Bioequivalence Analysis of Empagliflozin and Linagliptin in Human Plasma Using LC-MS/MS: Validation and Stability Studies

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ABSTRACT

This study presents a comprehensive bioequivalence analysis of Empagliflozin and Linagliptin (۱۰/۵ mg) in human plasma, utilizing a validated liquid chromatography-tandem mass spectrometry (LC-MS/MS) methodology. The method was validated according to EMEA and ICH M۱۰ guidelines to ensure specificity, precision, accuracy, matrix effect evaluation, and stability under various conditions. Calibration curves demonstrated excellent linearity over the concentration range of ۰.۵–۳۲۰ ng/mL for both compounds. The method's lower limit of quantification (LLOQ) was established at ۰.۵ ng/mL with a signal-to-noise ratio exceeding ۱۰, confirming the sensitivity of the assay.

Precision studies yielded within-run and between-run %RSD values within the acceptable limits, indicating high repeatability and reliability of the measurements. Matrix effects, assessed across six plasma sources, showed minimal interference, ensuring robustness of the results. Short-term and freeze-thaw stability assessments revealed deviations below ±۱۰٪, underscoring the suitability of the method for bioanalytical applications.

Plasma samples collected from healthy volunteers were analyzed to determine pharmacokinetic parameters, supporting the bioequivalence of test and reference formulations. This validated method enables accurate quantification of Empagliflozin and Linagliptin in clinical studies and reinforces its utility for therapeutic drug monitoring and bioequivalence assessments.

***Keywords*: Empagliflozin, Linagliptin, LC-MS/MS, Bioequivalence**

۱. *INTRODUCTION*

Type ۲ diabetes mellitus (T۲DM) is a chronic metabolic disorder characterized by insulin resistance and impaired insulin secretion, leading to persistent hyperglycemia. Effective management of T۲DM often necessitates combination pharmacotherapy to achieve optimal glycemic control. Empagliflozin, a sodium-glucose co-transporter ۲ (SGLT۲) inhibitor, and linagliptin, a dipeptidyl peptidase-٤ (DPP-٤) inhibitor, are commonly co-administered to enhance therapeutic outcomes.

Fixed-dose combinations (FDCs) of empagliflozin and linagliptin offer the advantages of reduced pill burden and improved patient adherence. However, the pharmacokinetic profiles of these FDCs must be rigorously evaluated to ensure bioequivalence with individual formulations. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is the preferred analytical technique for such assessments due to its high sensitivity and specificity.

Recent studies have developed and validated LC-MS/MS methods for the simultaneous quantification of empagliflozin and linagliptin in human plasma. For instance, Said et al. (۲۰۲٤) reported a method with linearity ranges of ۱.۵۰۰–۵۰۰.۰۰۰ ng/mL for empagliflozin and ۰.۰۵۰–۷.۰۰۰ ng/mL for linagliptin, demonstrating suitability for pharmacokinetic and bioequivalence studies.

Similarly, another study in ۲۰۲۳ employed a rapid and sensitive LC-MS/MS technique for the simultaneous determination of these drugs, highlighting the method's applicability in bioequivalence assessments.

Despite these advancements, challenges remain in ensuring the robustness of bioanalytical methods, particularly concerning matrix effects and analyte stability under various conditions. Addressing these issues is crucial for the accurate quantification of empagliflozin and linagliptin in clinical studies.

Objectives

General Objective:

- *To develop and validate a robust LC-MS/MS method for the simultaneous quantification of empagliflozin and linagliptin in human plasma, ensuring its suitability for bioequivalence and pharmacokinetic studies.*

Specific Objectives:

- *To assess the method's linearity, precision, accuracy, and sensitivity.*
- *To evaluate matrix effects and analyte stability under various storage and handling conditions.*
- *To apply the validated method in a bioequivalence study comparing an empagliflozin/linagliptin FDC with individual formulations.*

Research Problem

While existing LC-MS/MS methods for empagliflozin and linagliptin quantification have shown promise, there is a need for further validation to address potential matrix effects and stability concerns. Ensuring the robustness of these methods is essential for their application in bioequivalence studies, which are critical for the approval of FDCs.

Importance and Necessity of Research

Theoretical Perspective:

This research contributes to the analytical chemistry field by enhancing the reliability of LC-MS/MS methods for simultaneous drug quantification. Addressing matrix effects and stability issues will provide a more comprehensive understanding of the method's robustness.

Practical Perspective:

The validated method will facilitate bioequivalence studies necessary for regulatory approval of empagliflozin/linagliptin FDCs, ultimately aiding in the development of effective combination therapies for T۲DM patients.

Research Background

Previous studies have established LC-MS/MS methods for the simultaneous determination of empagliflozin and linagliptin in plasma. For example, Said et al. (۲۰۲٤) developed a method with high precision and accuracy, suitable for pharmacokinetic studies.

Another study in ۲۰۲۳ reported a rapid and sensitive LC-MS/MS technique for these analytes, emphasizing its applicability in bioequivalence assessments.

However, comprehensive evaluations of matrix effects and analyte stability remain limited, necessitating further research to ensure method robustness.

Hypotheses

- *The developed LC-MS/MS method will exhibit high precision, accuracy, and sensitivity for the simultaneous quantification of empagliflozin and linagliptin in human plasma.*
- *Matrix effects and analyte instability will not significantly impact the method's performance.*
- *The method will be suitable for application in bioequivalence studies comparing FDCs with individual drug formulations.*

Methodology

This section provides a comprehensive description of the materials, instrumentation, and procedures employed in the development and validation of the LC-MS/MS method for the simultaneous quantification of empagliflozin and linagliptin in human plasma. The methodology adheres to the guidelines set forth by the

European Medicines Agency (EMA) and the International Council for Harmonisation (ICH) M١٠ for bioanalytical method validation.

Materials and Reagents

The materials and reagents utilized in this study are detailed in Table ١.

Table ١: Materials and Reagents

Material	Quantity	Source
Empagliflozin standard	١٠٠ mg	Sigma-Aldrich
Linagliptin standard	١٠٠ mg	Sigma-Aldrich
Human plasma	٥٠٠ mL	BioreclamationIVT
Acetonitrile (HPLC grade)	٢ L	Fisher Scientific
Methanol (HPLC grade)	٢ L	Fisher Scientific
Ammonium acetate	٥٠٠ g	Sigma-Aldrich
Formic acid	١٠٠ mL	Sigma-Aldrich
Water (HPLC grade)	٢ L	Fisher Scientific
Internal standards (deuterated)	٥٠ mg	Toronto Research

Instrumentation

- ***Liquid Chromatography System:*** *Waters ACQUITY UPLC system equipped with a binary solvent manager, sample manager, and column oven.*
- ***Mass Spectrometry:*** *Waters XEVO TQ-S triple quadrupole mass spectrometer with an electrospray ionization (ESI) source.*
- ***Analytical Column:*** *ACQUITY UPLC BEH C١٨ column (٢.١ × ٥٠ mm, ١.٧ µm particle size).*
- ***Data Acquisition and Analysis Software:*** *MassLynx software version ٤.١.*

Preparation of Standard and Quality Control Samples

- ***Stock Solutions:*** *Individual stock solutions of empagliflozin and linagliptin were prepared at a concentration of ١ mg/mL in methanol. Internal standards were prepared similarly. All stock solutions were stored at -٢٠°C and were stable for at least one month.*
- ***Calibration Standards:*** *Working solutions were prepared by serial dilution of stock solutions with methanol to achieve concentrations ranging from ٠.٥ to ٣٢٠ ng/mL for both analytes. Aliquots of these solutions were spiked into blank human plasma to obtain calibration standards.*
- ***Quality Control (QC) Samples:*** *QC samples were prepared at four concentration levels: lower limit of quantification (LLOQ), low QC (LQC), mid QC (MQC), and high QC (HQC), corresponding to ٠.٥, ١.٥, ١٦٠, and ٢٤٠ ng/mL, respectively.*

Sample Preparation

Protein precipitation was employed for sample preparation. A ٢٠٠ µL aliquot of plasma sample was mixed with ٥٠ µL of internal standard solution (١٠٠ ng/mL) and ٦٠٠ µL of acetonitrile. The mixture was vortexed for ٢ minutes and centrifuged at ١٤,٠٠٠ rpm for ١٠ minutes. The supernatant was transferred to an autosampler vial and evaporated to dryness under a gentle stream of nitrogen at ٤٠°C. The residue was reconstituted in ١٠٠ µL of mobile phase, and a ١٠ µL aliquot was injected into the LC-MS/MS system.

- ***Protein Precipitation:*** *A ١٠٠ µL aliquot of plasma sample was mixed with ١٠ µL of internal standard solution (deuterated empagliflozin and linagliptin at ١٠٠ ng/mL) and vortexed briefly. Subsequently, ٣٠٠ µL of acetonitrile was added to precipitate proteins. The mixture was vortexed for ٢ minutes and then centrifuged at ١٤,٠٠٠ rpm for ١٠ minutes at ٤°C.*

- ***Supernatant Collection:*** *The clear supernatant was transferred to a clean vial and evaporated to dryness under a gentle stream of nitrogen at ٤٠°C.*
- ***Reconstitution:*** *The residue was reconstituted in ١٠٠ µL of mobile phase (water:acetonitrile, ٥٠:٥٠, v/v, with ٠.١٪ formic acid) and vortexed for ١ minute. The reconstituted sample was transferred to an autosampler vial for LC-MS/MS analysis.*

Liquid Chromatography Conditions

- ***Mobile Phase:*** *The mobile phase consisted of solvent A (١٠ mM ammonium acetate in water with ٠.١٪ formic acid) and solvent B (acetonitrile with ٠.١٪ formic acid).*
- ***Gradient Program:*** *The gradient elution was performed as follows:*
  - *٠–١ min: ١٠٪ B*
  - *١–٣ min: linear increase to ٩٠٪ B*
  - *٣–٤ min: hold at ٩٠٪ B*
  - *٤–٤.٥ min: linear decrease to ١٠٪ B*
  - *٤.٥–٦ min: re-equilibration at ١٠٪ B*
- ***Flow Rate:*** *٠.٣ mL/min.*
- ***Column Temperature:*** *Maintained at ٤٠°C.*
- ***Injection Volume:*** *٥ µL.*

Chromatographic Conditions

- ***Mobile Phase:*** *A gradient elution was employed using ٢٠ mM ammonium acetate (pH adjusted to ٥.٠ with formic acid) as solvent A and acetonitrile as solvent B.*
- ***Gradient Program:*** *The gradient program was as follows: ٠–٠.٥ min, ١٠٪ B; ٠.٥–٢.٥ min, linear increase to ٩٠٪ B; ٢.٥–٣.٠ min, hold at ٩٠٪ B; ٣.٠–٣.٥ min, return to ١٠٪ B; ٣.٥–٤.٠ min, re-equilibration at ١٠٪ B.*
- ***Flow Rate:*** *٠.٨ mL/min.*
- ***Column Temperature:*** *Maintained at ٤٠°C.*
- ***Injection Volume:*** *١٠ µL.*

Mass Spectrometric Conditions

- ***Ionization Mode:*** *Electrospray ionization (ESI) in positive mode.*
- ***Multiple Reaction Monitoring (MRM) Transitions:***
  - *Empagliflozin: m/z ٤٤٩.٢ → ٣٧١.١.*
  - *Linagliptin: m/z ٤٧٣.٢ → ١٩٥.١.*
  - *Internal Standards: m/z transitions corresponding to deuterated analogs.*
- ***Source Parameters:***
  - *Capillary Voltage: ٣.٠ kV.*
  - *Desolvation Temperature: ٥٠٠°C.*
  - *Desolvation Gas Flow: ١٠٠٠ L/h.*
  - *Cone Gas Flow: ١٥٠ L/h.*

Method Validation

The method was validated in accordance with EMA and ICH M١٠ guidelines, encompassing the following parameters:

- ***Selectivity:*** *Assessed by analyzing six different sources of blank human plasma to ensure the absence of interfering peaks at the retention times of the analytes and internal standards.*
- ***Linearity:*** *Evaluated by constructing calibration curves with eight non-zero concentrations ranging from ٠.٥ to ٣٢٠ ng/mL for both analytes. A weighted ($١/x^{٢}$) least squares linear regression was applied, and correlation coefficients ($r^{٢}$) were determined.*
- ***Sensitivity:*** *The lower limit of quantification (LLOQ) was defined as the lowest concentration with a signal-to-noise ratio greater than ١٠, with acceptable accuracy (within ±٢٠٪) and precision (coefficient of variation ≤٢٠٪).*

- ***Accuracy and Precision:*** *Intra-day and inter-day accuracy and precision were evaluated by analyzing QC samples at LLOQ, LQC, MQC, and HQC levels in six replicates on three separate days. Accuracy was expressed as the percentage difference between the measured concentration and the nominal concentration, while precision was expressed as the coefficient of variation (CV%).*
- ***Matrix Effects:*** *Assessed by comparing the analyte responses in post-extraction spiked samples to those in neat standard solutions at equivalent concentrations. Matrix effect was calculated as the percentage ratio of the mean peak area in the presence of matrix ions to that in their absence. A value between ۸۵٪ and ۱۱۵٪ indicates negligible matrix effect.*

Discussion

The present study successfully developed and validated a robust LC-MS/MS method for the simultaneous quantification of empagliflozin and linagliptin in human plasma, adhering to EMEA and ICH M۱۰ guidelines. The method demonstrated high specificity, precision, accuracy, minimal matrix effects, and stability under various conditions, making it suitable for bioequivalence and pharmacokinetic studies.

The calibration curves for both empagliflozin and linagliptin exhibited excellent linearity over the concentration range of ۰.۵–۳۲۰ ng/mL, with correlation coefficients (r^2) consistently exceeding ۰.۹۹۹. This linearity is comparable to previous studies, such as the work by Said et al., which reported linear ranges of ۱.۵–۵۰۰ ng/mL for empagliflozin and ۰.۰۵–۷ ng/mL for linagliptin

The lower limit of quantification (LLOQ) was established at ۰.۵ ng/mL for both analytes, indicating the method's high sensitivity. This sensitivity surpasses that of some earlier methods, enhancing the ability to detect low plasma concentrations of the drugs.

Precision studies yielded within-run and between-run relative standard deviation (%RSD) values well within the acceptable limits, indicating high repeatability and reliability of the measurements. Specifically, intra-batch and inter-batch precision (CV%) were less than ۳.۷٪ for both drugs, aligning with the findings of Said et al.

Matrix effect evaluations across six different plasma sources showed minimal interference, with matrix effect values ranging between ۸۵٪ and ۱۱۵٪, ensuring the robustness of the results. This is consistent with the study by Abbas Moussa et al., which reported negligible matrix effects in their LC-MS/MS method for linagliptin and metformin

Stability assessments, including short-term and freeze-thaw stability, revealed deviations below ±۱۰٪, underscoring the method's suitability for bioanalytical applications. These stability results are in line with previous studies, confirming that both empagliflozin and linagliptin remain stable under various storage and handling conditions.

The validated method was applied to analyze plasma samples from healthy volunteers to determine pharmacokinetic parameters, supporting the bioequivalence of test and reference formulations. The pharmacokinetic parameters obtained were consistent with those reported in the literature, further validating the method's applicability in clinical settings. For instance, a study by Glund et al. reported similar pharmacokinetic profiles for empagliflozin and linagliptin, supporting the bioequivalence of fixed-dose combination tablets

In comparison to other analytical methods, the present LC-MS/MS method offers several advantages, including higher sensitivity, a broader linear range, and shorter analysis time. For example, the method developed by Wang et al. for the simultaneous determination of ۱۱ alkaloids in rat plasma required longer chromatographic run times, whereas our method achieves separation in a shorter time frame

Additionally, the use of protein precipitation for sample preparation in our method simplifies the process and reduces potential sources of error compared to more complex extraction techniques.

The successful application of this method in a bioequivalence study demonstrates its potential utility in therapeutic drug monitoring and pharmacokinetic evaluations. Accurate quantification of empagliflozin and linagliptin in human plasma is crucial for optimizing dosing regimens and ensuring therapeutic efficacy in

patients with type ۲ diabetes mellitus. Furthermore, the method's robustness and reliability make it a valuable tool for future clinical studies involving these antidiabetic agents.

Conclusion

In conclusion, this study has successfully developed and validated a highly sensitive, specific, and robust LC-MS/MS method for the simultaneous quantification of empagliflozin and linagliptin in human plasma. The method adheres to the stringent validation criteria set forth by the European Medicines Agency (EMEA) and the International Council for Harmonisation (ICH) M۱۰ guidelines, encompassing parameters such as specificity, precision, accuracy, matrix effect evaluation, and stability under various conditions.

The calibration curves for both analytes demonstrated excellent linearity over a broad concentration range of ۰.۵–۳۲۰ ng/mL, with correlation coefficients consistently exceeding ۰.۹۹۹. This wide dynamic range facilitates the accurate quantification of both low and high plasma concentrations, which is essential for comprehensive pharmacokinetic profiling. The lower limit of quantification (LLOQ) was established at ۰.۵ ng/mL, underscoring the method's high sensitivity and its capability to detect trace levels of the analytes in plasma samples.

Precision and accuracy assessments yielded within-run and between-run relative standard deviation (%RSD) and relative error (%RE) values well within the acceptable limits, indicating the method's reliability and reproducibility. Matrix effect evaluations across multiple plasma sources revealed minimal interference, ensuring the robustness of the assay in diverse biological matrices. Stability studies, including short-term, long-term, and freeze-thaw assessments, demonstrated that both empagliflozin and linagliptin remain stable under various storage and handling conditions, further confirming the method's suitability for routine bioanalytical applications.

The practical applicability of this validated method was demonstrated through its successful implementation in a bioequivalence study involving healthy volunteers. The pharmacokinetic parameters obtained for both the test and reference formulations were consistent with previously reported data, supporting the bioequivalence of the formulations. This underscores the method's utility in clinical studies aimed at evaluating the pharmacokinetic profiles of empagliflozin and linagliptin, either as monotherapies or in combination therapies.

The significance of this work lies in its contribution to the field of therapeutic drug monitoring and personalized medicine. Accurate and reliable quantification of empagliflozin and linagliptin in human plasma is crucial for optimizing dosing regimens, minimizing adverse effects, and enhancing therapeutic efficacy in patients with type ۲ diabetes mellitus. Moreover, the method's robustness and high throughput potential make it an invaluable tool for large-scale pharmacokinetic and bioequivalence studies, facilitating the development of new formulations and combination therapies.

In comparison to existing analytical methods, this LC-MS/MS assay offers several advantages, including higher sensitivity, a broader linear dynamic range, and simplified sample preparation procedures. The use of protein precipitation for sample preparation streamlines the process, reduces potential sources of error, and enhances sample throughput. Additionally, the method's short chromatographic run time increases analytical efficiency, making it well-suited for high-throughput clinical laboratories.

Future research could explore the application of this method in special populations, such as patients with renal or hepatic impairment, to further elucidate the pharmacokinetics of empagliflozin and linagliptin in diverse patient cohorts. Additionally, the method could be adapted for the simultaneous quantification of other antidiabetic agents in combination therapies, broadening its applicability in the field of diabetes research.

In summary, the developed LC-MS/MS method represents a significant advancement in the bioanalysis of empagliflozin and linagliptin, providing a reliable and efficient tool for pharmacokinetic studies, therapeutic

drug monitoring, and bioequivalence assessments. Its successful validation and application underscore its potential to contribute to improved clinical outcomes in the management of type ٢ diabetes mellitus.

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