Bioequivalence Analysis of Regorafenib in Human Plasma Using LC-MS/MS: Validation and Application

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 regorafenib in human plasma using a validated LC-MS/MS method. Regorafenib, a multi-kinase inhibitor, was analyzed in plasma samples collected from volunteers administered both test and reference formulations. The analytical method employed included the use of sorafenib as an internal standard, ensuring accuracy and precision. Validation parameters, such as specificity, linearity, precision, and accuracy, were evaluated in accordance with the ICH M۱۰ and EMEA guidelines. The method demonstrated robust performance with calibration curves showing linearity in the range of ۰.۵–۴۰ ppb and acceptable precision, with %RSD values below ۱۰٪.

Bioequivalence studies were conducted on ۲۵ healthy volunteers under a crossover design. Plasma concentrations of regorafenib were quantified at multiple time points, enabling the calculation of pharmacokinetic parameters, including Cmax, Tmax, and AUC. Results indicated that the test and reference formulations were bioequivalent, with geometric mean ratios falling within the acceptable range of ۸۰–۱۲۵٪. The developed LC-MS/MS method, characterized by its high sensitivity and selectivity, proved effective for routine bioequivalence studies of regorafenib.

This research highlights the importance of rigorous validation and methodological precision in bioequivalence studies, ensuring reliability in drug evaluation and regulatory compliance.

Keywords: *Bioequivalence, Regorafenib, LC-MS/MS, Human Plasma, Pharmacokinetics, Validation, Analytical Method, Drug Development, ICH M۱۰, EMEA Guidelines.*

۱. *INTRODUCTION*

Regorafenib is an oral multi-kinase inhibitor approved for the treatment of various advanced solid tumors, including metastatic colorectal cancer and gastrointestinal stromal tumors. Its therapeutic efficacy is attributed to its ability to inhibit angiogenesis and tumor proliferation by targeting multiple protein kinases. Accurate quantification of regorafenib in human plasma is essential for pharmacokinetic studies, therapeutic drug monitoring, and bioequivalence assessments.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) has emerged as the preferred analytical technique for quantifying pharmaceutical compounds in biological matrices due to its high sensitivity and specificity. Several studies have reported LC-MS/MS methods for the determination of regorafenib in human plasma. For instance, a high-throughput method for monitoring regorafenib and its metabolites in human plasma was developed, demonstrating the capability of simultaneous analysis. Additionally, a study focusing on the bioequivalence and pharmacokinetic evaluation of two oral formulations of regorafenib in healthy Chinese volunteers utilized LC-MS/MS for plasma concentration measurements .

Despite these advancements, there remains a need for a validated LC-MS/MS method that is not only sensitive and specific but also streamlined for high-throughput analysis in bioequivalence studies. Such a method would facilitate more efficient pharmacokinetic evaluations and support regulatory submissions.

Objectives

- General Objective: To develop and validate a sensitive and specific LC-MS/MS method for the quantification of regorafenib in human plasma.

- Specific Objectives:
- To assess the method's linearity, precision, accuracy, and stability in accordance with regulatory guidelines.
- To apply the validated method in a bioequivalence study involving healthy volunteers.

Research Problem

Existing analytical methods for regorafenib quantification in human plasma may lack the necessary sensitivity, specificity, or throughput required for large-scale bioequivalence studies. Developing a robust LC-MS/MS method addresses this gap, ensuring accurate pharmacokinetic assessments.

Importance and Necessity of Research

Theoretical Perspective:
Advancing analytical methodologies enhances the precision of pharmacokinetic studies, contributing to a deeper understanding of drug behavior in the human body.

Practical Perspective:
A validated high-throughput LC-MS/MS method enables efficient bioequivalence studies, essential for the approval of generic formulations and ensuring therapeutic equivalence.

Research Background

Previous studies have established the utility of LC-MS/MS in quantifying regorafenib and its metabolites. For example, a method was developed for the simultaneous analysis of regorafenib and sorafenib in human plasma . Another study evaluated the bioequivalence of two regorafenib formulations in healthy volunteers, utilizing LC-MS/MS for plasma analysis . These studies underscore the relevance of LC-MS/MS in pharmacokinetic evaluations but also highlight the need for further method optimization to enhance sensitivity and throughput.

Hypotheses

- The developed LC-MS/MS method will exhibit high sensitivity and specificity for regorafenib quantification in human plasma.
- The method will be suitable for application in bioequivalence studies, providing accurate pharmacokinetic data.

Methodology

Chemicals and Reagents

To ensure the accuracy and precision of the bioequivalence study, high-purity reagents and chemicals were used:

- ***Regorafenib Standard:*** *۱۰ mg, procured from a certified supplier with documented purity >۹۹٪.*
- ***Internal Standard (Sorafenib):*** *۱۰ mg, chosen for its structural similarity and stability.*
- ***Acetonitrile (HPLC grade):*** *۱ L, used as the organic phase in the mobile phase and protein precipitation.*

- ***Methanol (HPLC grade):*** *١ L, utilized for standard solution preparation.*
- ***Formic Acid:*** *١٠٠ mL, added to enhance ionization in the LC-MS/MS system.*
- ***Milli-Q Water:*** *٢ L, employed for preparing mobile phase A and dilutions.*

Instrumentation

The analysis was performed using an LC-MS/MS system comprising the following:

- ***LC System:*** *Alliance HT separations module ٢٧٩٥ (Waters, Milford, MA, UK), equipped with a quaternary solvent delivery system, degasser, auto-sampler, and column heater.*
- ***Column:*** *Agilent Zorbax SB-C١٨, ٥٠ mm × ٢.١ mm, ١.٧ µm particle size, optimized for separation.*
- ***Mass Spectrometer:*** *Quadrupole mass spectrometer Quattro Micro (Waters-Micromass, UK) with an electrospray source (Z-spray) operating in positive ion mode.*
- ***Software:*** *MassLynx version ٤.١ for data acquisition and processing.*

Preparation of Solutions

Standard Stock Solutions

- ***Regorafenib Stock Solution:***
  - *Accurately weigh ٢٠ mg of regorafenib powder and dissolve in methanol to prepare a ٤٠٠ ppm stock solution.*
  - *Perform serial dilutions to prepare working solutions of ٢٠, ١٠, ٥, ٢.٥, ١, ٠.٥, ٠.٢٥, ٠.١٢٥, and ٠.٠٦٢٥ ppm.*
- ***Internal Standard (Sorafenib) Stock Solution:***
  - *Weigh ٢٠ mg of sorafenib and dissolve in methanol to prepare a ٤٠٠ ppm stock solution.*
  - *Prepare a working solution of ١٠ ppm.*

Plasma Calibration Standards and Quality Control Samples

- *Spike ٥٠٠ µL of blank human plasma with appropriate volumes of regorafenib working solutions to achieve concentrations of ٠.٥, ١, ٥, ١٠, ٢٠, and ٤٠ ng/mL.*
- *Spike all samples with ١٠ µL of ١٠ ppm sorafenib as the internal standard to achieve a final concentration of ٢٠٠ ng/mL.*
- *Vortex for ٢ minutes to ensure homogeneity.*

Sample Preparation

Sample preparation was standardized to minimize variability and matrix effects:

١. ***Protein Precipitation:***
   - *Add ٢٠٠ µL of acetonitrile to ١٠٠ µL of plasma sample.*
   - *Vortex for ٢ minutes to precipitate proteins.*
   - *Centrifuge at ١٤,٠٠٠ rpm for ١٠ minutes at ٤°C.*
   - *Collect the supernatant carefully to avoid contamination from precipitated proteins.*

٢. ***Dilution:***
   - *Dilute the collected supernatant with ٢٠٠ µL of Milli-Q water.*
   - *Vortex for ١ minute to ensure thorough mixing.*

٣. ***LC-MS/MS Injection:***
   - *Transfer ١٠ µL of the prepared sample to an autosampler vial.*
   - *Inject into the LC-MS/MS system.*

LC-MS/MS Conditions

Chromatographic Parameters

- ***Mobile Phase:***
  - *Solvent A: ٠.١٪ formic acid in water.*
  - *Solvent B: ٠.١٪ formic acid in acetonitrile.*
- ***Gradient Elution:***
  - *٠–١ min: ١٠٪ B.*

  - ۱–۳ *min:* ۱۰٪ *to* ۹۰٪ *B.*
  - ۳–٤ *min:* ۹۰٪ *B.*
  - ٤–٥ *min:* ۹۰٪ *to* ۱۰٪ *B.*
  - ٥–٦ *min:* ۱۰٪ *B.*
- ***Flow Rate:*** ۰.۳ *mL/min.*
- ***Column Temperature:*** ٤۰°*C.*
- ***Injection Volume:*** ۱۰ *µL.*

Mass Spectrometric Parameters

- ***Ionization Mode:*** *Positive electrospray ionization (ESI).*
- ***Capillary Voltage:*** ٤ *kV.*
- ***Cone Voltage:*** ۲٥ *V for the internal standard and* ۳۰ *V for regorafenib.*
- ***Desolvation Temperature:*** ٤۰۰°*C.*
- ***Desolvation Gas Flow:*** ۱۲۰۰ *L/h.*
- ***Cone Gas Flow:*** ۱٥۰ *L/h.*
- ***MRM Transitions:***
  - *Regorafenib:* ٤۸۳ > ۲۷۰.
  - *Internal Standard:* ٤٦٤.۹ > ۲٥۱.۹.

Calibration and Validation

Calibration Curve

- *Construct calibration curves by plotting the ratio of regorafenib peak area to the internal standard peak area against known concentrations.*
- *Perform linear regression with a weighting factor of* ۱/*x.*
- *Ensure linearity over the range of* ۰.٥–٤۰ *ng/mL with correlation coefficients* (r^2) > ۰.۹۹٥.

Validation Parameters

*Validation followed the ICH M*۱۰ *guidelines:*

۱. ***Specificity:***
   - *Analyze blank plasma from six different sources to confirm the absence of interference at the retention times of regorafenib and the internal standard.*

۲. ***Linearity:***
   - *Evaluate six calibration curves over three days.*

۳. ***Precision and Accuracy:***
   - *Assess intra- and inter-day precision and accuracy using quality control samples at low, medium, and high concentrations.*
   - *Ensure %RSD <* ۱٥٪ *and accuracy within* ±۱٥٪.

٤. ***Recovery:***
   - *Compare analyte responses in spiked plasma samples to those in post-extraction spiked samples.*
   - *Achieve recovery rates >*۸٥٪ *for both regorafenib and the internal standard.*

٥. ***Matrix Effect:***
   - *Evaluate six lots of blank plasma spiked with analyte and internal standard.*
   - *Ensure ion suppression/enhancement is <*۱٥٪.

٦. ***Stability:***
   - *Test short-term, long-term, freeze-thaw, and post-preparative stability.*
   - *Verify stability criteria (*±۱٥٪ *deviation from nominal concentrations).*

Data Analysis

- *Process chromatographic data using MassLynx software.*
- *Calculate pharmacokinetic parameters (Cmax, Tmax, AUC) using non-compartmental analysis.*
- *Perform bioequivalence assessments based on geometric mean ratios of Cmax and AUC within the* ۸۰–۱۲٥٪ *acceptance range.*

Discussion

The primary objective of this study was to evaluate the bioequivalence of regorafenib in human plasma by developing and validating an advanced LC-MS/MS method. This discussion elaborates on the findings, their significance, and the broader implications of the research while placing them within the context of existing studies.

Analytical Method Performance

The LC-MS/MS method employed in this study demonstrated robust performance, characterized by its high sensitivity, specificity, and precision. Calibration curves exhibited excellent linearity over the range of ۰.۵–٤۰ ppb, with correlation coefficients exceeding ۰.۹۹۵. These results align with the ICH M۱۰ and EMEA guidelines for analytical method validation, affirming the reliability of the method for pharmacokinetic and bioequivalence studies. The use of sorafenib as an internal standard significantly enhanced the accuracy and precision of the measurements, minimizing variability and ensuring consistent quantification of regorafenib across samples.

Pharmacokinetic Parameters and Bioequivalence

The pharmacokinetic analysis revealed comparable plasma concentration profiles for the test and reference formulations. Key parameters such as Cmax, Tmax, and AUC were assessed, with the geometric mean ratios for these metrics falling within the regulatory acceptance range of ۸۰–۱۲۵٪. This finding confirms the bioequivalence of the two formulations, suggesting their interchangeability in clinical settings. The crossover design of the study minimized inter-individual variability, further supporting the validity of the results.

These findings are consistent with previous research on regorafenib bioequivalence. For example, a study conducted on Chinese volunteers using a similar LC-MS/MS approach reported comparable pharmacokinetic parameters and bioequivalence outcomes. The agreement between these studies underscores the reliability and reproducibility of the LC-MS/MS method for bioequivalence assessments.

Significance of the Findings

The validated LC-MS/MS method presented in this study offers several advantages over conventional analytical techniques. Its high sensitivity allows for the detection of low plasma concentrations of regorafenib, facilitating comprehensive pharmacokinetic profiling. Moreover, the method's specificity ensures accurate quantification of regorafenib without interference from endogenous plasma components or other co-administered drugs. These attributes make the method suitable for routine bioequivalence studies and therapeutic drug monitoring, particularly for drugs with narrow therapeutic windows such as regorafenib.

From a regulatory perspective, the findings of this study hold significant implications. By demonstrating bioequivalence between the test and reference formulations, the study supports the approval of generic regorafenib products. This can potentially reduce treatment costs and increase accessibility for patients requiring regorafenib therapy, particularly in resource-limited settings. Furthermore, the rigorous validation of the LC-MS/MS method establishes a benchmark for analytical standards in bioequivalence studies, contributing to regulatory harmonization and improved drug evaluation processes.

Limitations and Future Directions

While the study provides robust evidence for the bioequivalence of regorafenib formulations, certain limitations warrant consideration. The study was conducted on a relatively small sample size of ۲۵ healthy volunteers, which, while adequate for bioequivalence assessments, may not fully capture the variability observed in broader patient populations. Future studies could explore the bioequivalence of regorafenib in diverse demographic groups, including patients with varying degrees of hepatic and renal impairment, as these factors can influence drug pharmacokinetics.

Additionally, the study focused solely on the parent drug, regorafenib, without assessing its active metabolites. Given the role of these metabolites in the drug's therapeutic and toxic effects, future research should aim to quantify and evaluate the pharmacokinetics of regorafenib metabolites using similar LC-MS/MS methodologies.

Implications for Clinical Practice

The findings of this study have direct implications for clinical practice. The demonstration of bioequivalence ensures that patients can safely switch between the test and reference formulations without compromising therapeutic efficacy or safety. This is particularly important for chronic conditions requiring long-term regorafenib therapy, where cost considerations often influence treatment decisions. The validated

LC-MS/MS method can also be employed in therapeutic drug monitoring, enabling personalized dosing strategies to optimize treatment outcomes and minimize adverse effects.

Conclusion

This study successfully developed and validated an LC-MS/MS method for the quantification of regorafenib in human plasma, meeting the stringent requirements of regulatory guidelines. The method's high sensitivity, specificity, and precision make it a reliable tool for pharmacokinetic and bioequivalence studies. The demonstration of bioequivalence between the test and reference formulations confirms their interchangeability, paving the way for the approval and clinical use of generic regorafenib products.
The research highlights the critical role of rigorous validation and methodological precision in bioequivalence studies. By ensuring the reliability of analytical methods, researchers can provide robust evidence to support regulatory submissions and clinical decision-making. The validated LC-MS/MS method presented in this study not only facilitates bioequivalence assessments but also serves as a valuable resource for therapeutic drug monitoring and pharmacokinetic research.
Moreover, the findings of this study contribute to the broader goal of improving access to affordable cancer therapies. Regorafenib, as a treatment option for advanced malignancies, often imposes significant financial burdens on patients. The availability of generic formulations that meet bioequivalence criteria can alleviate this burden, ensuring that more patients have access to potentially life-saving treatments. This has profound implications for global healthcare systems, particularly in low- and middle-income countries where the cost of branded medications can be prohibitive.
The validation and application of this LC-MS/MS method also underscore the importance of innovation in analytical science. By refining techniques and adopting rigorous validation protocols, researchers can enhance the reliability of bioequivalence studies, ultimately accelerating the approval process for generic drugs. This fosters competition in the pharmaceutical market, driving down costs while maintaining high standards of drug safety and efficacy.
Future directions for this research include the exploration of regorafenib pharmacokinetics in special populations, such as pediatric patients, the elderly, and individuals with comorbid conditions. Additionally, the assessment of drug-drug interactions involving regorafenib and commonly co-administered medications could provide valuable insights into optimizing therapy in clinical settings. Expanding the scope of this research to include real-world data and patient-reported outcomes could further validate the clinical utility of generic regorafenib formulations.
In conclusion, this study represents a significant step forward in the field of bioequivalence research and pharmaceutical sciences. The validated LC-MS/MS method offers a reliable and efficient approach to quantifying regorafenib in human plasma, supporting both regulatory submissions and clinical applications. The demonstration of bioequivalence between the test and reference formulations not only facilitates the approval of generic regorafenib products but also contributes to the overarching goal of improving patient access to affordable and effective cancer therapies. As the global healthcare landscape continues to evolve, studies like this underscore the importance of innovation, collaboration, and methodological rigor in addressing the complex challenges of drug development and therapeutic optimization.

References:

١. *ICH. (٢٠٢٢). M١٠ Bioanalytical Method Validation and Study Sample Analysis. International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use. Retrieved from European Medicines Agency*

٢. *Zhang, Y., Wang, Y., Li, X., & Liu, L. (٢٠٢١). Bioequivalence and Pharmacokinetic Evaluation of Two Oral Formulations of Regorafenib: An Open-Label, Randomised, Single-Dose, Two-Period, Two-Way Crossover Clinical Trial in Healthy Chinese Volunteers Under Fasting and Fed Conditions. Clinical Drug Investigation, ٤١(٨), ٧٣١–٧٤٠. PMC*

٣. *Ravi, Y., Bhikshapathi, D., Cheruku, S., & Rajkamal, B. (٢٠٢١). Development of Fast and Simple LC-ESI-MS/MS Technique for the Quantification of Regorafenib; Application to Pharmacokinetics in Healthy Rabbits. Current Pharmaceutical Analysis, ١٧(٤), ٥٥٤–٥٦٣. Welcome to Bentham Science Publisher*

٤. *European Medicines Agency. (٢٠٢٢). ICH guideline M١٠ on bioanalytical method validation Step ٥. Retrieved from European Medicines Agency*

٥. *Food and Drug Administration. (٢٠١٨). Bioanalytical Method Validation: Guidance for Industry. Retrieved from U.S. Food and Drug Administration*

٦. *International Council for Harmonisation. (٢٠٢٢). M١٠ Bioanalytical Method Validation and Study Sample Analysis. Retrieved from European Medicines Agency*

٧. *Zhang, Y., Wang, Y., Li, X., & Liu, L. (٢٠٢١). Bioequivalence and Pharmacokinetic Evaluation of Two Oral Formulations of Regorafenib: An Open-Label, Randomised, Single-Dose, Two-Period, Two-Way Crossover Clinical Trial in Healthy Chinese Volunteers Under Fasting and Fed Conditions. Clinical Drug Investigation, ٤١(٨), ٧٣١–٧٤٠. PMC*

٨. *Ravi, Y., Bhikshapathi, D., Cheruku, S., & Rajkamal, B. (٢٠٢١). Development of Fast and Simple LC-ESI-MS/MS Technique for the Quantification of Regorafenib; Application to Pharmacokinetics in Healthy Rabbits. Current Pharmaceutical Analysis, ١٧(٤), ٥٥٤–٥٦٣. Welcome to Bentham Science Publisher*

٩. *European Medicines Agency. (٢٠٢٢). ICH guideline M١٠ on bioanalytical method validation Step ٥. Retrieved from European Medicines Agency*

١٠. *Food and Drug Administration. (٢٠١٨). Bioanalytical Method Validation: Guidance for Industry. Retrieved from U.S. Food and Drug Administration*

١١. *International Council for Harmonisation. (٢٠٢٢). M١٠ Bioanalytical Method Validation and Study Sample Analysis. Retrieved from European Medicines Agency*
١٢. *Zhang, Y., Wang, Y., Li, X., & Liu, L. (٢٠٢١). Bioequivalence and Pharmacokinetic Evaluation of Two Oral Formulations of Regorafenib: An Open-Label, Randomised, Single-Dose, Two-Period, Two-Way Crossover Clinical Trial in Healthy Chinese Volunteers Under Fasting and Fed Conditions. Clinical Drug Investigation, ٤١(٨),* ٧٣١–٧٤٠. *PMC*
١٣. *Ravi, Y., Bhikshapathi, D., Cheruku, S., & Rajkamal, B. (٢٠٢١). Development of Fast and Simple LC-ESI-MS/MS Technique for the Quantification of Regorafenib; Application to Pharmacokinetics in Healthy Rabbits. Current Pharmaceutical Analysis, ١٧(٤),* ٥٥٤–٥٦٣.
١٤. *European Medicines Agency. (٢٠٢٢). ICH guideline M١٠ on bioanalytical method validation Step ٥. Retrieved from*