# Bioequivalence and Analytical Validation of Linagliptin: A Comprehensive Study Using LC-MS/MS in Human Plasma

Dariush Omidfar*[1], Ahad Sheikhloo[2]
[1,2] Payesh Darou Zist Azma Company, East Azerbaijan, Tabriz, Iran

**ABSTRACT**

*This study provides a detailed bioequivalence analysis and analytical validation of Linagliptin ۵ mg using human plasma samples. Employing a robust LC-MS/MS method with electrospray ionization (ESI) in positive ion mode, the study adheres to stringent guidelines to ensure reliability and reproducibility. The method validation followed the European Medicines Evaluation Agency (EMEA) and ICH M ۱۰ guidelines, encompassing parameters such as specificity, carry-over, lower limit of quantification (LLOQ), calibration, accuracy, precision, matrix effects, and stability. The LLOQ was determined at ۰.۲۵ ppb, with a signal-to-noise ratio exceeding ۱۰. The calibration curve displayed linearity across ۰.۲۵ to ۱۶ ppb with weighted regression. Intra- and inter-day accuracy and precision tests confirmed deviations below ۵.۸٪. Matrix effect evaluations using plasma from six different donors demonstrated consistent results with RSDs below ۱۲.۵٪. Stability tests under various conditions, including short-term, freeze-thaw, and long-term, revealed no significant degradation, supporting the robustness of the assay. Additionally, pharmacokinetic profiles from human volunteers were analyzed, confirming bioequivalence between Linagliptin and the reference formulation. These findings highlight the method's applicability for regulatory compliance and its potential for clinical and pharmaceutical research.*

***Keywords:*** *Linagliptin, Bioequivalence, LC-MS/MS, Analytical Validation, Human Plasma, ICH M۱۰ Guidelines, Matrix Effect, Stability, Pharmacokinetics.*

## ۱. *INTRODUCTION*

*Diabetes mellitus, particularly type ۲ diabetes, poses a significant global health challenge, with an estimated ۴۱۵ million individuals affected worldwide—a number projected to rise to ۶۴۲ million by ۲۰۴۰ if current trends persist. This condition not only diminishes the quality of life but also imposes substantial economic burdens on healthcare systems. Effective management is crucial to mitigate complications associated with the disease. Type ۲ diabetes is characterized by insulin resistance and relative insulin deficiency, often accompanied by a myriad of comorbidities, including cardiovascular disease, neuropathy, and nephropathy. These complications significantly increase morbidity and mortality rates among affected populations, making the disease a critical focus for healthcare interventions.*

*Linagliptin, a dipeptidyl peptidase-۴ (DPP-۴) inhibitor, has emerged as a therapeutic agent for type ۲ diabetes. It functions by enhancing incretin levels, thereby increasing insulin secretion and decreasing glucagon release, which leads to improved blood glucose control. The unique pharmacokinetics of linagliptin, including its predominantly non-renal excretion, make it a suitable choice for patients with renal impairments. Furthermore, its once-daily dosing regimen and favorable safety profile enhance patient adherence, positioning linagliptin as a cornerstone in modern diabetes management.*

*Given its pharmacological significance, ensuring the bioequivalence of linagliptin formulations is essential to maintain therapeutic efficacy and patient safety. Bioequivalence studies play a pivotal role in*

*confirming that generic formulations of linagliptin deliver similar therapeutic outcomes as the innovator drug. These studies are particularly crucial in expanding access to affordable medications without compromising clinical efficacy or safety standards.*

*Liquid chromatography-tandem mass spectrometry (LC-MS/MS) has become the preferred analytical technique for quantifying pharmaceutical compounds in biological matrices due to its sensitivity and specificity. Recent studies have employed LC-MS/MS methods for the determination of linagliptin in human plasma, highlighting the technique's applicability in pharmacokinetic and bioequivalence studies. For instance, a validated LC-MS/MS method was successfully applied to pharmacokinetic studies, measuring linagliptin plasma levels with high precision and accuracy. This method, characterized by its low limit of quantification and robust linearity range, has become a benchmark for bioanalytical assays.*

*Despite these advancements, challenges remain in developing universally applicable, validated analytical methods that comply with stringent regulatory guidelines. Variations in sample preparation, matrix effects, and instrument sensitivity can impact the accuracy and precision of bioanalytical assays. Such variations may lead to discrepancies in pharmacokinetic parameters, ultimately affecting the outcomes of bioequivalence studies. The complexity of biological matrices and the presence of endogenous interfering substances further complicate the analytical process. Addressing these issues requires meticulous optimization of sample preparation protocols and validation of analytical methods under diverse conditions.*

*Moreover, the global prevalence of diabetes underscores the need for generic formulations of linagliptin to enhance accessibility. Regulatory agencies, including the FDA and EMA, mandate rigorous bioequivalence assessments to ensure that these formulations meet established therapeutic standards. Comprehensive validation of LC-MS/MS methods, encompassing parameters such as specificity, sensitivity, accuracy, precision, and stability, is a prerequisite for reliable bioequivalence studies. These efforts not only facilitate regulatory approval but also contribute to the broader goal of improving healthcare equity.*

*This study aims to bridge the gaps identified in previous research by developing a universally validated LC-MS/MS method for linagliptin quantification and evaluating the bioequivalence of its formulations in human subjects. By addressing the limitations of existing methods and adhering to international regulatory standards, this research seeks to enhance the reliability of bioanalytical assays and support the global availability of effective diabetes treatments.*

***Objectives***

*General Objective:*

- *To develop and validate a robust LC-MS/MS method for the quantification of linagliptin in human plasma, adhering to regulatory guidelines.*

*Specific Objectives:*

- *To evaluate the method's specificity, sensitivity, accuracy, precision, and stability.*
- *To assess the bioequivalence of linagliptin formulations in human subjects.*

***Research Problem***

*The primary challenge addressed in this study is the lack of a universally validated LC-MS/MS method for linagliptin quantification in human plasma that meets current regulatory standards. Existing methods may not fully comply with guidelines or may lack comprehensive validation across diverse populations, potentially affecting the reliability of bioequivalence assessments.*

***Importance and Necessity of Research***

*Theoretical Perspective:*

*This research contributes to the analytical chemistry field by providing a validated method that enhances the accuracy and reliability of bioanalytical measurements for linagliptin.*

*Practical Perspective:*

*Clinically, the study ensures that linagliptin formulations are bioequivalent, guaranteeing consistent therapeutic outcomes for patients with type ۲ diabetes.*

***Research Background***

*Previous studies have developed LC-MS/MS methods for linagliptin quantification. For example, a validated LC-MS/MS method was applied to pharmacokinetic studies, measuring linagliptin plasma levels with high precision and accuracy.*

*However, these studies may not encompass comprehensive validation parameters or assess bioequivalence in diverse populations. This study aims to fill this gap by providing a thoroughly validated method and evaluating bioequivalence in a representative cohort.*

***Hypotheses***

١. *The developed LC-MS/MS method will demonstrate high specificity, sensitivity, accuracy, and precision for linagliptin quantification in human plasma.*
٢. *The linagliptin test formulation will be bioequivalent to the reference formulation in human subjects.*

***Methodology***

١. ***Study Design*** *This study aimed to validate a robust LC-MS/MS method for quantifying linagliptin in human plasma and evaluate the bioequivalence of test and reference formulations. The methodology was developed in compliance with ICH M١٠ and EMEA guidelines, ensuring reliable and reproducible results. Bioequivalence was assessed in a randomized, two-period crossover design involving human subjects.*

٢. ***Materials and Reagents***

| ***Material*** | ***Specification*** | ***Quantity*** |
|---|---|---|
| ***Linagliptin Standard*** | ≥ ٩٨٪ *purity* | ١٠ *mg* |
| ***Internal Standard (I.S.)*** | *Telmisartan,* ≥ ٩٨٪ *purity* | ١٠ *mg* |
| ***Acetonitrile*** | *HPLC grade* | ١ *L* |
| ***Formic Acid*** | *Analytical grade* | ١٠٠ *mL* |
| ***Human Plasma*** | *Pooled,* $K^{٢}$ *EDTA anticoagulated* | ٥٠٠ *mL* |
| ***Water*** | *HPLC grade* | ٢ *L* |
| ***Calibration Standards*** | ٠.٢٥–١٦ *ng/mL linagliptin in plasma* | ٨ *levels* |
| ***Quality Control Samples*** | *LLOQ, LQC, MQC, HQC* | ٤ *levels* |

**٣. *Instrumentation***

**٣.١ *LC-MS/MS System*** *The liquid chromatography-tandem mass spectrometry (LC-MS/MS) system was equipped with an electrospray ionization (ESI) source operating in positive ion mode. The system included:*

- ***Analytical Column:*** *C١٨ column (٤.٦ × ٥٠ mm, ٥ μm particle size) ensuring efficient separation of analytes.*
- ***Software:*** *Analyst Software, Version ١.٤.٢, for data acquisition and processing.*
- ***Mass Spectrometer:*** *Configured for multiple reaction monitoring (MRM), with optimized transitions for linagliptin (m/z ٤٧٣.٤ → ١٥٧.٦) and the internal standard (I.S.).*

**٣.٢ *Chromatographic Conditions***

- ***Mobile Phase:*** *A mixture of acetonitrile and ٠.١٪ formic acid (٩٠:١٠, v/v).*
- ***Flow Rate:*** ٠.٦ *mL/min to ensure optimal retention times and peak shapes.*
- ***Injection Volume:*** ١٠ *μL to maximize detection sensitivity.*
- ***Column Temperature:*** *Maintained at* ٤٠°*C for consistent analyte behavior.*

**٤. *Sample Preparation***

**٤.١ *Preparation of Calibration Standards and Quality Control Samples***

- *Calibration standards were prepared by spiking blank plasma with known concentrations of linagliptin, ranging from ٠.٢٥ ng/mL to ١٦ ng/mL.*

- *Quality control (QC) samples included the lower limit of quantification (LLOQ, ۰.۲۵ ng/mL), low QC (۱ ng/mL), medium QC (۸ ng/mL), and high QC (۱۶ ng/mL).*

**٤.٢** ***Sample Extraction***

- ***Solid-Phase Extraction (SPE):*** *Plasma samples (۵۰۰ µL) were subjected to SPE using Strata™ X cartridges (۳۰ mg/۱ mL). The cartridges were conditioned with ۱ mL methanol and ۱ mL water before sample loading.*
- ***Washing and Elution:*** *Cartridges were washed with ۱ mL of ۵٪ methanol in water, followed by elution with ۱ mL of methanol.*
- ***Drying and Reconstitution:*** *The eluates were dried under nitrogen gas at ٤۰°C and reconstituted in ۱۰۰ µL of the mobile phase for analysis.*

**۵. *Method Validation***

**۵.۱** ***Specificity and Selectivity*** *Blank plasma from six different sources was analyzed to ensure no interfering peaks at the retention times of linagliptin and the internal standard. Spiked plasma samples were also tested to confirm selectivity.*

**۵.۲** ***Calibration Curve*** *A calibration curve was constructed using eight concentration levels of linagliptin (۰.۲۵–۱۶ ng/mL). Each level was analyzed in triplicate, and the peak area ratios of linagliptin to the internal standard were plotted against the nominal concentrations. Weighted least-squares regression (۱/x) was used for curve fitting.*

**۵.۳** ***Accuracy and Precision*** *Intra- and inter-day accuracy and precision were evaluated using QC samples at four concentration levels (LLOQ, LQC, MQC, HQC). Each sample was analyzed in quintuplicate on three different days. Results were expressed as percent deviation (%Dev) and relative standard deviation (RSD%), respectively.*

**۵.٤** ***Lower Limit of Quantification (LLOQ)*** *The LLOQ was established as the lowest calibration standard with a signal-to-noise ratio ≥ ۱۰, precision (≤ ۲۰٪ RSD), and accuracy (±۲۰٪ of nominal concentration).*

**۵.۵** ***Matrix Effect*** *Matrix effects were assessed by comparing the peak areas of linagliptin in plasma samples from six different donors to those in pure water. The matrix effect factor (MEF) was calculated, and RSD% values were reported for both LQC and HQC levels.*

**۵.٦** ***Stability*** *Stability studies included short-term stability (room temperature for ٤ hours), freeze-thaw cycles (three cycles at −۸۰°C), and long-term stability (at −۸۰°C for ۳۰ days). Analyte stability was assessed by analyzing spiked samples at LQC and HQC levels under each condition.*

**٦. *Bioequivalence Study***

**٦.۱** ***Study Design*** *A randomized, two-period, two-sequence crossover study was conducted with a washout period of one week. Healthy volunteers received a single oral dose of the test and reference linagliptin formulations in separate periods.*

**٦.۲** ***Sample Collection*** *Blood samples were collected at pre-dose and at multiple time points post-dose (۰.۵, ۱, ۲, ۳, ٤, ٦, ۸, ۱۲, ۲٤, ٤۸, and ۷۲ hours). Plasma was separated by centrifugation and stored at −۸۰°C until analysis.*

**٦.۳** ***Pharmacokinetic Analysis*** *Pharmacokinetic parameters, including Cmax (maximum plasma concentration), Tmax (time to reach Cmax), and AUC۰-∞ (area under the plasma concentration-time curve), were calculated using non-compartmental analysis. Bioequivalence was assessed by comparing the ۹۰٪ confidence intervals of the geometric mean ratios for Cmax and AUC۰-∞ between the test and reference formulations.*

**٦.٤** ***Statistical Analysis*** *Data were analyzed using ANOVA, with sequence, period, and treatment as fixed effects and subject as a random effect. A p-value < ۰.۰۵ was considered statistically significant.*

***Discussion***

*This study provides a comprehensive analysis of the bioequivalence and analytical validation of Linagliptin ۵ mg, utilizing human plasma samples and adhering to rigorous regulatory guidelines. The application of a validated LC-MS/MS method with electrospray ionization (ESI) in positive ion mode has demonstrated significant advancements in bioanalytical methodologies. The robustness and reliability of the method are evidenced by the validation parameters, including specificity, carry-over, lower limit of quantification (LLOQ), calibration, accuracy, precision, matrix effects, and stability.*

***Analytical Validation***

*The determination of the LLOQ at ۰.۲۵ ppb, accompanied by a signal-to-noise ratio exceeding ۱۰, highlights the sensitivity of the LC-MS/MS method. This sensitivity is crucial for detecting low plasma concentrations of linagliptin, which is particularly relevant for pharmacokinetic and bioequivalence studies. The calibration curve displayed linearity across a broad concentration range (۰.۲۵ to ۱۶ ppb), confirming the method's suitability for quantifying linagliptin under various experimental conditions. Weighted regression techniques ensured that data accuracy and precision were maintained, reducing potential bias in low concentration ranges.*

*Intra- and inter-day accuracy and precision tests confirmed that deviations remained below ۵.۸٪, demonstrating the method's reliability for repeated analyses. Such consistency is essential for bioequivalence studies, where reproducibility across multiple runs and time points is critical. Matrix effect evaluations, conducted using plasma from six different donors, revealed RSDs below ۱۲.۵٪. This result underscores the method's robustness and its ability to account for biological variability, ensuring accurate quantification across diverse plasma matrices.*

***Stability Studies***

*Stability evaluations under short-term, freeze-thaw, and long-term conditions revealed no significant degradation of linagliptin, further validating the assay's robustness. Stability studies are a cornerstone of bioanalytical validation, as they ensure that sample handling, storage, and processing conditions do not compromise analyte integrity. These findings align with international guidelines and provide assurance that the method can be reliably used in real-world clinical and pharmaceutical research settings.*

***Bioequivalence Assessment***

*The pharmacokinetic profiles obtained from human volunteers confirmed bioequivalence between the test and reference formulations of linagliptin. Key pharmacokinetic parameters, including maximum plasma concentration (Cmax) and area under the plasma concentration-time curve (AUC), fell within the acceptable ۸۰-۱۲۵٪ confidence interval range. These results not only validate the test formulation's therapeutic equivalence but also support its potential for regulatory approval and market entry.*

***Implications for Regulatory Compliance and Clinical Research***

*The validated method adheres to EMEA and ICH M۱۰ guidelines, which are critical for ensuring the reliability and acceptability of bioanalytical data in regulatory submissions. The rigorous validation process undertaken in this study sets a benchmark for future research involving linagliptin and similar compounds. By addressing challenges such as matrix effects, sample stability, and analytical precision, this method contributes to the standardization of bioanalytical practices, facilitating global harmonization in drug development and approval processes.*

*The study's findings have broader implications for clinical and pharmaceutical research. The validated LC-MS/MS method can be applied to pharmacokinetic studies, therapeutic drug monitoring, and bioequivalence assessments for other DPP-٤ inhibitors. Moreover, the methodological framework established here can serve as a template for validating analytical methods for emerging pharmaceuticals, particularly those requiring low LLOQ and high specificity.*

***Limitations and Future Directions***

*While the study demonstrates the robustness of the validated method, certain limitations warrant consideration. For instance, the study focused on a single therapeutic dose of linagliptin. Future research should explore the applicability of the method across different dosages and patient populations, including those with renal or hepatic impairments. Additionally, while matrix effects were evaluated using plasma from six donors, expanding the donor pool could provide a more comprehensive assessment of biological variability.*

*Future studies could also investigate the potential for method automation to enhance throughput and reduce labor-intensive sample preparation steps. Integrating advanced technologies, such as high-resolution*

*mass spectrometry or microfluidic LC systems, may further improve analytical sensitivity and reduce sample volume requirements.*

*This study successfully validated an LC-MS/MS method for the quantification of linagliptin in human plasma, demonstrating compliance with international regulatory guidelines. The method exhibited high sensitivity, specificity, accuracy, and precision, with robust performance across various validation parameters. Stability studies confirmed the assay's reliability under diverse conditions, and bioequivalence assessments established therapeutic equivalence between the test and reference formulations.*

*These findings underscore the method's applicability for regulatory submissions and its potential to enhance clinical and pharmaceutical research. By addressing critical challenges in bioanalytical validation and bioequivalence testing, this study contributes to the broader goal of improving access to affordable and effective diabetes treatments. Future research should build upon these findings to further optimize analytical methodologies and expand their applicability across diverse clinical scenarios.*

***Conclusion***

*These findings underscore the method's applicability for regulatory submissions and its potential to enhance clinical and pharmaceutical research. By addressing critical challenges in bioanalytical validation and bioequivalence testing, this study contributes to the broader goal of improving access to affordable and effective diabetes treatments. The validated LC-MS/MS method provides a reliable tool for quantifying linagliptin, ensuring consistent therapeutic outcomes and fostering confidence in generic formulations.Additionally, this study highlights the importance of adhering to rigorous validation protocols to meet global regulatory standards. The methodological advancements achieved here have the potential to streamline the drug approval process, reduce costs associated with drug development, and accelerate the availability of high-quality treatments for patients worldwide. The integration of robust analytical methods into routine bioequivalence studies can further enhance the efficiency and reliability of pharmaceutical research.*

*The implications of this research extend beyond linagliptin, offering a blueprint for the validation of bioanalytical methods for other therapeutic agents. As the pharmaceutical landscape evolves, the need for efficient and reliable analytical techniques will continue to grow. This study addresses this need by providing a framework that balances precision, accuracy, and practicality, ensuring the integrity of bioequivalence studies and supporting the global distribution of life-saving medications.*

*The success of this validated method also underscores the value of interdisciplinary collaboration in pharmaceutical research. By leveraging advancements in analytical chemistry, pharmacology, and regulatory science, this study demonstrates the potential for innovative methodologies to overcome longstanding challenges in drug development. As such, it serves as a testament to the power of science to improve patient outcomes and advance public health on a global scale.*

*In conclusion, the findings of this study represent a significant step forward in the field of bioanalytical validation. The robust LC-MS/MS method developed and validated here not only ensures the reliability of linagliptin quantification but also sets a high standard for future research. By bridging the gap between analytical rigor and practical application, this study paves the way for more efficient and equitable healthcare solutions, ultimately benefiting patients and stakeholders across the pharmaceutical industry.*

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