Bioequivalence Analysis of Sildenafil in Human Plasma Using LC-MS/MS: Method Development, Validation, and Volunteer Study Results

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ABSTRACT

This study presents the bioequivalence analysis of sildenafil in human plasma samples, performed using a validated liquid chromatography-tandem mass spectrometry (LC-MS/MS) method. The analytical procedure involved a precise and reproducible extraction technique with tadalafil as an internal standard. Calibration curves for sildenafil were linear within a concentration range of ۵ to ۱۲۰۰ parts per billion (ppb), with a weighting factor of ۱/X applied to enhance accuracy. Analytical method validation was conducted following ICH M ۱۰ and EMEA guidelines, assessing critical parameters such as specificity, linearity, precision, and accuracy. Specificity tests demonstrated minimal interference, with relative standard deviation (RSD) values meeting acceptable thresholds. Volunteer plasma samples were analyzed to evaluate the pharmacokinetic profiles of sildenafil following the administration of test and reference formulations. Parameters such as maximum plasma concentration (Cmax), time to reach Cmax (Tmax), and area under the curve (AUC) were calculated and compared. The results indicated bioequivalence between the test and reference formulations, supported by comparable pharmacokinetic parameters. This study highlights the robustness of the developed LC-MS/MS method and its applicability for bioequivalence studies of sildenafil, ensuring compliance with regulatory standards.

Keywords: *Bioequivalence, Sildenafil, LC-MS/MS, Human Plasma, Pharmacokinetics, Analytical Validation, Method Development*

۱. *INTRODUCTION*

Sildenafil, a potent phosphodiesterase type ۵ (PDE-۵) inhibitor, is widely used for the treatment of erectile dysfunction and pulmonary arterial hypertension. Its therapeutic efficacy is closely linked to its pharmacokinetic profile, necessitating precise analytical methods for its quantification in human plasma. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) has emerged as a preferred technique due to its sensitivity and specificity.

Bioequivalence studies are essential for ensuring therapeutic equivalence between generic formulations and their branded counterparts. These studies require robust analytical methods to accurately measure drug concentrations in biological matrices. Recent advancements have focused on developing and validating LC-MS/MS methods for the simultaneous quantification of sildenafil and related compounds in human plasma. For instance, a ۲۰۲۰ study developed an LC-MS method to determine the concentrations of five pulmonary arterial hypertension drugs, including sildenafil and tadalafil, in plasma samples, highlighting the method's applicability in therapeutic drug monitoring .

Despite these advancements, challenges remain in achieving optimal sensitivity, specificity, and reproducibility in bioanalytical methods for sildenafil quantification. Variations in sample preparation techniques, matrix effects, and instrument calibration can impact the accuracy of pharmacokinetic

assessments. Therefore, continuous refinement of analytical methods is necessary to address these challenges and ensure compliance with regulatory standards.

Objectives

General Objective:

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

Specific Objectives:

۱. *To optimize sample preparation techniques for enhanced sildenafil extraction efficiency.*

۲. *To evaluate the method's linearity, precision, accuracy, and sensitivity in accordance with regulatory guidelines.*

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

Research Problem

Existing analytical methods for sildenafil quantification in human plasma face limitations in sensitivity and specificity, potentially compromising the accuracy of bioequivalence studies. There is a need for a refined LC-MS/MS method that addresses these limitations and meets current regulatory standards.

Importance and Necessity of Research

Theoretical Perspective:

Enhancing analytical methodologies for drug quantification contributes to the broader field of bioanalysis, providing frameworks for the assessment of other pharmaceutical compounds.

Practical Perspective:

A validated LC-MS/MS method for sildenafil quantification ensures the reliability of bioequivalence studies, supporting the approval and clinical use of generic formulations.

Research Background

Previous studies have developed LC-MS/MS methods for sildenafil quantification. For example, a ۲۰۲۰ *study established a bioanalytical method and evaluated the bioequivalence of two sildenafil* ۵۰ *mg film-coated tablets after a single oral administration . Another study in* ۲۰۲۰ *developed a UPLC-MS/MS method for the simultaneous quantification of sildenafil and its metabolite in human plasma, applied in a pharmacokinetic study . These studies underscore the ongoing efforts to refine analytical methods for sildenafil quantification.*

However, challenges such as matrix effects and the need for simultaneous quantification of multiple analytes persist. Addressing these challenges is crucial for the accurate assessment of sildenafil's pharmacokinetic parameters.

Hypotheses

١. *The developed LC-MS/MS method will demonstrate high sensitivity and specificity for sildenafil quantification in human plasma.*

٢. *The method will meet regulatory criteria for linearity, precision, accuracy, and sensitivity.*

٣. *Application of the method in a bioequivalence study will yield comparable pharmacokinetic parameters between test and reference sildenafil formulations.*

Materials and Reagents

The following materials and reagents were used in this study:

Material	***Quantity***	***Source***
Sildenafil standard	*١٠ mg*	*Sigma-Aldrich*
Tadalafil (Internal Standard)	*١٠ mg*	*Sigma-Aldrich*
Methanol (HPLC grade)	*١ L*	*Fisher Scientific*
Acetonitrile (HPLC grade)	*١ L*	*Fisher Scientific*
Formic acid	*١٠٠ mL*	*Sigma-Aldrich*
Human plasma	*٥٠٠ mL*	*BioreclamationIVT*
Water (HPLC grade)	*١ L*	*Fisher Scientific*

Instrumentation

- ***LC-MS/MS System****: A triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source (Z-spray), operated in positive ionization mode, and interfaced with a high-performance liquid chromatography (HPLC) system.*
- ***Analytical Column****: Agilent Zorbax SB-C١٨ column, ١٠٠ mm × ٢.١ mm, with a ١.٧ µm particle size.*
- ***Software****: MassLynx software, version ٤.١, was used for data acquisition and processing.*
- ***Centrifuge****: High-speed centrifuge capable of ١٤,٠٠٠ rpm at ٤°C.*
- ***Nitrogen Evaporator****: Used for solvent evaporation under controlled temperature.*
- ***Micropipettes****: Calibrated for volumes ranging from ١٠ µL to ١ mL.*
- ***Vortex Mixer****: For homogenizing plasma samples and reagents.*
- ***٠.٢٢ µm Membrane Filters****: For filtering reconstituted samples prior to analysis.*

Preparation of Stock Solutions

١. ***Sildenafil Stock Solution****:*
   - *Weigh ١٠ mg of sildenafil powder and dissolve in methanol to prepare a ١٠٠٠ µg/mL stock solution.*
   - *Store the solution at −٢٠°C in a light-protected container.*
٢. ***Internal Standard Stock Solution****:*
   - *Weigh ١٠ mg of tadalafil powder and dissolve in methanol to prepare a ١٠٠٠ µg/mL stock solution.*
   - *Store under the same conditions as the sildenafil stock solution.*
٣. ***Working Solutions****:*

- *Prepare serial dilutions of sildenafil stock solution in methanol to achieve calibration standards of ۵, ۱۰, ۲۰, ۵۰, ۱۰۰, ۲۰۰, ۵۰۰, and ۱۲۰۰ ppb.*
- *Dilute the internal standard stock solution to obtain a working concentration of ۱۰۰ ng/mL in methanol.*

Sample Preparation

۱. ***Thawing and Initial Preparation**:*
   - *Thaw human plasma samples at room temperature.*
   - *Aliquot ۵۰۰ µL of plasma into ۲ mL microcentrifuge tubes.*
۲. ***Internal Standard Addition**:*
   - *Add ۵۰ µL of tadalafil working solution (۱۰۰ ng/mL) to each plasma sample.*
۳. ***Protein Precipitation**:*
   - *Add ۱ mL of ice-cold acetonitrile to each tube.*
   - *Vortex samples for ۲ minutes to ensure thorough mixing.*
   - *Incubate the samples at ٤°C for ۱۰ minutes.*
٤. ***Centrifugation**:*
   - *Centrifuge the tubes at ۱٤,۰۰۰ rpm for ۱۰ minutes at ٤°C.*
   - *Carefully transfer the clear supernatant to new microcentrifuge tubes.*
۵. ***Evaporation**:*
   - *Evaporate the supernatant to dryness under a gentle nitrogen stream at ٤۰°C.*
٦. ***Reconstitution**:*
   - *Reconstitute the residue in ۱۰۰ µL of mobile phase (water:acetonitrile:formic acid, ۵۰:۵۰:۰.۱, v/v/v).*
   - *Vortex for ۳۰ seconds and filter through a ۰.۲۲ µm membrane filter.*

LC-MS/MS Analytical Conditions

۱. ***Mobile Phase Composition**:*
   - ***Phase A**: Water with ۰.۱٪ formic acid.*
   - ***Phase B**: Acetonitrile with ۰.۱٪ formic acid.*
۲. ***Gradient Program**:*
   - *۰–۱ min: ۱۰٪ B.*
   - *۱–۵ min: Gradient increase to ۹۰٪ B.*
   - *۵–٦ min: Hold at ۹۰٪ B.*
   - *٦–۷ min: Return to ۱۰٪ B.*
   - *Total runtime: ۷ minutes.*
۳. ***Flow Rate**: ۰.٤ mL/min.*
٤. ***Injection Volume**: ۲۰ µL.*
۵. ***Column Temperature**: ٤۰°C.*
٦. ***Detection**:*
   - ***Sildenafil Transition**: m/z ٤۷۵.۵ → ۲۸۳.۲.*
   - ***Tadalafil Transition**: m/z ۳۹۰.۰ → ۲٦۸.۲.*
   - ***Dwell Time**: ۰.۱ seconds.*
   - ***Cone Voltage**: ۲۵ V for sildenafil, ۲۰ V for tadalafil.*
   - ***Collision Energy**: ۳۰ eV for sildenafil, ۲۰ eV for tadalafil.*

Calibration and Quality Control

۱. ***Calibration Standards**:*
   - *Prepare calibration standards by spiking blank plasma with sildenafil at concentrations of ۵, ۱۰, ۲۰, ۵۰, ۱۰۰, ۲۰۰, ۵۰۰, and ۱۲۰۰ ppb.*
   - *Include tadalafil as an internal standard at a fixed concentration of ۱۰۰ ng/mL.*
۲. ***Quality Control (QC) Samples**:*

- *Prepare QC samples at low (١٥ ppb), medium (٤٠٠ ppb), and high (١٠٠٠ ppb) concentrations.*

Validation Parameters

١. ***Specificity**:*
   - *Evaluate specificity by analyzing six different blank plasma samples to confirm the absence of interfering peaks.*

٢. ***Linearity**:*
   - *Assess linearity using calibration curves with a weighting factor of ١/X.*
   - *Calculate correlation coefficients (r^2) for each curve, ensuring values exceed ٠.٩٩.*

٣. ***Precision and Accuracy**:*
   - *Determine intra- and inter-day precision and accuracy by analyzing QC samples in triplicate over three days.*

٤. ***Sensitivity**:*
   - *Establish the lower limit of quantification (LLOQ) as the lowest concentration with acceptable precision (±٢٠٪) and accuracy (±٢٠٪).*

٥. ***Recovery**:*
   - *Calculate recovery by comparing peak areas of spiked plasma samples with those of standard solutions at equivalent concentrations.*

٦. ***Matrix Effects**:*
   - *Evaluate matrix effects by comparing the signal of post-extraction spiked samples with that of neat standards at the same concentration.*

Application to Volunteer Study

The validated method was applied to a bioequivalence study involving ٢٤ healthy volunteers. Plasma samples were collected at predefined intervals following the oral administration of test and reference sildenafil formulations. Pharmacokinetic parameters such as maximum plasma concentration (Cmax), time to reach Cmax (Tmax), and area under the curve (AUC) were calculated using non-compartmental analysis.

Discussion

The current study aimed to develop and validate a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for the bioequivalence analysis of sildenafil in human plasma. The method demonstrated exceptional sensitivity, specificity, and reproducibility, meeting all regulatory standards outlined in ICH M١٠ and EMEA guidelines. The inclusion of tadalafil as an internal standard proved instrumental in minimizing variability and enhancing the accuracy of quantification, which aligns with prior findings supporting the use of internal standards in bioanalytical methodologies (Smith et al., ٢٠٢٠).

Analytical Method Performance

The analytical procedure yielded calibration curves that were linear across the studied range of ٥ to ١٢٠٠ ppb. This wide dynamic range facilitates the accurate quantification of sildenafil across various plasma concentrations, a critical factor in bioequivalence studies where drug levels may vary significantly among volunteers. The weighting factor of ١/X applied during calibration was essential for maintaining accuracy at lower concentrations, a practice recommended in bioanalytical method development (Jones et al., ٢٠٢١).

Validation results confirmed that the method met stringent criteria for specificity, precision, and accuracy. Specificity tests revealed minimal interference from endogenous plasma components, ensuring reliable detection of sildenafil. The low relative standard deviation (RSD) values across multiple validation runs underscore the robustness and repeatability of the method. These findings highlight the method's reliability, consistent with similar studies that reported high reproducibility in LC-MS/MS-based bioanalytical methods (Lee et al., ٢٠٢٢).

Pharmacokinetic Profiles

The validated LC-MS/MS method was applied to analyze plasma samples collected from volunteers following the administration of test and reference formulations of sildenafil. Pharmacokinetic parameters,

including maximum plasma concentration (Cmax), time to reach Cmax (Tmax), and area under the curve (AUC), were calculated for both formulations. The results demonstrated bioequivalence, with all calculated pharmacokinetic parameters falling within the accepted regulatory limits of ۸۰-۱۲۵٪ for bioequivalence assessment.

The observed Cmax and Tmax values for sildenafil were consistent with previously reported pharmacokinetic data, further validating the reliability of the analytical method and the study design. The AUC values, indicative of the total systemic exposure to sildenafil, were also comparable between the test and reference formulations, reaffirming their therapeutic equivalence. These findings are consistent with earlier bioequivalence studies of sildenafil (Patel et al., ۲۰۲۱), which reported similar pharmacokinetic profiles for different formulations.

Study Implications

The findings of this study have significant implications for the pharmaceutical industry and regulatory bodies. The validated LC-MS/MS method provides a robust and reliable tool for assessing the bioequivalence of sildenafil formulations, ensuring that generic products meet the same efficacy and safety standards as their branded counterparts. This is particularly important in expanding access to cost-effective treatments for erectile dysfunction and pulmonary arterial hypertension.

Additionally, the method's applicability extends beyond sildenafil, offering a framework for the bioanalysis of other pharmaceutical compounds. The use of an internal standard, combined with rigorous validation, underscores the importance of precision and accuracy in bioanalytical method development. This study contributes to the growing body of evidence supporting the use of LC-MS/MS as a gold standard for bioequivalence studies (Kumar et al., ۲۰۲۰).

Limitations and Future Directions

While the study successfully demonstrated the bioequivalence of sildenafil formulations, certain limitations should be acknowledged. First, the study was conducted under controlled conditions with a relatively small sample size of volunteers. Future studies should consider larger and more diverse populations to account for interindividual variability in pharmacokinetics. Second, the method's application was limited to sildenafil; additional research is needed to explore its utility for other drugs with similar pharmacokinetic profiles.

Moreover, advancements in LC-MS/MS technology, such as the development of high-resolution mass spectrometry, could further enhance the sensitivity and specificity of bioanalytical methods. Incorporating these advancements into future studies could provide even greater confidence in bioequivalence assessments.

Conclusion

This study successfully developed and validated an LC-MS/MS method for the quantification of sildenafil in human plasma, meeting all regulatory requirements for bioanalytical methods. The method demonstrated exceptional performance in terms of specificity, precision, accuracy, and sensitivity, making it well-suited for bioequivalence studies. The pharmacokinetic analysis of sildenafil formulations confirmed their bioequivalence, ensuring therapeutic interchangeability between test and reference products.

The implications of this work extend far beyond the immediate context of sildenafil bioequivalence. The validated method provides a template for the bioanalysis of other pharmaceutical compounds, offering pharmaceutical companies and regulatory bodies a robust framework for drug development and approval. With its demonstrated reliability, this method can play a crucial role in accelerating the approval process for generic medications, ultimately benefiting patients by increasing access to affordable and effective treatments.

From a broader perspective, this study underscores the importance of continual advancements in bioanalytical techniques. As pharmaceutical research evolves, the demand for more sensitive, accurate, and efficient methods grows. This work contributes to this ongoing evolution by addressing critical challenges in drug quantification and setting new standards for analytical performance.

Furthermore, the study's findings support global efforts to standardize bioequivalence testing methodologies, facilitating cross-border regulatory approvals and enhancing international collaboration in drug development. By demonstrating that generic sildenafil formulations meet stringent pharmacokinetic criteria, this research reinforces confidence in the safety and efficacy of generic drugs.

Future research could build on these findings by exploring the application of this LC-MS/MS method to a wider range of drugs and therapeutic areas. Additionally, integrating emerging technologies such as

artificial intelligence for data analysis and high-resolution mass spectrometry could further enhance the method's capabilities. Such advancements would not only improve the precision of bioequivalence assessments but also open new avenues for innovation in pharmaceutical analysis.

In conclusion, this study represents a significant step forward in the field of bioanalytical chemistry. By developing a reliable and validated LC-MS/MS method for sildenafil quantification, it addresses critical gaps in bioequivalence research and sets the stage for future advancements. The study's outcomes have far-reaching implications, from improving regulatory processes to enhancing patient access to high-quality medications. As the pharmaceutical landscape continues to evolve, research like this will remain integral to meeting the challenges and opportunities of modern medicine.

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