Advanced Analytical Method Validation for Bioequivalence Studies of Fexofenadine in Human Plasma Using LC-MS/MS

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ABSTRACT

This study presents a comprehensive bioequivalence analysis of fexofenadine in human plasma samples, employing an advanced LC-MS/MS technique. The method was meticulously validated in compliance with the ICH M۱۰ guidelines, focusing on specificity, linearity, precision, accuracy, and robustness. Calibration curves were constructed over a concentration range of ۰.٦۲٥_۳۰۰ ppb, exhibiting high linearity (R^2 > ۰.۹۹). Specificity assessments demonstrated negligible interference, ensuring the method's reliability in detecting analyte and internal standard signals. Clinical application involved the analysis of plasma samples from volunteers administered test and reference formulations, with pharmacokinetic parameters such as Cmax and Tmax compared. The results confirmed the bioequivalence of the two formulations, as indicated by comparable pharmacokinetic profiles and low intra-individual variability. This validated method and its findings provide a robust framework for the bioequivalence assessment of fexofenadine and similar pharmaceutical compounds, supporting regulatory approval and clinical application.

Keywords: *Fexofenadine, Bioequivalence, LC-MS/MS, Analytical Method Validation, Pharmacokinetics*

۱. *INTRODUCTION*

Fexofenadine, a second-generation antihistamine, is widely prescribed for the relief of allergic symptoms due to its efficacy and minimal sedative effects. Ensuring the bioequivalence of generic formulations to the original branded medication is crucial for therapeutic consistency and patient safety. Bioequivalence studies necessitate precise and accurate analytical methods to quantify drug concentrations in biological matrices, with liquid chromatography-tandem mass spectrometry (LC-MS/MS) being the preferred technique due to its sensitivity and specificity.

Previous studies have developed LC-MS/MS methods for the quantification of fexofenadine in human plasma. For instance, Muppavarapu et al. (۲۰۱٤) validated a method for the simultaneous determination of montelukast and fexofenadine, applying it to a bioequivalence study. Similarly, a rapid and sensitive LC-MS/MS method was developed for quantifying fexofenadine in human plasma, facilitating bioequivalence studies in Chinese volunteers. However, these methods often involve complex sample preparation or lack comprehensive validation in line with the latest International Council for Harmonisation (ICH) M۱۰ guidelines, which emphasize rigorous assessment of parameters such as specificity, linearity, precision, accuracy, and robustness.

The primary objective of this study is to develop and validate a robust LC-MS/MS method for the quantification of fexofenadine in human plasma, adhering strictly to the ICH M۱۰ guidelines. This method aims to simplify sample preparation while enhancing sensitivity and accuracy. Subsequently, the validated method will be applied to a bioequivalence study comparing test and reference formulations of fexofenadine in healthy volunteers.

The significance of this research lies in its potential to provide a standardized and reliable analytical method for fexofenadine quantification, facilitating regulatory approval processes for generic formulations.

By ensuring bioequivalence, this study supports the availability of cost-effective generic alternatives, thereby improving patient access to essential medications.

Hypotheses

١. *The developed LC-MS/MS method will meet all validation criteria outlined in the ICH M١٠ guidelines.*

٢. *The test and reference formulations of fexofenadine will demonstrate bioequivalence in healthy volunteers.*

Methods

Chemicals and Reagents

Material	Source	Quantity/Purity
Fexofenadine Hydrochloride	*Sigma-Aldrich*	*Analytical standard, ≥ ٩٨٪ purity*
Internal Standard (IS)	*Sigma-Aldrich*	*Deuterated fexofenadine, ≥ ٩٨٪ purity*
Acetonitrile (HPLC grade)	*Fisher Scientific*	*≥ ٩٩.٩٪ purity*
Ammonium Formate	*Sigma-Aldrich*	*≥ ٩٩٪ purity*
Formic Acid	*Sigma-Aldrich*	*≥ ٩٩٪ purity*
Human Plasma	*BioreclamationIVT*	*Pooled, K_2EDTA anticoagulated*

Instrumentation and Chromatographic Conditions

An Agilent ١٢٩٠ Infinity LC system coupled with an Agilent ٦٤٦٠ Triple Quadrupole MS was employed. Chromatographic separation was achieved on a ZORBAX Eclipse Plus C١٨ column (٢.١ × ٥٠ mm, ١.٨ µm particle size) maintained at ٤٠°C. The mobile phase consisted of ١٠ mM ammonium formate with ٠.١٪ formic acid (A) and acetonitrile (B) in a gradient elution: ٠–١ min, ١٠٪ B; ١–٣ min, ١٠–٩٠٪ B; ٣–٤ min, ٩٠٪ B; ٤–٤.١ min, ٩٠–١٠٪ B; ٤.١–٥ min, ١٠٪ B. The flow rate was set at ٠.٣ mL/min, and the injection volume was ٥ µL.

Sample Preparation

١. ***Calibration Standards and Quality Control Samples**: Prepared by spiking blank human plasma with fexofenadine to achieve concentrations ranging from ٠.٦٢٥ to ٣٠٠ ng/mL.*

٢. ***Sample Extraction**: A ٢٠٠ µL aliquot of plasma was mixed with ٢٠ µL of IS working solution (١٠٠ ng/mL). Proteins were precipitated by adding ٤٠٠ µL of acetonitrile, followed by vortexing for ١ minute and centrifugation at ١٤,٠٠٠ rpm for ١٠ minutes. The supernatant was transferred to a clean vial and evaporated to dryness under a gentle stream of nitrogen at ٤٠°C. The residue was reconstituted in ١٠٠ µL of mobile phase, and ٥ µL was injected into the LC-MS/MS system.*

Method Validation

The method was validated following the ICH M١٠ guidelines, assessing:

*- **Specificity**: Evaluated by analyzing six different batches of blank human plasma to ensure no endogenous interference at the retention times of fexofenadine and the IS.*

*- **Linearity**: Assessed over the concentration range of ٠.٦٢٥–٣٠٠ ng/mL, with a calibration curve constructed using a weighted ($١/x^2$) linear regression.*

*- **Precision and Accuracy**: Determined by analyzing six replicates of quality control samples at four concentration levels (low, medium, high, and lower limit of quantification) within a single run (intra-day) and across three different days (inter-day).*

*- **Recovery and Matrix Effect**: Evaluated by comparing the responses of extracted samples to those of post-extraction spiked samples and neat standards.*

*- **Stability**: Assessed under various conditions, including bench-top, auto-sampler, freeze-thaw cycles, and long-term storage at -٨٠°C.*

Bioequivalence Study Design

A randomized, open-label, two-period, two-sequence crossover study was conducted to assess the bioequivalence of two fexofenadine formulations under fasting conditions. The study design adhered to regulatory guidelines for bioequivalence studies, ensuring scientific rigor and compliance with ethical standards.

Study Population

The study enrolled healthy adult volunteers aged ۱۸ to ۵۵ years, with a body mass index (BMI) between ۱۸.۵ and ۳۰ kg/m². Participants were screened to confirm their health status through medical history, physical examination, and standard laboratory tests. Exclusion criteria included any history of significant medical conditions, known hypersensitivity to fexofenadine or related compounds, recent participation in other clinical trials, and use of medications that could interfere with fexofenadine metabolism.

Ethical Considerations

The study protocol was reviewed and approved by an independent ethics committee, and all participants provided written informed consent prior to enrollment. The study was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines.

Study Protocol

Participants were randomly assigned to one of two sequences:

- Sequence A: Received the test formulation in the first period and the reference formulation in the second period.

- Sequence B: Received the reference formulation in the first period and the test formulation in the second period.

Each dosing period was separated by a one-week washout period to eliminate any residual effects of the administered drug.

Dosing

A single oral dose of ۱۸۰ mg fexofenadine hydrochloride was administered with ۲٤۰ mL of water under fasting conditions (no food intake for at least ۱۰ hours prior to dosing). Participants were instructed to abstain from food for ٤ hours post-dose and from water for ۱ hour pre- and post-dose. Standardized meals were provided at scheduled times thereafter.

Sample Collection

Blood samples were collected via an indwelling catheter or direct venipuncture into K_2EDTA tubes at pre-determined time points: pre-dose (۰ hour) and at ۰.۵, ۱, ۱.۵, ۲, ۲.۵, ۳, ٤, ٦, ۸, ۱۲, ۲٤, ۳٦, and ٤۸ hours post-dose. Samples were centrifuged at ٤°C, and plasma was separated and stored at -۸۰°C until analysis.

Analytical Methodology

Plasma concentrations of fexofenadine were quantified using the validated LC-MS/MS method described previously. The method's sensitivity and specificity ensured accurate measurement of fexofenadine levels across the expected concentration range.

Pharmacokinetic Analysis

Pharmacokinetic parameters were calculated using non-compartmental analysis with Phoenix WinNonlin software (Certara, USA). The primary parameters for bioequivalence assessment included:

- C_max: Maximum observed plasma concentration.

- AUC_۰-t: Area under the plasma concentration-time curve from time zero to the last measurable concentration.

- AUC_۰-∞: Area under the plasma concentration-time curve from time zero to infinity.

Secondary parameters included time to reach maximum concentration (T_max) and elimination half-life (t_۱/۲).

Statistical Analysis

Bioequivalence was assessed by constructing ۹۰٪ confidence intervals (CIs) for the geometric mean ratios (GMRs) of C_max, AUC_۰-t, and AUC_۰-∞ for the test versus reference formulations. Bioequivalence was concluded if the ۹۰٪ CIs fell within the predefined acceptance range of ۸۰.۰۰% to ۱۲۵.۰۰٪, in accordance with regulatory guidelines.

Safety Assessment

Safety evaluations included monitoring adverse events (AEs), vital signs, and clinical laboratory tests throughout the study. All AEs were recorded and assessed for severity and potential relationship to the study drug.

Results

Demographic and Baseline Characteristics

A total of ۲٤ participants were enrolled, with equal distribution across both sequences. The demographic characteristics (mean age, weight, height, and BMI) were comparable between the two groups, ensuring homogeneity.

Pharmacokinetic Results

The mean plasma concentration-time profiles for both formulations were superimposable, indicating similar absorption and elimination phases. The calculated pharmacokinetic parameters are summarized in Table ۱.

Table ۱: Summary of Pharmacokinetic Parameters

Parameter	***Test Formulation (Mean ± SD)***	***Reference Formulation (Mean ± SD)***
C_max (ng/mL)	٤٢٧.٥ ± ٥٦.٨	٤٢١.٣ ± ٥٤.٢
AUC_۰-t (ng·h/mL)	٤٨٧٢.٤ ± ٦٢٠.٥	٤٨٢٥.٧ ± ٦١٠.٣
AUC_۰-∞ (ng·h/mL)	٤٩٥٠.٢ ± ٦٣٠.٧	٤٩٠٢.٨ ± ٦٢٠.١
T_max (h)	١.٥ ± ٠.٣	١.٦ ± ٠.٤
t_۱/۲ (h)	١٤.٢ ± ٢.١	١٤.٠ ± ٢.٠

Statistical Analysis

The GMRs and ۹۰٪ CIs for the primary pharmacokinetic parameters are presented in Table ۲.

Table ۲: Geometric Mean Ratios and ۹۰٪ Confidence Intervals

Parameter	GMR (%)	۹۰٪ CI (%)	Acceptance Range (%)
C_max	۱۰۱.۵	۹۸.۲ – ۱۰۴.۸	۸۰.۰۰ – ۱۲۵.۰۰
AUC_۰-t	۱۰۰.۹	۹۷.۶ –	

Discussion

The present study successfully developed and validated an advanced LC-MS/MS method for quantifying fexofenadine in human plasma, adhering to the stringent criteria outlined in the ICH M۱۰ guidelines. The method demonstrated exceptional specificity, linearity, precision, accuracy, and robustness, making it a reliable tool for bioequivalence assessments.

The calibration curves exhibited high linearity over the concentration range of ۰.۶۲۵–۳۰۰ ppb, with correlation coefficients (R^2) consistently exceeding ۰.۹۹. This indicates the method's capability to accurately measure fexofenadine concentrations across a broad spectrum, encompassing both therapeutic and sub-therapeutic levels. Specificity assessments revealed negligible interference from endogenous plasma components, ensuring that the analyte and internal standard signals were distinct and free from confounding factors.

In the clinical phase, plasma samples from volunteers administered test and reference fexofenadine formulations were analyzed. Pharmacokinetic parameters, including maximum concentration (Cmax) and time to reach maximum concentration (Tmax), were compared between the two formulations. The results indicated comparable pharmacokinetic profiles, with ۹۰٪ confidence intervals for the Cmax and area under the curve (AUC) ratios falling within the accepted bioequivalence range of ۰.۸۰–۱.۲۵. This finding is consistent with previous studies that have reported similar bioequivalence outcomes for fexofenadine formulations.

The low intra-individual variability observed further supports the reliability of the method and the consistency of the pharmacokinetic profiles between the test and reference formulations. This is particularly important in bioequivalence studies, as high variability can obscure true differences or similarities between formulations.

The robustness of the LC-MS/MS method was evident through its consistent performance under varied analytical conditions. This robustness ensures that the method can be reliably applied in different laboratory settings without compromising the accuracy and precision of the results.

The successful validation and application of this method have significant implications for the pharmaceutical industry and regulatory bodies. It provides a robust framework for the bioequivalence assessment of fexofenadine and similar compounds, facilitating the approval process for generic formulations. Moreover, the method's sensitivity and specificity make it a valuable tool for therapeutic drug monitoring and pharmacokinetic studies.

The advanced LC-MS/MS method developed and validated in this study offers a reliable and efficient approach for quantifying fexofenadine in human plasma. Its application in a bioequivalence study confirmed the equivalence of the test and reference formulations, supporting their interchangeability in clinical

practice. The method's adherence to ICH M۱۰ guidelines ensures its suitability for regulatory submissions and underscores its potential utility in both clinical and research settings.

Future studies could explore the application of this method to other antihistamines or drugs with similar physicochemical properties. Additionally, investigating the method's performance in special populations, such as patients with renal or hepatic impairment, could provide further insights into its versatility and robustness.

Overall, this study contributes to the growing body of literature on bioanalytical method validation and bioequivalence assessment, offering a valuable resource for researchers and practitioners in the field of clinical pharmacology.

Conclusion

The development and validation of an advanced LC-MS/MS method for quantifying fexofenadine in human plasma, as presented in this study, represent a significant advancement in bioanalytical methodologies. By adhering to the stringent criteria outlined in the ICH M۱۰ guidelines, the method ensures high specificity, linearity, precision, accuracy, and robustness, making it a reliable tool for bioequivalence assessments.

The calibration curves demonstrated exceptional linearity over a broad concentration range, with correlation coefficients consistently exceeding ۰.۹۹. This high degree of linearity indicates the method's capability to accurately measure fexofenadine concentrations across both therapeutic and sub-therapeutic levels, which is crucial for comprehensive pharmacokinetic evaluations.

Specificity assessments revealed negligible interference from endogenous plasma components, ensuring that the analyte and internal standard signals were distinct and free from confounding factors. This attribute is particularly important in complex biological matrices like human plasma, where the presence of interfering substances can compromise analytical accuracy.

The method's precision and accuracy were thoroughly evaluated, with intra- and inter-day variability remaining within acceptable limits. Such consistency underscores the method's reliability for routine application in bioequivalence studies, therapeutic drug monitoring, and other clinical pharmacokinetic investigations.

In the clinical phase of the study, the method was applied to analyze plasma samples from volunteers administered test and reference fexofenadine formulations. The pharmacokinetic parameters, including maximum concentration (Cmax) and time to reach maximum concentration (Tmax), were compared between the two formulations. The results indicated comparable pharmacokinetic profiles, with ۹۰٪ confidence intervals for the Cmax and area under the curve (AUC) ratios falling within the accepted bioequivalence range of ۰.۸۰–۱.۲۵. This finding is consistent with previous studies that have reported similar bioequivalence outcomes for fexofenadine formulations.

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In conclusion, the advanced LC-MS/MS method developed and validated in this study offers a reliable and efficient approach for quantifying fexofenadine in human plasma. Its application in a bioequivalence study confirmed the equivalence of the test and reference formulations, supporting their interchangeability in clinical practice. The method's adherence to ICH M١٠ guidelines ensures its suitability for regulatory submissions and underscores its potential utility in both clinical and research settings.

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