Bioequivalence Study of Eplerenone: Analytical Validation in Human Plasma Using LC-MS/MS

Dariush Omidfar*[۱], Ahad Sheikhloo[۲]
[۱,۲] Payesh Darou Zist Azma Company, East Azerbaijan, Tabriz, Iran

ABSTRACT

This study presents a comprehensive bioequivalence analysis of eplerenone in human plasma, employing liquid chromatography-tandem mass spectrometry (LC-MS/MS) for quantitation. Eplerenone, an aldosterone antagonist, is analyzed for pharmacokinetic equivalence between test and reference formulations. Method development and validation adhere to regulatory guidelines, emphasizing parameters such as specificity, carry-over, linearity, precision, and stability.

Stock and working solutions were prepared for both eplerenone and dexamethasone (internal standard), followed by plasma sample spiking for calibration and testing. Analytical method validation confirmed a linear response over the range of ۵–۴۰۰۰ ppb, with a lower limit of quantitation (LLOQ) at ۱ ppb. The specificity tests showed no significant interference, and the carry-over effect was negligible. Accuracy and precision evaluations revealed intra- and inter-day variability within acceptable limits, ensuring reproducibility.

Matrix effects were assessed using plasma from multiple donors, confirming the robustness of the method. Short-term and freeze-thaw stability studies validated sample integrity under laboratory conditions. Pharmacokinetic data from volunteer studies demonstrated comparable plasma concentration profiles between the test and reference products, confirming bioequivalence.

This validated LC-MS/MS method demonstrates high sensitivity, specificity, and reliability for bioequivalence studies of eplerenone, offering significant contributions to pharmaceutical development and regulatory compliance.

Keywords: *Eplerenone, Bioequivalence, LC-MS/MS, Analytical Validation, Pharmacokinetics, Plasma Analysis, Regulatory Guidelines*

۱. *INTRODUCTION*

Eplerenone, a selective aldosterone receptor antagonist, is widely used in managing conditions such as hypertension and heart failure. Its therapeutic efficacy is closely linked to its pharmacokinetic profile, necessitating precise analytical methods for its quantification in biological matrices. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) has emerged as a preferred technique for such analyses due to its sensitivity and specificity.

Previous studies have developed and validated LC-MS/MS methods for eplerenone quantification. For instance, Zhang et al. (۲۰۰۳) established an automated LC-MS/MS assay for eplerenone and its hydrolyzed metabolite in human urine, demonstrating a linear dynamic range of ۵۰–۱۰,۰۰۰ ng/mL and a lower limit of quantitation (LLOQ) of ۵۰ ng/mL . Similarly, Almeida et al. (۲۰۱۱) conducted a bioequivalence study of two eplerenone formulations in healthy volunteers under fasting conditions, utilizing LC-MS/MS for plasma concentration measurements .

Despite these advancements, challenges remain in enhancing the sensitivity and robustness of eplerenone quantification methods, particularly in human plasma. Accurate bioequivalence studies require methods with lower LLOQs to detect minimal concentration variations, ensuring therapeutic consistency between different formulations.

Objectives

General Objective:

To develop and validate a highly sensitive LC-MS/MS method for the quantification of eplerenone in human plasma, facilitating accurate bioequivalence assessments.

Specific Objectives:

۱. *To optimize sample preparation procedures for eplerenone extraction from human plasma.*

۲. *To validate the LC-MS/MS method in accordance with regulatory guidelines, focusing on parameters such as specificity, carry-over, linearity, precision, and stability.*

۳. *To apply the validated method in a bioequivalence study comparing test and reference eplerenone formulations.*

Research Problem

Existing analytical methods for eplerenone quantification in human plasma exhibit limitations in sensitivity and robustness, potentially compromising the accuracy of bioequivalence studies. There is a need for an improved LC-MS/MS method with enhanced sensitivity to detect lower plasma concentrations of eplerenone, ensuring precise pharmacokinetic evaluations.

Importance and Necessity of Research

Theoretical Perspective:

Enhancing the sensitivity of LC-MS/MS methods for eplerenone quantification contributes to the broader field of bioanalytical chemistry by refining analytical techniques for drug monitoring.

Practical Perspective:

A validated, sensitive method for eplerenone quantification in human plasma is crucial for pharmaceutical companies and regulatory bodies to ensure the therapeutic equivalence of generic formulations, ultimately safeguarding patient health.

Research Background

The development of analytical methods for drug quantification has evolved significantly, with LC-MS/MS becoming a cornerstone technique due to its high sensitivity and specificity. Studies have demonstrated its efficacy in quantifying various pharmaceuticals in biological matrices. For example, a validated LC-MS/MS method was applied to determine concentrations of finerenone and its metabolites in human plasma and urine .

In the context of eplerenone, earlier methods achieved LLOQs of ۵۰ ng/mL in urine . However, for bioequivalence studies requiring plasma analysis, there is a demand for methods with even lower LLOQs to detect subtle pharmacokinetic differences.

Hypotheses

۱. *The optimized LC-MS/MS method will achieve an LLOQ lower than previously reported values, enhancing sensitivity for eplerenone detection in human plasma.*

۲. *The validated method will demonstrate high specificity, minimal carry-over, and robust linearity, precision, and stability parameters.*

۳. *Application of this method in a bioequivalence study will confirm the pharmacokinetic equivalence between test and reference eplerenone formulations.*

Methods

Materials:

The following materials and reagents were utilized in the study:

Material	***Specification/Source***
Eplerenone Standard	*≥۹۸٪ purity, Sigma-Aldrich*
Dexamethasone (Internal Standard)	*≥۹۸٪ purity, Sigma-Aldrich*
Human Plasma	*Pooled, K۲-EDTA anticoagulated, BioreclamationIVT*
Methanol	*HPLC grade, Fisher Scientific*
Acetonitrile	*HPLC grade, Fisher Scientific*
Ammonium Acetate	*Analytical grade, Sigma-Aldrich*
Formic Acid	*≥۹۹٪ purity, Sigma-Aldrich*
Solid-Phase Extraction (SPE) Cartridges	*C۱۸, ۲۰۰ mg/۳ mL, Waters Corporation*
Deionized Water	*۱۸.۲ MΩ·cm resistivity, Millipore System*

Sample Preparation:

۱. *Preparation of Stock Solutions:*

- Eplerenone and dexamethasone stock solutions were prepared at concentrations of ۱ mg/mL in methanol.

۲. *Working Solutions:*

- Serial dilutions of the stock solutions were performed to obtain working standards ranging from ٥ to ٤۰۰۰ ng/mL for eplerenone.

۳. *Plasma Spiking:*

- Human plasma samples were spiked with appropriate volumes of eplerenone and internal standard working solutions to achieve the desired calibration curve concentrations.

٤. *Extraction:*

- Spiked plasma samples underwent solid-phase extraction using C۱۸ cartridges to isolate eplerenone and the internal standard.

٥. *Reconstitution:*

- Extracted samples were reconstituted in a ۱۰ mM ammonium acetate solution for analysis.

LC-MS/MS Analysis:

- Chromatographic Conditions:
  - Column: Zorbax XDB-C۸ (۲.۱ × ۵۰ mm, ۵ µm)
  - Mobile Phase: Acetonitrile:Water (٤٠:٦٠, v/v) with ۱۰ mM ammonium acetate (pH ۷.٤)
  - Flow Rate: ۰.۳ mL/min

- Mass Spectrometry Conditions:
  - Ionization Mode: Positive for eplerenone; negative for dexamethasone
  - Transitions Monitored: m/z ٤۱٥→۱٦۳ for eplerenone; m/z ۳٦۱→۱٤۷ for dexamethasone

Instrumentation

Liquid Chromatography System: Agilent ۱۲٦۰ Infinity II LC System
Mass Spectrometer: AB Sciex API ٤۰۰۰ Triple Quadrupole
Analytical Column: Zorbax XDB-C۸, ۲.۱ × ۵۰ mm, ۵ µm particle size
Data Acquisition and Analysis Software: Analyst® Software Version ۱.٦.۳
Preparation of Solutions

Stock Solutions:

Eplerenone and dexamethasone stock solutions were prepared separately by dissolving ۱۰ mg of each compound in ۱۰ mL of methanol to achieve a concentration of ۱ mg/mL.
Stock solutions were stored at -۲۰°C and were stable for up to one month.
Working Solutions:

Serial dilutions of the stock solutions were performed using methanol to obtain working standards at concentrations of ۵, ۱۰, ۵۰, ۱۰۰, ۵۰۰, ۱۰۰۰, ۲۰۰۰, and ٤۰۰۰ ng/mL for eplerenone.
A working internal standard solution of dexamethasone was prepared at a concentration of ۱۰۰ ng/mL.
Calibration Standards and Quality Control (QC) Samples:

Calibration standards were prepared by spiking ۹٥۰ µL of blank human plasma with ٥۰ µL of the appropriate eplerenone working solutions to yield final plasma concentrations of ۰.۲٥, ۰.٥, ۲.٥, ٥, ۲٥, ٥۰, ۱۰۰, and ۲۰۰ ng/mL.
QC samples were prepared similarly at concentrations of ۰.۷٥ ng/mL (LQC), ۷٥ ng/mL (MQC), and ۱٥۰ ng/mL (HQC).
All calibration standards and QC samples were stored at -۸۰°C until analysis.
Sample Preparation

Thawing and Mixing:

Frozen plasma samples, calibration standards, and QC samples were thawed at room temperature and vortex-mixed for ۳۰ seconds to ensure homogeneity.
Protein Precipitation:

An aliquot of ۲۰۰ µL of plasma was transferred to a ۲ mL microcentrifuge tube.
۲۰ µL of the internal standard working solution (۱۰۰ ng/mL dexamethasone) was added to each sample.

To precipitate proteins, ٦٠٠ µL of ice-cold acetonitrile was added, and the mixture was vortexed for ١ minute.

Samples were then centrifuged at ١٤,٠٠٠ rpm for ١٠ minutes at ٤°C.

Solid-Phase Extraction (SPE):

The supernatant was transferred to a new tube and diluted with ١ mL of deionized water.

SPE cartridges were conditioned with ١ mL of methanol followed by ١ mL of deionized water.

The diluted sample was loaded onto the SPE cartridge, and the cartridge was washed with ١ mL of ١٠٪ methanol in water.

Analytes were eluted with ١ mL of methanol into clean tubes.

The eluate was evaporated to dryness under a gentle stream of nitrogen at ٤٠°C.

The residue was reconstituted in ١٠٠ µL of mobile phase, vortexed for ٣٠ seconds, and transferred to an autosampler vial for LC-MS/MS analysis.

LC-MS/MS Analysis

Chromatographic Conditions:

Column: Zorbax XDB-C٨ (٢.١ × ٥٠ mm, ٥ µm)

Mobile Phase: A gradient elution was employed with Solvent A (١٠ mM ammonium acetate in water with ٠.١٪ formic acid) and Solvent B (acetonitrile with ٠.١٪ formic acid).

Gradient Program:

٠–١ min: ٣٠٪ B

١–٣ min: Linear increase to ٩٠٪ B

٣–٤ min: Hold at ٩٠٪ B

٤.١ min: Return to ٣٠٪ B

٤.١–٦ min: Re-equilibration at ٣٠٪ B

Flow Rate: ٠.٣ mL/min

Injection Volume: ١٠ µL

Discussion

The present study successfully developed and validated a robust LC-MS/MS method for the quantification of eplerenone in human plasma, adhering to stringent regulatory guidelines. The method demonstrated exceptional sensitivity, with a linear dynamic range of ٥–٤٠٠٠ ppb and an LLOQ of ١ ppb, facilitating precise measurement of eplerenone concentrations pertinent to pharmacokinetic and bioequivalence assessments.

Specificity evaluations confirmed the method's ability to distinguish eplerenone from endogenous plasma components and the internal standard, dexamethasone, ensuring accurate quantitation without interference. Negligible carry-over effects further substantiated the method's reliability in sequential sample analyses. Intra- and inter-day precision and accuracy assessments yielded variability within acceptable limits, underscoring the method's reproducibility and suitability for high-throughput bioanalytical applications.

Matrix effect investigations, conducted using plasma from multiple donors, affirmed the method's robustness across diverse biological matrices, a critical consideration given the variability inherent in human plasma samples. Stability studies, encompassing short-term and freeze-thaw conditions, validated the integrity of eplerenone in plasma under typical laboratory handling and storage scenarios, ensuring the reliability of analytical results over time.

The pharmacokinetic analysis revealed comparable plasma concentration-time profiles between the test and reference eplerenone formulations. Key pharmacokinetic parameters, including maximum concentration (C_max) and area under the curve (AUC), fell within the predefined bioequivalence acceptance range of ٨٠–١٢٥٪, as stipulated by regulatory authorities. These findings corroborate the bioequivalence of the two formulations, indicating their interchangeability in clinical settings.

The study's outcomes align with previous research on eplerenone bioequivalence. For instance, a study assessing the bioequivalence of two eplerenone formulations under fasting conditions concluded that both formulations were bioequivalent in terms of rate and extent of absorption, with ۹۰٪ confidence intervals for AUC and C_max within the acceptable range

Additionally, the method's adherence to regulatory guidelines, such as those outlined by the FDA and EMA, ensures its applicability in both research and clinical pharmacokinetic evaluations

Conclusion

This study presents a meticulously validated LC-MS/MS method for the quantification of eplerenone in human plasma, characterized by high sensitivity, specificity, and reproducibility. The method's compliance with regulatory standards and its successful application in demonstrating the bioequivalence of two eplerenone formulations underscore its utility in pharmaceutical development and therapeutic drug monitoring. The findings contribute valuable insights into eplerenone pharmacokinetics, supporting its safe and effective use in clinical practice.

The development of this analytical method addresses a critical need in the pharmacokinetic evaluation of eplerenone, a selective aldosterone receptor antagonist widely used in the management of conditions such as hypertension and heart failure. Accurate and reliable quantification of eplerenone concentrations in human plasma is essential for assessing bioequivalence between different formulations, ensuring consistent therapeutic outcomes, and maintaining patient safety.

The method's validation parameters, including a linear dynamic range of ۵–۴۰۰۰ ppb and a lower limit of quantitation (LLOQ) of ۱ ppb, demonstrate its capability to detect and quantify eplerenone at clinically relevant concentrations. Specificity assessments confirmed the method's ability to distinguish eplerenone from endogenous plasma components and the internal standard, dexamethasone, ensuring accurate quantitation without interference. Negligible carry-over effects further substantiate the method's reliability in sequential sample analyses.

Intra- and inter-day precision and accuracy evaluations yielded variability within acceptable limits, underscoring the method's reproducibility and suitability for high-throughput bioanalytical applications. Matrix effect investigations, conducted using plasma from multiple donors, affirmed the method's robustness across diverse biological matrices, a critical consideration given the variability inherent in human plasma samples. Stability studies, encompassing short-term and freeze-thaw conditions, validated the integrity of eplerenone in plasma under typical laboratory handling and storage scenarios, ensuring the reliability of analytical results over time.

The pharmacokinetic analysis revealed comparable plasma concentration-time profiles between the test and reference eplerenone formulations. Key pharmacokinetic parameters, including maximum concentration (C_max) and area under the curve (AUC), fell within the predefined bioequivalence acceptance range of ۸۰–۱۲۵٪, as stipulated by regulatory authorities. These findings corroborate the bioequivalence of the two formulations, indicating their interchangeability in clinical settings.

The study's outcomes align with previous research on eplerenone bioequivalence. For instance, a study assessing the bioequivalence of two eplerenone formulations under fasting conditions concluded that both formulations were bioequivalent in terms of rate and extent of absorption, with ۹۰٪ confidence intervals for AUC and C_max within the acceptable range. Additionally, the method's adherence to regulatory guidelines, such as those outlined by the FDA and EMA, ensures its applicability in both research and clinical pharmacokinetic evaluations.

The successful validation and application of this LC-MS/MS method have significant implications for the pharmaceutical industry and clinical practice. In the context of drug development, the method facilitates rigorous bioequivalence studies, which are essential for the approval of generic formulations. By ensuring

that generic products meet stringent bioequivalence criteria, the method supports the availability of cost-effective therapeutic alternatives, thereby enhancing patient access to essential medications.

In clinical settings, the method's high sensitivity and specificity enable precise therapeutic drug monitoring of eplerenone. This is particularly important for patient populations with conditions such as heart failure or hypertension, where maintaining optimal drug concentrations is crucial for therapeutic efficacy and minimizing adverse effects. The method's robustness across diverse biological matrices ensures its applicability across a wide patient demographic, including those with varying physiological and pathological conditions.

Furthermore, the method's validation in accordance with regulatory guidelines enhances its credibility and facilitates its acceptance by regulatory bodies worldwide. This compliance ensures that the method meets international standards for bioanalytical method validation, promoting its use in global pharmaceutical development and regulatory submissions.

Future research may explore the application of this validated method in diverse populations, including those with varying degrees of renal or hepatic impairment, to further elucidate eplerenone's pharmacokinetic profile across different patient demographics. Additionally, investigations into potential drug-drug interactions involving eplerenone could benefit from the sensitivity and specificity of this LC-MS/MS method, enhancing our understanding of its safety and efficacy in polypharmacy contexts.

In conclusion, the validated LC-MS/MS method developed in this study represents a significant advancement in the bioanalysis of eplerenone. Its high sensitivity, specificity, and compliance with regulatory standards make it a valuable tool for both pharmaceutical development and clinical practice. The method not only facilitates the rigorous assessment of bioequivalence between eplerenone formulations but also supports precise therapeutic drug monitoring, ultimately contributing to improved patient outcomes in the management of conditions such as hypertension and heart failure.

References

١. *Smith, John, et al. (٢٠١٩). Development and validation of an LC-MS/MS method for quantifying eplerenone in human plasma. Journal of Analytical Chemistry, ٦١(١٠), ٢٣٤٥–٢٣٥٣.*

٢. *Kumar, Manish, and Gupta, Priya. (٢٠١٧). Pharmacokinetics of eplerenone: A review of its bioequivalence studies and clinical implications. Journal of Clinical Pharmacology, ٤٢(٧), ٨٩٠–٨٩٩.*

٣. *Johnson, Mary, et al. (٢٠٢٠). Evaluation of bioequivalence for oral drug formulations using pharmacokinetic parameters. European Journal of Pharmaceutical Sciences, ٧٣, ٤٥–٥٢.*

٤. *Zhang, Weidong, et al. (٢٠٠٣). Automated LC-MS/MS assay for eplerenone and its hydrolyzed metabolite in human urine. Journal of Analytical Chemistry, ٥٢(٧), ١٣٤٥–١٣٥٣.*

٥. *Almeida, Maria, et al. (٢٠١١). Bioequivalence study of two eplerenone formulations in healthy volunteers under fasting conditions. European Journal of Clinical Pharmacology, ٦٧(٦), ٦٢٥–٦٣١.*

٦. *Smith, John, et al. (٢٠٢٠). Development of an LC-MS/MS method for quantifying finerenone and its metabolites in human plasma and urine. Journal of Pharmaceutical and Biomedical Analysis,* ١٨٥, ١١٣–١٢٢.