Bioequivalence and Analytical Validation of Tizanidine 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 detailed bioequivalence analysis and method validation for quantifying tizanidine hydrochloride (TIZ) in human plasma using liquid chromatography-tandem mass spectrometry (LC-MS/MS). The analytical method incorporates internal standardization with amlodipine besylate, achieving high sensitivity and specificity through multiple reaction monitoring (MRM). A robust linear calibration curve (۵۰–۳۲۰۰ ppt) was established, weighted by ۱/x, with a correlation coefficient exceeding ۰.۹۹. Analytical method validation adhered to ICH M۱۰ guidelines, ensuring accuracy, precision, specificity, and robustness. The liquid-liquid extraction protocol optimized sample preparation, while system suitability was confirmed through key performance parameters, including retention time stability and signal-to-noise ratios.

Bioequivalence was evaluated in a single-dose crossover study with healthy volunteers, comparing test and reference formulations. Pharmacokinetic parameters such as maximum plasma concentration (Cmax), time to reach Cmax (Tmax), and area under the curve (AUC) were statistically analyzed, meeting regulatory acceptance criteria. The methodology demonstrated negligible matrix effects and interference, enabling precise quantification of TIZ at therapeutic concentrations. These findings confirm the reliability of this validated LC-MS/MS method for bioequivalence studies and therapeutic drug monitoring of tizanidine.

Keywords: *Tizanidine hydrochloride , LC-MS/MS , Bioequivalence , Analytical method validation , Pharmacokinetics , Liquid-liquid extraction.*

۱. *INTRODUCTION*

Tizanidine hydrochloride (TIZ) is a centrally acting α۲-adrenergic agonist widely prescribed for the management of spasticity associated with neurological disorders such as multiple sclerosis and spinal cord injuries. Its therapeutic efficacy is attributed to the inhibition of excitatory neurotransmitter release, leading to muscle relaxation. Given its narrow therapeutic index and significant interindividual variability in pharmacokinetics, precise quantification of TIZ in human plasma is essential for bioequivalence studies and therapeutic drug monitoring.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) has emerged as the preferred analytical technique for quantifying pharmaceuticals in biological matrices due to its high sensitivity, specificity, and rapid throughput. Previous studies have developed LC-MS/MS methods for TIZ quantification; however, many of these methods lack comprehensive validation or fail to meet the latest regulatory guidelines, such as the International Council for Harmonisation (ICH) M۱۰ guidelines introduced in ۲۰۱۹. These guidelines emphasize the importance of method validation parameters, including accuracy, precision, selectivity, sensitivity, reproducibility, and stability, to ensure the reliability of bioanalytical data.

Recent advancements in LC-MS/MS technology and sample preparation techniques, such as liquid-liquid extraction, have enhanced the detection capabilities for analytes like TIZ. Despite these advancements,

there remains a need for a fully validated, robust, and reproducible analytical method that complies with current regulatory standards and can be effectively applied in bioequivalence studies.

Objectives

- ***General Objective:*** *To develop and validate a sensitive and specific LC-MS/MS method for the quantification of tizanidine hydrochloride in human plasma, adhering to ICH M۱۰ guidelines.*
- ***Specific Objectives:***
  - *To optimize sample preparation using liquid-liquid extraction to achieve high recovery rates of TIZ from human plasma.*
  - *To establish a linear calibration curve for TIZ within a therapeutically relevant concentration range.*
  - *To evaluate the method's accuracy, precision, selectivity, sensitivity, reproducibility, and stability in accordance with regulatory requirements.*
  - *To apply the validated method in a bioequivalence study involving healthy volunteers to assess pharmacokinetic parameters.*

Research Problem

The primary challenge addressed in this study is the lack of a fully validated LC-MS/MS method for TIZ quantification in human plasma that complies with the latest ICH M۱۰ guidelines. Existing methods may not meet these stringent validation criteria, potentially compromising the reliability of bioequivalence assessments and therapeutic drug monitoring.

Importance and Necessity of Research

Theoretical Perspective: This research contributes to the analytical chemistry field by providing a validated method that ensures accurate and precise quantification of TIZ, facilitating better understanding of its pharmacokinetics and pharmacodynamics.

Practical Perspective: Clinicians and researchers will benefit from a reliable analytical tool for monitoring TIZ levels in patients, optimizing therapeutic outcomes, and ensuring patient safety.

Research Background

Several studies have attempted to quantify TIZ in human plasma using various analytical techniques. For instance, Nirogi et al. (۲۰۰۶) developed an LC-MS/MS method for TIZ quantification; however, this method was validated prior to the establishment of the ICH M۱۰ guidelines and may not fully comply with current regulatory standards. Additionally, Bhadauria and Joshi (۲۰۱۹) reported a bioanalytical method for TIZ estimation in human plasma, but the study lacked comprehensive validation data required by contemporary guidelines. These gaps highlight the necessity for developing a method that aligns with current regulatory expectations and offers enhanced sensitivity and specificity.

Hypotheses

۱. *The developed LC-MS/MS method will demonstrate high sensitivity and specificity for TIZ quantification in human plasma.*
۲. *The method will comply with ICH M۱۰ guidelines, exhibiting acceptable accuracy, precision, selectivity, sensitivity, reproducibility, and stability.*
۳. *Application of the validated method in a bioequivalence study will yield reliable pharmacokinetic data for TIZ.*

Purpose of the Study

This study aims to address the existing gap in analytical methodologies by developing and validating an LC-MS/MS method for TIZ quantification in human plasma that adheres to the latest regulatory guidelines. The successful validation and application of this method in bioequivalence studies will provide a reliable tool for therapeutic drug monitoring and support the development of generic formulations of tizanidine hydrochloride.

Methodology

Materials and Reagents

The materials and reagents used for the development and validation of the LC-MS/MS method for tizanidine hydrochloride (TIZ) quantification are summarized in the table below:

Material	***Quantity***	***Source***
Tizanidine Hydrochloride Standard	۱۰ *mg*	*Sigma-Aldrich*
Amlodipine Besylate (Internal Standard)	۱۰ *mg*	*Sigma-Aldrich*
Human Plasma	۱ *L*	*BioreclamationIVT*
Methanol (HPLC Grade)	۲ *L*	*Fisher Scientific*
Acetonitrile (HPLC Grade)	۲ *L*	*Fisher Scientific*
Formic Acid	۱۰۰ *mL*	*Sigma-Aldrich*
Ammonium Formate	۵۰ *g*	*Sigma-Aldrich*
Ethyl Acetate	۱ *L*	*Fisher Scientific*
Water (Milli-Q)	۱۰ *L*	*In-house Laboratory Supply*

Instrumentation

- ***LC-MS/MS System:*** *Agilent ۱۲۹۰ Infinity LC system coupled with an Agilent ٦٤٦۰ Triple Quadrupole Mass Spectrometer.*
- ***Analytical Column:*** *ZORBAX Eclipse Plus C۱۸ column (۲.۱ × ۵۰ mm, ۱.۸ µm particle size).*
- ***Software:*** *Agilent MassHunter Workstation Software for data acquisition and analysis.*

Sample Preparation

۱. ***Preparation of Calibration Standards and Quality Control Samples:***
   - *Stock solutions of TIZ and the internal standard (IS) amlodipine besylate were prepared at a concentration of ۱ mg/mL in methanol.*
   - *Calibration standards were prepared by serial dilution of the TIZ stock solution with blank human plasma to achieve concentrations of ۵۰, ۱۰۰, ۲۰۰, ٤۰۰, ۸۰۰, ۱٦۰۰, and ۳۲۰۰ pg/mL.*
   - *QC samples were prepared at low (۱۵۰ pg/mL), medium (۸۰۰ pg/mL), and high (۲٤۰۰ pg/mL) concentrations within the calibration range.*
   - *All solutions were stored at -۲۰°C until use.*

۲. ***Sample Extraction:***
   - *Liquid-liquid extraction was employed for sample preparation. To ۵۰۰ µL of plasma sample, ۱۰ µL of IS solution (۱.۵ µg/mL) was added.*
   - *The mixture was vortexed for ۲ minutes and allowed to stand for ۵ minutes.*

- *Five milliliters of ethyl acetate were added, and the mixture was vortexed for an additional ۵ minutes.*
- *The samples were centrifuged at ۴۰۰۰ rpm for ۵ minutes, and the organic layer was transferred to a clean glass tube.*
- *The organic solvent was evaporated under a nitrogen stream at ۵۰°C.*
- *The residue was reconstituted in ۲۵۰ µL of a ۵۰:۵۰ (v/v) mixture of methanol and water, vortexed, and transferred to autosampler vials for LC-MS/MS analysis.*

LC-MS/MS Conditions

۱. ***Chromatographic Conditions:***
   - *Mobile Phase A: ۰.۲٪ formic acid in water.*
   - *Mobile Phase B: Methanol.*
   - *Gradient Program:*

Time (min)	% Mobile Phase A	% Mobile Phase B
۰.۰	۳۰	۷۰
۵.۰	۱۰	۹۰
۷.۰	۳۰	۷۰

   - *Flow Rate: ۰.۴ mL/min.*
   - *Column Temperature: ۴۰°C.*
   - *Injection Volume: ۱۰ µL.*
   -

۲. ***Mass Spectrometry Conditions:***
   - *Ionization Mode: Positive electrospray ionization (ESI).*
   - *Multiple Reaction Monitoring (MRM) Transitions:*
     - *TIZ: m/z ۲۵۴.۱ → ۴۴.۱.*
     - *IS: m/z ۴۰۹.۲ → ۲۳۹.۲.*
   - *Capillary Voltage: ۴۰۰۰ V.*
   - *Cone Voltage: ۳۵ V for TIZ, ۲۰ V for IS.*
   - *Source Temperature: ۱۲۰°C.*
   - *Desolvation Temperature: ۴۰۰°C.*
   - *Desolvation Gas Flow Rate: ۸۰۰ L/h.*
   - *Collision Energy: ۲۰ eV for both TIZ and IS.*

Method Validation

The analytical method was validated following ICH M۱۰ guidelines, encompassing the following parameters:

۱. ***Selectivity:***
   - *Six blank plasma samples from different sources were analyzed to evaluate the absence of interfering peaks at the retention times of TIZ and IS.*

۲. ***Linearity:***
   - *Calibration curves were constructed using seven concentrations ranging from ۵۰ to ۳۲۰۰ pg/mL.*
   - *Weighted least squares regression (۱/x weighting) was applied to calculate the slope, intercept, and correlation coefficient.*

۳. ***Accuracy and Precision:***
   - *Intra- and inter-day accuracy and precision were assessed by analyzing QC samples at three concentrations (low, medium, high) in triplicate over three days.*
   - *Accuracy was expressed as the percent deviation from the nominal concentration, and precision was calculated as the percent relative standard deviation (%RSD).*

۴. ***Recovery:***

- *The recovery of TIZ and IS was determined by comparing the peak areas of extracted QC samples to those of unextracted standards at equivalent concentrations.*

۵. ***Matrix Effects:***
   - *Matrix effects were assessed by comparing the peak areas of analytes spiked into post-extracted plasma to those of analytes spiked into neat solutions.*

۶. ***Stability:***
   - *Stability tests included freeze-thaw stability, short-term stability, long-term stability, and post-preparative stability.*
   - *QC samples were analyzed after storage under specific conditions, and concentrations were compared to freshly prepared standards.*

Application to Bioequivalence Study

The validated method was applied to a bioequivalence study involving ۲۶ healthy volunteers in a single-dose, crossover design. Plasma samples were collected at predetermined time points and analyzed for TIZ concentrations. Pharmacokinetic parameters, including Cmax, Tmax, and AUC, were calculated using non-compartmental analysis to evaluate bioequivalence between test and reference formulations.

Results Summary

- ***Linearity:*** *Correlation coefficient (r) > ۰.۹۹.*
- ***Accuracy:*** *Within ±۱۰٪ of nominal concentrations.*
- ***Precision:*** *%RSD < ۱۵٪ for all QC levels.*
- ***Recovery:*** *>۸۵٪ for both TIZ and IS.*
- ***Matrix Effects:*** *<۱۵٪ variability.*
- ***Stability:*** *TIZ was stable under all tested conditions.*

This comprehensive methodology ensured the reliability of the LC-MS/MS method for both analytical validation and bioequivalence studies.

Discussion

The study successfully developed and validated an LC-MS/MS method for quantifying tizanidine hydrochloride (TIZ) in human plasma. The method's adherence to ICH M۱۰ guidelines ensures its reliability for bioequivalence studies and therapeutic drug monitoring. Incorporating internal standardization with amlodipine besylate significantly enhanced accuracy and precision by compensating for variability in sample preparation and analysis.

The linear calibration range (۵۰–۳۲۰۰ ppt) and the correlation coefficient exceeding ۰.۹۹ demonstrate the robustness of the analytical method. These results align with recent advancements in LC-MS/MS methodologies, which emphasize high sensitivity and specificity for pharmacokinetic analyses.

Sample preparation using liquid-liquid extraction was optimized to achieve high recovery rates (>۸۵٪) while minimizing matrix effects (<۱۵٪). This optimization underscores the importance of efficient extraction techniques in enhancing the method's performance. The negligible matrix effects observed indicate that the method is suitable for complex biological matrices, a crucial requirement for pharmacokinetic studies.

The bioequivalence study conducted using this validated method demonstrated that the test and reference formulations of TIZ met regulatory acceptance criteria for pharmacokinetic parameters, including Cmax, Tmax, and AUC. These findings confirm the interchangeability of the two formulations, thereby supporting their clinical application.

The stability studies ensured that TIZ remains stable under various conditions, including freeze-thaw cycles and long-term storage. This stability is critical for the practical application of the method in clinical and research settings.

Compared to previous studies, this method offers significant improvements in sensitivity, specificity, and compliance with regulatory standards. For instance, earlier methods often lacked comprehensive

validation data or did not adhere to contemporary guidelines, limiting their applicability in bioequivalence studies.

Conclusion

This study presents a validated LC-MS/MS method for the quantification of TIZ in human plasma, offering high sensitivity, specificity, and robustness. By adhering to ICH M١٠ guidelines, the method ensures reliable pharmacokinetic assessments necessary for bioequivalence studies and therapeutic drug monitoring. The method's high sensitivity enables precise quantification even at low concentrations, which is essential for therapeutic drug monitoring and ensuring accurate dose adjustments.

The bioequivalence study successfully demonstrated the interchangeability of the test and reference formulations of TIZ, meeting all regulatory criteria. This is a significant advancement, as it provides confidence in the use of generic formulations, potentially reducing healthcare costs while maintaining therapeutic efficacy.

The comprehensive stability tests conducted reinforce the practicality of the method for routine clinical and laboratory use. Stability under various conditions ensures that the method can be reliably used across different clinical and research scenarios, providing consistent and accurate results.

Future research could expand on this work by exploring its applicability to other similar pharmaceutical compounds. Additionally, the method could be adapted for use with high-throughput automation systems to further enhance its utility in large-scale bioequivalence studies.

In conclusion, the validated LC-MS/MS method developed in this study sets a benchmark for analytical methods in pharmacokinetics, providing a reliable tool for bioequivalence studies and therapeutic monitoring of tizanidine. This advancement not only addresses current challenges but also paves the way for improved patient care and drug development processes. Its adoption in clinical and research settings is expected to have a significant impact on enhancing drug safety, efficacy, and accessibility.

References

١. *Bhadauria, R., and Joshi, A. (٢٠١٩). Bioanalytical method development and validation for estimation of tizanidine in human plasma using LC-MS/MS. Journal of Pharmaceutical Analysis. ٩(٤), ٢٥٣–٢٦٠.*

٢. *Chandra, R., and Kumar, M. (٢٠١٤). Advances in pharmacokinetics: Applications in bioequivalence studies. International Journal of Drug Development and Research. ٦(٣), ١١–٢٥.*

٣. *International Council for Harmonisation. (٢٠١٩). ICH M١٠: Guideline for Bioanalytical Method Validation. ICH Official Publication. Retrieved from [ICH Website].*

٤. *Nirogi, R., Kandikere, V., Shukla, M., Mudigonda, K., Maurya, S., and Boosi, R. (٢٠٠٦). Liquid chromatography–tandem mass spectrometry method for quantification of tizanidine in human plasma. Biomedical Chromatography. ٢٠(٩), ٩٠٣–٩١١.*

٥. *Singh, A., and Chauhan, R. (٢٠٢٠). Recent advances in bioanalytical method validation: A regulatory perspective. Journal of Pharmaceutical Sciences. ١٠٩(١), ٥–١٦.*

٦. *Villaverde, J., and Sánchez, C. (٢٠٢١). Modern approaches in LC-MS/MS for quantification of small molecules in biological matrices. Analytical Chemistry Research. ٣٠, ١٠٠٣٨.*

٧. *Waters, P., and Griffith, L. (٢٠٢٠). Application of high-performance liquid chromatography in pharmaceutical analysis: A decade of advancements. Journal of Chromatographic Science. ٥٨(٧), ٤٥٣–٤٦٨.*