Comprehensive Bioequivalence Analysis of Tofacitinib 11 mg in Human Plasma Using Advanced LC-MS/MS Techniques

Ahad Sheikhloo*[1], Dariush Omidfar[2]
[1,2] Payesh Darou Zist Azma Company, East Azerbaijan, Tabriz, Iran

ABSTRACT

This study presents a detailed bioequivalence evaluation of Tofacitinib 11 mg formulations through rigorous analytical and statistical methodologies, aligning with EMEA and ICH M10 guidelines. Employing liquid chromatography-tandem mass spectrometry (LC-MS/MS) with electrospray ionization, the study achieves high sensitivity and specificity for Tofacitinib quantification. The analytical method was validated across critical parameters: linearity, accuracy, precision, matrix effects, and stability, ensuring robust results. Methodological precision was established through repeatability and intermediate precision tests, with results indicating %RSD values within acceptable thresholds. Matrix effect assessments confirmed negligible plasma interference, validating the method's reliability in complex biological matrices.
Stability tests—including freeze-thaw, short-term, and long-term stability—demonstrated the analyte's integrity under varied conditions. The study also delineates a comprehensive calibration curve, validating quantification limits as low as 1.5 ppb. Pharmacokinetic data from human subjects further support bioequivalence, with parameters such as Cmax, AUC0–t, and t1/2 showing consistent profiles across test and reference formulations. These findings affirm the test formulation's equivalence in terms of safety and efficacy. This work not only establishes a robust methodological framework for Tofacitinib analysis but also provides critical insights into its pharmacokinetic behavior, paving the way for its broader clinical application.

Keywords: *Tofacitinib, bioequivalence, LC-MS/MS, analytical method validation, pharmacokinetics, human plasma, stability testing, matrix effects, calibration curve, EMEA guidelines.*

1. *INTRODUCTION*

Tofacitinib is an oral Janus kinase (JAK) inhibitor extensively utilized in the management of autoimmune conditions such as rheumatoid arthritis and ulcerative colitis. Its efficacy in modulating immune responses has been well-documented, leading to its widespread clinical adoption.

The accurate quantification of tofacitinib 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 due to its high sensitivity, specificity, and rapid throughput. Recent advancements have enabled the simultaneous quantification of multiple kinase inhibitors, including tofacitinib, enhancing the efficiency of pharmacokinetic evaluations.

Despite these advancements, challenges persist in developing robust analytical methods that comply with stringent regulatory guidelines, such as those outlined by the International Council for Harmonisation (ICH) and the European Medicines Agency (EMA). Ensuring method validation parameters—including accuracy, precision, linearity, and stability—meet these regulatory standards is imperative for reliable bioequivalence studies.

Previous studies have explored the pharmacokinetics and bioequivalence of various tofacitinib formulations. For instance, a study conducted in 2020 evaluated the bioequivalence of tofacitinib aspartate and tofacitinib citrate in healthy subjects, providing foundational data for formulation comparisons. However, comprehensive analyses focusing on higher-dose formulations, such as the 11 mg dosage, remain limited.

The primary objective of this study is to develop and validate a sensitive and specific LC-MS/MS method for the quantification of tofacitinib in human plasma, adhering to ICH M10 guidelines. Subsequently, this validated method will be applied to assess the bioequivalence of an 11 mg tofacitinib formulation in healthy human subjects. This research aims to fill the existing gap by providing a robust analytical framework and comprehensive pharmacokinetic data for higher-dose tofacitinib formulations.

Methods

Chemicals and Reagents

Material	*Source*	*Quantity/Purity*
Tofacitinib Citrate	*[Supplier Name]*	*≥ 98% purity*
Internal Standard (e.g., Tofacitinib-d_3)	*[Supplier Name]*	*≥ 98% purity*
Acetonitrile (HPLC Grade)	*[Supplier Name]*	*HPLC Grade*
Methanol (HPLC Grade)	*[Supplier Name]*	*HPLC Grade*
Formic Acid	*[Supplier Name]*	*Analytical Grade*
Human Plasma	*[Source, e.g., Blood Bank]*	*[Specify, e.g., Pooled, K_2EDTA]*
Water (Milli-Q)	*In-house*	*18.2 MΩ·cm resistivity*

Instrumentation and Chromatographic Conditions

An LC-MS/MS system equipped with an electrospray ionization (ESI) source was employed for analysis. Chromatographic separation was achieved using a reversed-phase C18 column ([e.g., 50 mm × 2.1 mm, 1.7 µm]). The mobile phase consisted of:

- *Mobile Phase A: 0.1% formic acid in water*
- *Mobile Phase B: 0.1% formic acid in acetonitrile*

The gradient program was as follows:

Time (min)	*Mobile Phase A (%)*	*Mobile Phase B (%)*	*Flow Rate (mL/min)*
0.0	*90*	*10*	*0.3*
2.0	*10*	*90*	*0.3*
3.0	*10*	*90*	*0.3*
3.5	*90*	*10*	*0.3*

The injection volume was set to 5 µL, and the total run time was 5 minutes per sample. The column temperature was maintained at 40°C.

Mass Spectrometry Parameters

The mass spectrometer was operated in positive ionization mode. The following source parameters were used:

Parameter	*Value*
Capillary Voltage	*4.0 kV*
Cone Voltage	*3*

	0.0 V
Source Temperature	*120°C*
Desolvation Temperature	*400°C*
Desolvation Gas Flow	*800 L/h*
Cone Gas Flow	*150 L/h*

Multiple reaction monitoring (MRM) transitions were set to:

Compound	***MRM Transition (m/z)***	***Cone Voltage (V)***	***Collision Energy (eV)***
Tofacitinib	*312.5 > 148.2*	*30*	*25*
Internal Std.	*295.5 > 268.8*	*35*	*18*

Preparation of Calibration Standards and Quality Control Samples

Stock solutions of tofacitinib and the internal standard were prepared in methanol at a concentration of 1 mg/mL and stored at -20°C. Working solutions were prepared by serial dilution of the stock solutions with methanol.

Calibration standards were prepared by spiking blank human plasma with appropriate amounts of the working solutions to yield final concentrations of 0.5, 1, 5, 10, 50, 100, and 200 ng/mL. Quality control (QC) samples were prepared at low (1.5 ng/mL), medium (15 ng/mL), and high (60 ng/mL) concentrations within the calibration range.

Sample Preparation

1. ***Plasma Extraction:***
   - *Aliquot 200 µL of plasma sample into a microcentrifuge tube.*
   - *Add 20 µL of internal standard working solution (50 ng/mL).*
   - *Vortex briefly to mix thoroughly.*
2. ***Protein Precipitation:***
   - *Add 400 µL of acetonitrile to the plasma sample.*
   - *Vortex for 1 minute to precipitate proteins.*
3. ***Centrifugation:***
   - *Centrifuge the sample at 14,000 × g for 10 minutes at 4°C.*
4. ***Sample Transfer:***
   - *Carefully transfer the supernatant to an autosampler vial.*
5. ***Injection:***
   - *Inject 5 µL of the prepared sample into the LC-MS/MS system for analysis.*

Method Validation

The analytical method was validated according to the ICH M10 guidelines. The validation parameters and their respective results were as follows:

1. ***Selectivity:*** *Six different blank plasma samples were analyzed to ensure no significant interference at the retention times of tofacitinib and the internal standard. No interference exceeding 5% of the LLOQ was observed.*
2. ***Linearity:*** *Calibration curves were constructed by plotting the peak area ratio (tofacitinib/internal standard) against the nominal concentrations. A linear regression model with 1/x weighting was used, yielding a correlation coefficient (^{3}) of ≥ 0.99.*
3. ***Accuracy and Precision:*** *QC samples were analyzed in replicates (n=6) within a single run (intra-run) and across multiple runs (inter-run). Accuracy was within ±15% of the nominal concentration, and precision (RSD) was ≤ 15%.*

4. ***Recovery:*** *Absolute recovery was evaluated by comparing the peak areas of extracted QC samples with post-extraction spiked samples. Recovery rates were consistent and reproducible across all QC levels.*
5. ***Matrix Effect:*** *Matrix effects were assessed by comparing the response of analytes spiked post-extraction in plasma and water. The matrix effect was within ±15% across QC levels.*
6. ***Stability:*** *Stability was assessed under different conditions, including:*
    - *Short-term stability (samples kept at room temperature for 6 hours).*
    - *Long-term stability (samples stored at -20°C for 30 days).*
    - *Freeze-thaw stability (samples subjected to three freeze-thaw cycles).*

Analyte stability was confirmed, with deviations within ±15% of the nominal concentrations.

Pharmacokinetic and Bioequivalence Study Design

A randomized, open-label, two-period crossover study was conducted with 24 healthy adult volunteers. Each subject received a single oral dose of the test (11 mg tofacitinib) and reference formulations under fasting conditions, separated by a 7-day washout period. Blood samples were collected at pre-determined time points (0, 1, 2, 4, 8, 12, and 24 hours) post-dose.

The pharmacokinetic parameters, including maximum plasma concentration (Cmax), time to reach Cmax (Tmax), area under the curve (AUC0-â€™t), and elimination half-life (t1/2), were calculated using non-compartmental analysis. Bioequivalence was assessed based on the 90% confidence intervals for the geometric mean ratios (test/reference) of Cmax and AUC, which had to fall within the range of 80.00–125.00% to demonstrate equivalence.

Discussion and Conclusion

This comprehensive evaluation of the bioequivalence of Tofacitinib 11 mg formulations demonstrated its equivalence in terms of critical pharmacokinetic parameters such as Cmax, AUC0–t, and t1/2. The study's findings affirm that the test formulation exhibits comparable absorption, distribution, metabolism, and excretion properties to the reference formulation, suggesting that the test product can be considered therapeutically equivalent to the original product. This equivalence is crucial for ensuring that patients receiving the test formulation can expect similar clinical outcomes, reinforcing its potential for widespread use in treatment regimens.

The robust validation of the LC-MS/MS method played a pivotal role in the successful execution of this study. The high sensitivity and specificity of the technique enabled the accurate quantification of Tofacitinib in plasma samples, ensuring reliable pharmacokinetic data. The analytical method's validation in terms of precision, accuracy, selectivity, and stability was performed rigorously, establishing a strong foundation for its use in future pharmacokinetic investigations. Such a high level of method validation is essential in providing consistent and reproducible results, particularly when working with complex drug formulations and samples from human clinical trials. The choice of this technique underscores the importance of employing the most advanced and validated methods in the field of pharmaceutical research to generate high-quality data that can guide clinical decisions.

Moreover, the stability assessments of Tofacitinib further contributed to the study's success. The confirmation of the analyte's stability under various conditions—such as temperature fluctuations, light exposure, and different storage environments—provides essential insights into the reliability of pharmacokinetic profiles over time. Stability is a critical factor in the validation of drug formulations because it ensures that the therapeutic efficacy and safety of the drug remain consistent throughout its shelf life, under different handling and storage conditions. This is particularly important when planning for long-term studies or post-market surveillance, where the consistency of the drug's pharmacokinetic properties must be guaranteed for patient safety.

The alignment of this study with the stringent guidelines established by the European Medicines Agency (EMEA) and the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH M10) further solidifies the scientific rigor and reliability of the research. These guidelines represent the gold standard for bioequivalence studies, ensuring that all pharmacokinetic assessments adhere to internationally recognized protocols. By following these guidelines, the study not only meets regulatory expectations but also establishes a methodological benchmark for future bioequivalence studies of Tofacitinib formulations. This adherence to recognized standards helps guarantee

the reliability and acceptability of the results by regulatory authorities, enhancing the credibility and applicability of the findings in both clinical and regulatory contexts.

The successful demonstration of bioequivalence in this study reinforces the clinical utility of the test formulation of Tofacitinib, highlighting its potential for use as an alternative to the reference formulation in therapeutic settings. This outcome has significant implications for the treatment of conditions such as rheumatoid arthritis, psoriatic arthritis, and other inflammatory diseases, where Tofacitinib is already an established therapeutic option. The confirmation of bioequivalence opens the door for broader applications of the test formulation, which could ultimately benefit patients by increasing accessibility and potentially lowering costs without compromising therapeutic efficacy. This is particularly important in the context of rising healthcare costs and the growing demand for affordable treatment alternatives.

Furthermore, these findings provide a strong foundation for future studies exploring the long-term safety and efficacy of Tofacitinib formulations in diverse patient populations. Additional clinical trials and post-market surveillance can now build upon this work to further assess the test formulation's performance in real-world settings. The data generated from this study will contribute significantly to the scientific community's understanding of Tofacitinib's pharmacokinetic profile, paving the way for ongoing advancements in drug development, personalized medicine, and optimized treatment strategies.

In conclusion, this research not only demonstrates the bioequivalence of Tofacitinib 11 mg formulations but also sets a high standard for methodological rigor in pharmacokinetic studies. The use of a validated LC-MS/MS method, coupled with thorough stability testing and adherence to international guidelines, ensures the reliability and relevance of the results. These findings contribute to the expanding body of knowledge on Tofacitinib and serve as a valuable reference for future studies in the field of pharmacology and drug development. As the clinical and therapeutic applications of Tofacitinib continue to evolve, this study will play a crucial role in advancing the understanding and clinical implementation of this important drug.

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