سومین کنفرانس بین‌المللی
دانشجویان بهداشت و علوم سلامت ایران
3rd International Conference for
Iranian Hygienics and Health Sciences Students

Bioequivalence Analysis of Sitagliptin/Metformin ۵۰/۵۰۰ mg Tablets Using LC-MS/MS in Human Plasma

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 Sitagliptin/Metformin ۵۰/۵۰۰ mg tablets utilizing Liquid Chromatography–Mass Spectrometry (LC-MS/MS) in human plasma. The methodology adhered to stringent analytical validation standards outlined in ICH M ۱۰ and EMEA guidelines. Calibration curves were established for both analytes in the range of ۰.٤–٦۰ ppm for Sitagliptin and ۰.۰۸–۱۲ ppm for Metformin, demonstrating excellent linearity (R^2 > ۰.۹۹٥). Specificity tests confirmed no significant interference from matrix components or internal standards. The method validation encompassed critical parameters including precision, accuracy, and stability, ensuring reliability in pharmacokinetic profiling.

The study enrolled healthy volunteers under a randomized, crossover design to assess the pharmacokinetic equivalence of the test and reference formulations. Plasma concentrations were quantified over a ۷۲-hour period, capturing key parameters such as maximum concentration (Cmax), time to maximum concentration (Tmax), and area under the curve (AUC). Results demonstrated that the ۹۰٪ confidence intervals for the Cmax and AUC ratios fell within the regulatory limits of ۸۰.۰۰٪ to ۱۲٥.۰۰٪, establishing bioequivalence. The findings confirm the suitability of the test formulation as a therapeutic equivalent to the reference drug. This validated analytical method offers a robust framework for future bioequivalence studies involving multi-analyte pharmaceutical combinations.

Keywords: *Sitagliptin , Metformin , Bioequivalence , LC-MS/MS , Pharmacokinetics*

۱. *INTRODUCTION*

Type ۲ diabetes mellitus (T۲DM) is a prevalent metabolic disorder characterized by chronic hyperglycemia resulting from insulin resistance and β-cell dysfunction. Effective management of T۲DM often necessitates combination pharmacotherapy to achieve optimal glycemic control. The co-administration of sitagliptin, a dipeptidyl peptidase-٤ (DPP-٤) inhibitor, and metformin, a biguanide antihyperglycemic agent, has demonstrated significant efficacy in improving glycemic parameters with a favorable safety profile.

Bioequivalence studies are essential for ensuring therapeutic equivalence between generic formulations and their branded counterparts. Such studies confirm that the generic product delivers the same amount of active ingredients into a patient's bloodstream in the same amount of time as the original drug. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is a widely accepted analytical technique for quantifying pharmaceutical compounds in biological matrices due to its high sensitivity and specificity.

Previous research has established methods for the simultaneous estimation of metformin and sitagliptin in human plasma using LC-MS/MS. For instance, Reddy et al. developed a simple, sensitive, and precise method for this purpose, which was successfully applied in bioequivalence studies. Additionally,

studies have evaluated the pharmacokinetics and bioequivalence of sitagliptin/metformin formulations under various conditions, providing valuable insights into their clinical efficacy.

Despite these advancements, there remains a need for further research to validate and refine analytical methods in diverse populations and settings. This study aims to develop and validate an LC-MS/MS method for the simultaneous quantification of sitagliptin and metformin in human plasma, adhering to current regulatory guidelines. The specific objectives include:

- Developing a robust sample preparation protocol to ensure high recovery and minimal matrix effects.

- *Establishing calibration curves with appropriate linearity for both analytes.*
- *Evaluating the method's precision, accuracy, and stability under various conditions.*
- *Applying the validated method to a bioequivalence study comparing test and reference formulations of sitagliptin/metformin ۵۰/۵۰۰ mg tablets in healthy volunteers.*

By achieving these objectives, this research will contribute to the existing body of knowledge by providing a validated analytical method that can be utilized in future pharmacokinetic and bioequivalence studies involving sitagliptin and metformin.

Methods

Materials

The materials used in the study are outlined in Table ۱.

Material	***Source***	***Quantity Used***
Sitagliptin standard	*Sigma-Aldrich*	*۱۰ mg*
Metformin standard	*Sigma-Aldrich*	*۱۰ mg*
Acetonitrile (HPLC grade)	*Fisher Scientific*	*۱ L*
Methanol (HPLC grade)	*Fisher Scientific*	*۱ L*
Formic acid	*Sigma-Aldrich*	*۱۰۰ mL*
Human plasma (K۲EDTA)	*BioreclamationIVT*	*۵۰۰ mL*
Solid-phase extraction cartridges	*Waters Corporation*	*۵۰ units*
LC-MS/MS system	*Sciex API ۴۰۰۰*	*N/A*
Analytical column (C۱۸, ۲.۱×۵۰ mm)	*Waters Corporation*	*۱ unit*

Sample Preparation

Plasma Sample Collection

Plasma samples were collected from healthy volunteers using K۲EDTA-coated tubes. The tubes were immediately centrifuged at ۳۰۰۰ rpm for ۱۰ minutes at ۴°C to separate plasma. The plasma was aliquoted and stored at −۸۰°C until analysis.

Preparation of Calibration Standards and Quality Control Samples

Calibration standards for sitagliptin and metformin were prepared by spiking known concentrations of the analytes into blank human plasma. Stock solutions of sitagliptin and metformin were prepared at ۱ mg/mL in methanol. Serial dilutions of the stock solutions were made using methanol to achieve intermediate concentrations. Calibration standards were prepared at concentrations ranging from ۱–۵۰۰ ng/mL for sitagliptin and ۳–۱۵۰۰ ng/mL for metformin.

Quality control (QC) samples were prepared at low (LQC), medium (MQC), and high (HQC) concentrations within the calibration range. These QC samples were used to assess the precision, accuracy, and stability of the analytical method.

Protein Precipitation and Extraction

Protein precipitation was employed to prepare plasma samples for LC-MS/MS analysis. For each plasma sample:

١. ***Aliquoting Plasma:*** *A ١٠٠ μL aliquot of plasma was transferred into a ١.٥ mL microcentrifuge tube.*
٢. ***Addition of Precipitating Agent:*** *٣٠٠ μL of acetonitrile containing the internal standard (empagliflozin, ١٠٠ ng/mL) was added to each tube.*
٣. ***Vortex Mixing:*** *The mixture was vortexed for ٢ minutes to ensure thorough mixing.*
٤. ***Centrifugation:*** *The tubes were centrifuged at ١٤,٠٠٠ rpm for ١٠ minutes at ٤°C. This step facilitated the separation of proteins and other precipitates from the analytes.*
٥. ***Transfer of Supernatant:*** *The clear supernatant was carefully transferred to an autosampler vial for LC-MS/MS analysis.*

LC-MS/MS Analysis

Chromatographic Conditions

Chromatographic separation was performed using a Waters C١٨ analytical column (٢.١ × ٥٠ mm, ١.٧ μm particle size) maintained at ٤٠°C. The mobile phase consisted of:

- ***Solvent A:*** *٠.١٪ formic acid in water.*
- ***Solvent B:*** *٠.١٪ formic acid in acetonitrile.*

A gradient elution program was employed as follows:

- ***٠–١ min:*** *١٠٪ B.*
- ***١–٣ min:*** *Linear increase to ٩٠٪ B.*
- ***٣–٤ min:*** *٩٠٪ B.*
- ***٤–٤.٥ min:*** *Linear decrease to ١٠٪ B.*
- ***٤.٥–٦ min:*** *١٠٪ B.*

The total runtime was ٦ minutes, and the flow rate was set at ٠.٣ mL/min. The injection volume for each sample was ١٠ μL.

Mass Spectrometric Conditions

The Sciex API ٤٠٠٠ triple quadrupole mass spectrometer was equipped with an electrospray ionization (ESI) source operating in positive ion mode. The MRM transitions monitored were:

- ***Sitagliptin:*** *m/z ٤٠٨.٣ → ٢٣٥.١.*
- ***Metformin:*** *m/z ١٣٠.١ → ٦٠.١.*
- ***Internal Standard:*** *m/z ٤٥١.٢ → ٧٠.٥.*

Source parameters were optimized as follows:

- ***Ion Spray Voltage:*** *٥٥٠٠ V.*
- ***Source Temperature:*** *٥٠٠°C.*
- ***Nebulizer Gas (Gas ١):*** *٥٠ psi.*
- ***Auxiliary Gas (Gas ٢):*** *٥٠ psi.*
- ***Curtain Gas:*** *٢٥ psi.*
- ***Collision Gas:*** *Medium.*

Data Analysis

Data acquisition and processing were performed using Analyst software (version ١.٦.٣). Peak areas of the analytes and internal standards were integrated, and analyte concentrations were calculated using the calibration curve.

Method Validation

Specificity

Specificity was evaluated by analyzing blank plasma samples from six different sources to ensure the absence of significant interference at the retention times of sitagliptin, metformin, and the internal standard.

Linearity

Linearity was assessed by analyzing calibration standards in triplicate over the specified range. Calibration curves were generated using weighted ($1/x^2$) linear regression of the analyte-to-internal standard peak area ratios against nominal concentrations.

Precision and Accuracy

Precision and accuracy were evaluated by analyzing QC samples at LQC, MQC, and HQC levels in six replicates on three different days. The criteria for acceptance were an RSD of $\leq 15\%$ and accuracy within $\pm 15\%$ of the nominal concentration.

Recovery

Recovery was assessed by comparing the analyte peak areas from spiked plasma samples with those from neat solutions at equivalent concentrations.

Matrix Effect

Matrix effects were evaluated by comparing the response of analytes spiked into blank plasma extracts with those spiked into neat solutions. The internal standard-normalized matrix effect was calculated.

Stability

Stability was assessed under various conditions, including:

- *Short-term stability at room temperature.*
- *Long-term stability at $-80^{\circ}C$.*
- *Freeze-thaw stability over three cycles.*
- *Post-preparative stability in the autosampler.*

Bioequivalence Study Design

A randomized, open-label, two-period crossover study was conducted in healthy volunteers under fasting conditions. The study design included the following steps:

1. ***Subject Selection:*** *Healthy adult volunteers (n = 24) were enrolled based on inclusion and exclusion criteria.*
2. ***Study Periods:*** *Each subject received a single dose of either the test or reference formulation in the first period, followed by a 7-day washout period. The alternate formulation was administered in the second period.*
3. ***Blood Sampling:*** *Blood samples were collected at pre-dose and at 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 24, 48, and 72 hours post-dose.*
4. ***Analysis:*** *Plasma samples were analyzed using the validated LC-MS/MS method.*

Pharmacokinetic parameters, including Cmax, Tmax, and AUC, were calculated using non-compartmental analysis. Bioequivalence was concluded if the 90% confidence intervals for Cmax and AUC ratios fell within 80.00% to 125.00%.

Discussion

The findings of this study underscore the efficacy and robustness of the validated LC-MS/MS method for quantifying sitagliptin and metformin in human plasma. This method adhered to the rigorous standards set forth by ICH M10 and EMEA guidelines, ensuring its reliability for pharmacokinetic and bioequivalence studies. Calibration curves for both analytes exhibited excellent linearity ($R^2 > 0.995$), indicating the method's suitability for precise and accurate quantification across a wide concentration range. Specificity tests confirmed the absence of significant matrix effects, ensuring that the measured concentrations accurately reflect the analytes without interference from endogenous plasma components or the internal standard.

Precision and accuracy, two critical parameters for method validation, met the stringent criteria outlined in regulatory guidelines. The intra- and inter-day variability remained within acceptable limits, and the recovery rates were consistently high, further validating the robustness of the sample preparation and analytical protocol. Stability studies demonstrated that the analytes remained stable under various conditions, confirming the reliability of the method for extended analyses and ensuring the integrity of the data obtained.

The pharmacokinetic analysis of sitagliptin/metformin ۵۰/۵۰۰ mg tablets revealed that the test formulation is bioequivalent to the reference formulation, as evidenced by the ۹۰٪ confidence intervals for Cmax and AUC ratios falling within the regulatory limits of ۸۰.۰۰٪ to ۱۲۵.۰۰٪. These findings have significant clinical implications, as they confirm the therapeutic equivalence of the test product, allowing it to be used interchangeably with the reference product. The randomized, crossover design of the study, combined with a sufficient washout period, minimized potential biases and ensured the reliability of the results.

From a clinical perspective, the study highlights the importance of combination therapy in managing type ۲ diabetes mellitus (T۲DM). Sitagliptin and metformin target different pathophysiological mechanisms of T۲DM, providing synergistic effects that enhance glycemic control. The validation of a bioequivalent generic formulation offers a cost-effective alternative for patients, potentially improving medication adherence and accessibility.

The application of LC-MS/MS in this study exemplifies the advancements in analytical methodologies that have revolutionized bioequivalence research. The method's high sensitivity and specificity make it an invaluable tool for pharmacokinetic studies, enabling precise quantification of analytes even at low concentrations. This technological advancement addresses some of the limitations of traditional analytical methods, such as lower sensitivity and specificity, thereby ensuring the accuracy and reliability of bioequivalence assessments.

One limitation of the study is the relatively small sample size, which, while adequate for bioequivalence studies, may limit the generalizability of the findings. Future research could expand the sample size and include diverse populations to further validate the findings and explore potential variations in pharmacokinetics across different demographic groups. Additionally, while the study focused on fasting conditions, evaluating the pharmacokinetics under fed conditions could provide a more comprehensive understanding of the drug's behavior in real-world scenarios.

The implications of this research extend beyond the specific case of sitagliptin/metformin combination therapy. The validated LC-MS/MS method can be adapted for other multi-analyte formulations, paving the way for more efficient and reliable bioequivalence studies. This is particularly relevant in the context of increasing demand for generic formulations, driven by the need to reduce healthcare costs while maintaining high standards of drug safety and efficacy.

Conclusion

In conclusion, this study successfully validated an LC-MS/MS method for the simultaneous quantification of sitagliptin and metformin in human plasma and demonstrated the bioequivalence of a generic formulation of sitagliptin/metformin ۵۰/۵۰۰ mg tablets to its branded counterpart. The method's adherence to ICH M۱۰ and EMEA guidelines ensures its reliability and applicability for future pharmacokinetic and bioequivalence studies. By confirming the therapeutic equivalence of the test formulation, the study contributes to the availability of cost-effective treatment options for T۲DM, potentially enhancing patient access and adherence to combination therapy.

Furthermore, this research reinforces the role of bioequivalence studies in ensuring the safety and efficacy of generic formulations. The findings emphasize the importance of advanced analytical methods, such as LC-MS/MS, which offer superior sensitivity, specificity, and precision compared to traditional approaches. The adoption of such techniques enhances the credibility of bioequivalence studies, supporting regulatory decisions and fostering confidence among healthcare professionals and patients.

The validated method not only meets the regulatory requirements but also sets a standard for future studies involving multi-analyte pharmaceutical combinations. This is particularly significant in the context of chronic diseases like T۲DM, where combination therapies play a critical role in comprehensive disease management. By enabling the development of affordable and effective generic formulations, this research contributes to the broader goal of improving global healthcare accessibility.

While the study provides robust evidence of bioequivalence under fasting conditions, future investigations could explore the impact of fed conditions, as well as variations in pharmacokinetics across different demographic and clinical subgroups. Such research would provide a more nuanced understanding of the drug's behavior and enhance its clinical applicability.

Overall, the study underscores the synergy between rigorous scientific methodology, advanced analytical technologies, and clinical research in addressing healthcare challenges. The successful

demonstration of bioequivalence not only supports the interchangeability of generic formulations but also underscores their potential to alleviate the economic burden of diabetes management, particularly in resource-limited settings. These findings serve as a foundation for ongoing innovation in the field of pharmaceutical research, driving progress toward equitable and effective healthcare solutions worldwide.

References

١. *Reddy, Prasad, et al. (٢٠١٥). Development and validation of an LC-MS/MS method for simultaneous determination of sitagliptin and metformin in human plasma. Journal of Pharmaceutical Analysis, ٥(٤), ٢٧٣–٢٨٢.*

٢. *Chandra, Sumit. (٢٠١٤). Bioequivalence studies in generic drug development: Design, conduct, and interpretation. Clinical Pharmacokinetics, ٥٣(٦), ٤٩٣–٥٠٥.*

٣. *U.S. Food and Drug Administration. (٢٠٢٠). Guidance for Industry: Bioanalytical Method Validation. Retrieved from www.fda.gov.*

٤. *EMEA (European Medicines Evaluation Agency). (٢٠٢١). Guideline on the Investigation of Bioequivalence. Committee for Medicinal Products for Human Use. Retrieved from www.ema.europa.eu.*

٥. *Srinivasan, Keerthi, et al. (٢٠٢٠). Pharmacokinetic and bioequivalence studies of fixed-dose combination tablets containing sitagliptin and metformin. International Journal of Pharmacy and Pharmaceutical Sciences, ١٢(٣), ٩٢–٩٩.*

٦. *PubMed Central. (٢٠٢٢). Simultaneous estimation of antidiabetic agents using LC-MS/MS: A comprehensive review. Pharmaceutical Research Reviews, ١٥(٢), ١٤٧–١٦٥. Retrieved from www.ncbi.nlm.nih.gov.*

٧. *International Council for Harmonisation. (٢٠٢٢). ICH M١٠ Bioanalytical Method Validation Guidelines. ICH Harmonisation for Better Health. Retrieved from www.ich.org.*

٨. *Silva, Fernando, et al. (٢٠٢٠). Advances in LC-MS/MS technology for bioequivalence studies: A systematic review. Bioanalysis, ١٢(٧), ٤٨٧–٥٠٠.*