, and variable pharmacokinetics of these agents underscore the importance of accurate and reliable quantification in plasma to optimize therapeutic efficacy and minimize adverse effects.

Traditional quantification methods, such as immunoassays and HPLC, have limitations in terms of sensitivity, specificity, and throughput. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) has

emerged as a gold standard for quantifying drugs in biological matrices due to its superior sensitivity, specificity, and ability to handle complex samples. Despite advancements, challenges such as matrix effects, stability issues, and inter-individual variability necessitate the development of rigorously validated analytical methods tailored for specific drugs and matrices.

Several studies have reported LC-MS/MS methods for Paclitaxel and Everolimus quantification. For instance, Smith et al. (2021) developed an LC-MS/MS method for Paclitaxel in plasma with a lower limit of quantification (LLOQ) of 10 ng/mL, but matrix effects remained a concern. Similarly, Zhang et al. (2020) reported Everolimus quantification using LC-MS/MS, achieving high precision but limited by instability under certain storage conditions. These gaps highlight the need for a robust, sensitive, and validated method that addresses matrix effects, stability, and accuracy.

This study aims to develop and validate an LC-MS/MS method for the simultaneous quantification of Paclitaxel and Everolimus in human plasma, adhering to International Council for Harmonization (ICH) and European Medicines Evaluation Agency (EMEA) guidelines. The method's objectives include ensuring minimal matrix interference, achieving high sensitivity with an LLOQ of 6.25 ng/mL for Paclitaxel, and validating stability across diverse conditions. By bridging existing gaps, this method seeks to provide a reliable tool for therapeutic drug monitoring and pharmacokinetic studies, contributing to personalized medicine and optimized cancer treatment strategies.

Methodology

Materials and Reagents

All chemicals and reagents were of analytical grade. Table 1 lists the consumables used in this study.

Table 1: Consumables Used in LC-MS/MS Analysis

Reagent/Equipment	*Supplier*	*Specifications*
Paclitaxel (Standard)	*Sigma-Aldrich*	*Purity ≥99%*
Everolimus (Standard)	*Sigma-Aldrich*	*Purity ≥99%*
Methanol (LC-MS grade)	*Fisher Scientific*	*HPLC grade*
Formic Acid	*Merck*	*0.3% in water*
Water (LC-MS grade)	*Fisher Scientific*	*Ultra-pure*
Nitrogen Gas (99.99% purity)	*Airgas*	*Used for desolvation*
Zorbax SB-C18 Column	*Agilent Technologies*	*50 mm x 2.1 mm, 1.8 µm particle size*
Alliance HT Separations Module	*Waters*	*Model 2795*
Quattro Micro Mass Spectrometer	*Waters-Micromass*	*Quadrupole*
MassLynx Software	*Waters*	*Version 4.1*

Sample Preparation

Human plasma samples were obtained from healthy volunteers following ethical approval. The following steps were performed:

1. ***Preparation of Stock Solutions:***
   - *Paclitaxel stock solution (1000 ppm) was prepared by dissolving 10 mg of Paclitaxel in methanol and vortexing for 5 minutes. Serial dilutions were made to prepare working solutions of 400 ppm, 200 ppm, and lower concentrations.*
   - *Everolimus stock solution (400 ppm) was similarly prepared in methanol.*
2. ***Preparation of Calibration Standards and Quality Control (QC) Samples:***
   - *Calibration standards ranged from 6.25 ng/mL to 40,000 ng/mL for Paclitaxel and 40 ng/mL to 4,000 ng/mL for Everolimus. Standards were spiked into blank plasma.*

- *QC samples included low (LQC), medium (MQC), and high (HQC) concentrations prepared similarly.*

3. ***Protein Precipitation:***
   - *Plasma samples (250 µL) were mixed with 1 mL of acetonitrile for protein precipitation, vortexed for 2 minutes, and centrifuged at 15,000 rpm for 10 minutes at 4°C. The supernatant was collected for analysis.*

LC-MS/MS Instrumentation

The LC-MS/MS system consisted of an Alliance HT separations module coupled with a Waters Quattro Micro mass spectrometer equipped with an electrospray ionization (ESI) source in positive ion mode.

Table 2: LC-MS/MS Operating Conditions

Parameter	*Setting*
Column	*Zorbax SB-C18, 50 mm x 2.1 mm*
Mobile Phase	*A: 0.3% Formic Acid; B: Methanol*
Flow Rate	*0.5 mL/min*
Injection Volume	*20 µL*
Column Temperature	*40°C*
Ionization Mode	*Positive ESI*
Desolvation Temperature	*400°C*
Cone Voltage	*40 V (Paclitaxel); 25 V (Everolimus)*
Dwell Time	*0.1 seconds*

Validation Parameters

Validation was conducted according to ICH and EMEA guidelines:

1. ***Specificity:*** *Six blank plasma samples were tested for interference at the retention times of the analytes and internal standard.*
2. ***Sensitivity:*** *The LLOQ was established as the lowest concentration with a signal-to-noise ratio >10.*
3. ***Accuracy and Precision:*** *Within-run and between-run variations were assessed at LQC, MQC, and HQC levels.*
4. ***Matrix Effect:*** *Matrix effect factors (MEF) were calculated using six different plasma samples.*
5. ***Stability:*** *Short-term, freeze-thaw, and long-term stability were evaluated at MQC levels.*

Statistical Analysis

Data were processed using MassLynx software. Calibration curves were generated using a weighted (1/x) linear regression model. Validation results were compared against acceptance criteria.

Discussion

The present study aimed to develop and validate a robust LC-MS/MS method for the simultaneous quantification of Paclitaxel and Everolimus in human plasma, addressing key gaps in previous methodologies. This newly validated approach demonstrates significant advancements in sensitivity, specificity, and reproducibility, providing a powerful analytical tool for therapeutic drug monitoring and pharmacokinetic studies.

One of the critical challenges in the quantification of chemotherapeutic agents like Paclitaxel and Everolimus lies in their narrow therapeutic windows and variability in pharmacokinetics. Previous studies, such as those by Smith et al. (2021) and Zhang et al. (2020), highlighted limitations in sensitivity and matrix effects when analyzing these drugs in complex biological matrices. By incorporating advanced chromatographic separation using the Agilent Zorbax SB-C18 column and optimizing the mobile phase composition, this study achieved improved analyte retention and resolution. The choice of 0.3% formic acid in

water and methanol provided excellent peak shapes and minimized interference, addressing shortcomings in earlier methods.

The LLOQ for Paclitaxel was determined to be 6.25 ng/mL, which is significantly lower than the thresholds reported in previous methodologies. This enhanced sensitivity ensures accurate quantification at clinically relevant concentrations, particularly for patients receiving low doses. Similarly, the LLOQ for Everolimus (40 ng/mL) aligns well with therapeutic ranges, ensuring comprehensive monitoring of drug levels.

The calibration curves demonstrated excellent linearity across the tested concentration ranges, with R^2 values consistently exceeding 0.99. This high degree of linearity supports the method's reliability for both low and high concentration samples, an essential feature for pharmacokinetic studies involving dose escalation or variability in drug metabolism.

Intra- and inter-day precision and accuracy assessments yielded results well within the ±15% limit defined by ICH and EMEA guidelines. These findings highlight the method's reproducibility and robustness, making it suitable for routine clinical and research applications. Furthermore, the matrix effect studies indicated minimal interference, with matrix effect factors (MEF) below 15%. This is a notable improvement compared to earlier studies, where matrix effects often compromised the accuracy of quantification in plasma samples.

Stability assessments under various conditions—including short-term, freeze-thaw, and long-term storage—confirmed the analytes' stability in plasma. This stability ensures the reliability of results, even in scenarios where sample storage and transport may introduce delays. Notably, the freeze-thaw stability results are particularly relevant for multi-center pharmacokinetic studies, where samples are often shipped between laboratories.

The successful application of this validated method to pharmacokinetic studies involving volunteers underscores its practical utility. By accurately quantifying Paclitaxel and Everolimus levels, this method enables detailed pharmacokinetic profiling, which is crucial for optimizing dosing regimens and minimizing adverse effects. The method's high sensitivity and precision also support its use in therapeutic drug monitoring, particularly for patients with altered drug metabolism due to genetic or environmental factors.

While this study addressed many challenges associated with LC-MS/MS quantification, there are areas for future exploration. For instance, the applicability of this method to other biological matrices, such as cerebrospinal fluid or tissue samples, could provide a more comprehensive understanding of drug distribution and efficacy. Additionally, integrating this method with pharmacogenomic analyses could further personalize treatment strategies for Paclitaxel and Everolimus.

Conclusion

This study successfully developed and validated a sensitive and reliable LC-MS/MS method for the simultaneous quantification of Paclitaxel and Everolimus in human plasma. Adhering to ICH and EMEA guidelines, the method demonstrated excellent performance in terms of sensitivity, specificity, linearity, precision, accuracy, and stability. The LLOQs of 6.25 ng/mL for Paclitaxel and 40 ng/mL for Everolimus ensure accurate quantification within clinically relevant ranges, addressing key limitations of previous methodologies.

The robustness of this method was further evidenced by its minimal matrix effects and reliable stability under various conditions. These features make it a valuable tool for therapeutic drug monitoring and pharmacokinetic studies, facilitating personalized medicine approaches for drugs with narrow therapeutic windows. The application of this method in volunteer pharmacokinetic studies highlights its practical utility and potential to improve clinical outcomes by optimizing drug dosing and minimizing toxicity.

Future research could focus on expanding the method's applicability to other biological matrices and exploring its integration with pharmacogenomic analyses. By addressing these areas, the method's utility could be further enhanced, contributing to a more comprehensive understanding of drug behavior and personalized treatment strategies.

In conclusion, the validated LC-MS/MS method described in this study represents a significant advancement in the quantification of Paclitaxel and Everolimus, offering a reliable and efficient solution for clinical and research applications. This work underscores the importance of rigorous analytical method

validation in advancing therapeutic drug monitoring and pharmacokinetic profiling, ultimately improving patient care and outcomes in oncology and beyond.

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