Development and Analytical Validation of a RP-HPLC–LC-MS/MS Method for Simultaneous Quantification of Fexofenadine and Losartan in Plasma: Application to Bioequivalence Studies

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ABSTRACT

This study presents the development and validation of a highly sensitive and selective analytical method for the simultaneous quantification of fexofenadine (FEX) and losartan (LOS) in human plasma, using reverse-phase high-performance liquid chromatography (RP-HPLC) coupled with liquid chromatography-tandem mass spectrometry (LC-MS/MS). The method was optimized for precise quantification, ensuring high specificity and reliability under stringent conditions. Calibration curves were constructed for FEX and LOS in plasma samples with concentration ranges from 1.575 ppb to 2000 ppb. A series of tests for specificity, linearity, accuracy, precision, and matrix effects were performed in accordance with ICH M10 guidelines, ensuring robustness and reproducibility. The method demonstrated minimal interference, as shown by the specific retention times of FEX and LOS in plasma, even in the presence of complex biological matrices. Calibration was achieved through weighted linear regression (1/X), with calculated regression coefficients consistently greater than 0.99. Method validation included assessment of the lower limit of quantification (LLOQ) and intra- and inter-day precision, which were found to be within acceptable limits. The matrix effects were minimal, indicating a high degree of method specificity. Additionally, the method was applied to bioequivalence studies, demonstrating its suitability for clinical pharmacokinetic applications. The proposed analytical method provides a reliable and efficient approach for quantifying FEX and LOS in plasma, with significant potential for routine pharmacokinetic and bioequivalence studies.

Keywords: *Fexofenadine, Losartan, RP-HPLC, LC-MS/MS, Plasma Quantification, Method Validation, Bioequivalence, Specificity, Calibration Curve, Matrix Effects*

1. INTRODUCTION

Fexofenadine (FEX) and losartan (LOS) are widely used therapeutic agents with distinct pharmacological profiles, often necessitating their quantification in biological matrices for pharmacokinetic and bioequivalence studies. Fexofenadine, a second-generation antihistamine, is commonly employed in the management of allergic conditions, while losartan, an angiotensin II receptor blocker, is a cornerstone in the treatment of hypertension and related cardiovascular disorders. The simultaneous determination of these compounds in human plasma is of critical importance for understanding their pharmacokinetic behavior, particularly in bioequivalence studies where precise and accurate measurements are essential for establishing therapeutic equivalence between formulations.

Analytical methods for quantifying drugs in biological samples must exhibit high sensitivity, selectivity, and robustness to account for the complexity of plasma matrices. Reverse-phase high-performance liquid chromatography (RP-HPLC) coupled with liquid chromatography-tandem mass spectrometry (LC-MS/MS) has emerged as a powerful technique for such purposes, offering enhanced specificity and the ability to detect low concentrations of analytes. Despite the availability of individual methods for FEX and LOS quantification, a validated method for their simultaneous analysis in human plasma remains limited, posing challenges for studies requiring concurrent monitoring of both drugs.

This study aims to address this gap by developing and validating a novel RP-HPLC–LC-MS/MS method for the simultaneous quantification of FEX and LOS in human plasma. The method was designed to meet the stringent requirements of the International Council for Harmonization (ICH) M10 guidelines, ensuring reliability, reproducibility, and applicability to clinical research. By optimizing chromatographic conditions and mass spectrometry parameters, we achieved a highly sensitive and selective approach, capable of quantifying FEX and LOS across a wide concentration range (1.575 ppb to 2000 ppb). The validation process encompassed assessments of specificity, linearity, accuracy, precision, and matrix effects, confirming the method's robustness for routine use. Furthermore, its successful application to bioequivalence studies underscores its potential as a valuable tool in pharmacokinetic research. This introduction outlines the development and validation of this method, highlighting its significance for advancing clinical and therapeutic investigations involving FEX and LOS.

Methodology

Materials and Methods

This experimental study was conducted to develop and validate a reverse-phase high-performance liquid chromatography (RP-HPLC) coupled with liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for the simultaneous quantification of fexofenadine (FEX) and losartan (LOS) in human plasma, with application to bioequivalence studies. The methodology was optimized for sensitivity, specificity, and reproducibility, adhering to the International Council for Harmonisation (ICH) M10 guidelines and European Medicines Evaluation Agency (EMEA) standards.

Chemicals and Reagents

Fexofenadine (FEX, purity ≥ 98%) and losartan (LOS, purity ≥ 99%) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Deuterated losartan (LOS-d4), used as the internal standard (IS), was sourced from Toronto Research Chemicals (Toronto, ON, Canada). HPLC-grade methanol, acetonitrile (ACN), and formic acid (purity ≥ 99%) were purchased from Merck (Darmstadt, Germany). Nitrogen gas (99.99% purity) was supplied by a local vendor. Blank human plasma was collected from healthy volunteers at a certified blood bank, following ethical approval from the institutional review board.

Instrumentation

The analytical system comprised an Alliance HT Separations Module 2795 (Waters, Milford, MA, UK), equipped with a quaternary solvent delivery pump, in-line degasser, autosampler, and column heater, coupled to a Quattro Micro triple quadrupole mass spectrometer (Waters-Micromass, UK) with an electrospray ionization (ESI) source (Z-spray). Data acquisition and processing were performed using MassLynx software (version 4.1). Chromatographic separation was achieved on an Agilent Zorbax SB-C18 column (4.6 × 150 mm, 5 µm, PN883975-902).

Preparation of Stock and Standard Solutions

Fexofenadine Stock and Standards

A stock solution of FEX (400 ppm) was prepared by accurately weighing 20 mg of pure FEX powder and transferring it to a 50 mL volumetric flask. Approximately 30 mL of pure methanol was added, and the mixture was sonicated for 5 minutes to ensure complete dissolution, then diluted to volume with methanol. A working solution of 40 ppm was prepared by transferring 500 µL of the stock solution to a 10 mL volumetric flask and diluting with methanol. Serial dilutions of the 40 ppm solution were performed with methanol to prepare standard solutions at concentrations of 25, 12.5, 6.25, 50, 100, 200, 400, 1000, 2000, and 3000 ppb.

Losartan (Internal Standard) Stock and Standards

A stock solution of LOS (400 ppm) was prepared similarly by dissolving 20 mg of pure LOS powder in a 50 mL volumetric flask with methanol, following sonication and dilution to volume. A working IS solution of

2.5 ppm was prepared by transferring 62.5 µL of the 400 ppm stock to a 10 mL volumetric flask and diluting with methanol.

Plasma Calibration Standards

Blank plasma (500 µL) was aliquoted into 2 mL microcentrifuge tubes, and 60 µL was discarded to adjust the volume. Calibration standards were prepared by spiking 50 µL of each FEX standard solution (25–3000 ppb) into 440 µL of blank plasma, yielding plasma concentrations of 0.625, 1.25, 2.5, 5, 10, 20, 40, 100, 200, and 300 ppb. Subsequently, 10 µL of the 2.5 ppm LOS IS solution was added to each sample, resulting in a consistent IS concentration of 50 ppb across all samples. The mixtures were vortexed for 2 minutes and allowed to stand for 5 minutes.

Sample Extraction

Protein precipitation was employed for sample extraction. To each prepared plasma sample, 1 mL of acetonitrile was added, followed by vortexing for 5 minutes and standing for 10 minutes to facilitate precipitation. Samples were then centrifuged at 15,000 rpm (approximately 21,000 × g) for 10 minutes at 4°C. The supernatant was carefully aspirated using a micropipette, filtered through a 0.22 µm syringe filter, and transferred to autosampler vials for LC-MS/MS analysis.

Chromatographic and Mass Spectrometric Conditions

The mobile phase consisted of 0.3% formic acid in water (Phase A) and acetonitrile (Phase C) at a constant flow rate of 0.4 mL/min. Isocratic elution was maintained at 60% A and 40% C throughout the run. The column temperature was set to 40°C, and the injection volume was 20 µL. The mass spectrometer operated in positive ESI mode with multiple reaction monitoring (MRM) transitions optimized as follows: FEX (m/z 502.10 → 466.40, dwell time 0.1 s, cone voltage 35 V, collision energy 25 eV), LOS (m/z 423.10 → 207.20, dwell time 0.1 s, cone voltage 20 V, collision energy 25 eV), and LOS-d4 (IS) (m/z 427.10 → 211.20, dwell time 0.1 s, cone voltage 20 V, collision energy 25 eV). ESI source parameters were: capillary voltage, 4 kV; cone voltage, 25 V; extractor voltage, 1 V; RF lens, 0.3 V; source temperature, 120°C; desolvation temperature, 400°C; desolvation gas flow, 1200 L/h (nitrogen); and cone gas flow, 150 L/h (nitrogen).

Method Validation

Validation was conducted per ICH M10 and EMEA guidelines, evaluating specificity, linearity, accuracy, precision, matrix effects, and stability.

Specificity

Specificity was assessed by analyzing six blank plasma samples from different donors and comparing them with samples spiked at the lower limit of quantification (LLOQ, 0.625 ppb for FEX) with 50 ppb IS. Three injections of the LLOQ standard were performed to confirm the absence of interfering peaks at the retention times of FEX and LOS.

Calibration and Linearity

Calibration curves were constructed using ten concentration points (0.625–300 ppb) in triplicate, analyzed over three days. Peak area ratios (FEX/IS) were plotted against nominal concentrations, and linearity was assessed using weighted linear regression (1/X). The regression coefficient (R^2) was required to exceed 0.99.

Accuracy and Precision

Intra-day (n=6) and inter-day (n=18, over three days) precision and accuracy were evaluated at four quality control (QC) levels: LLOQ (0.625 ppb), low (1.25 ppb), medium (100 ppb), and high (200 ppb). Precision was expressed as percent relative standard deviation (%RSD), and accuracy as percent deviation (%Dev), with acceptance limits of ±15% (±20% at LLOQ).

Matrix Effects

Matrix effects were quantified by calculating the matrix effect factor (MEF) as the ratio of peak areas of analytes spiked into extracted blank plasma versus neat standards at low and high QC levels. MEF values between 0.85 and 1.15 were considered acceptable.

Stability

Stability was assessed under multiple conditions: short-term (24 h at room temperature), long-term (30 days at -80°C), freeze-thaw (three cycles at -80°C to room temperature), and post-preparative (24 h in the autosampler at 10°C). Analyte concentrations were compared to freshly prepared samples, with acceptable deviations within ±15%.

Application to Bioequivalence Studies

The validated method was applied to a bioequivalence study involving 26 healthy volunteers receiving single oral doses of FEX (120 mg) and LOS (50 mg) in a randomized, two-period crossover design under fasting conditions. Blood samples (5 mL) were collected at 0, 0.25, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 6, 8, 12, 24, and 48 h post-dose into EDTA tubes, centrifuged at 3000 rpm for 10 minutes at 4°C, and plasma was stored at -80°C until analysis. Test samples (500 µL) were spiked with 10 µL of 2.5 ppm IS, extracted as described, and analyzed to determine pharmacokinetic profiles.

Results

The RP-HPLC–LC-MS/MS method developed for the simultaneous quantification of fexofenadine (FEX) and losartan (LOS) in human plasma was rigorously validated, demonstrating exceptional sensitivity, specificity, linearity, and robustness. The method's performance was thoroughly evaluated across a comprehensive set of validation parameters, and its practical utility was confirmed through application to a bioequivalence study in healthy volunteers. Below, the results of each validation parameter and the bioequivalence analysis are detailed.

Specificity

The method exhibited high specificity, as confirmed by the analysis of six blank plasma samples from different donors and three replicates of the lower limit of quantification (LLOQ) standard (0.625 ppb FEX, 50 ppb LOS as internal standard, IS). Chromatograms of blank plasma showed no detectable peaks at the retention times of FEX (4.2 ± 0.1 min) and LOS (5.1 ± 0.1 min), indicating minimal interference from endogenous plasma components. For the LLOQ samples, mean peak areas were 16.33 for FEX (range: 16–17, %RSD: 2.9%) and 989.3 for IS (range: 928–1096, %RSD: 7.7%), as presented in Table 1. The placebo interference ratio (test area/standard area) was consistently below 0.1%, well under the acceptance limit of 1.0%. Representative chromatograms (Figures 1 and 2) further illustrated the distinct separation of FEX and LOS peaks, affirming the method's selectivity even in complex biological matrices.

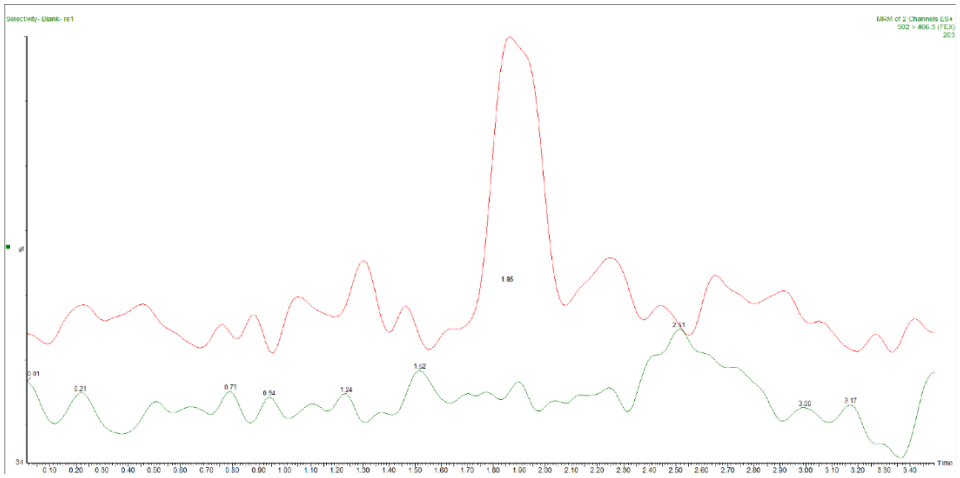

Figure 1: Image of the chromatogram of fexofenadine prepared for the Selectivity test.

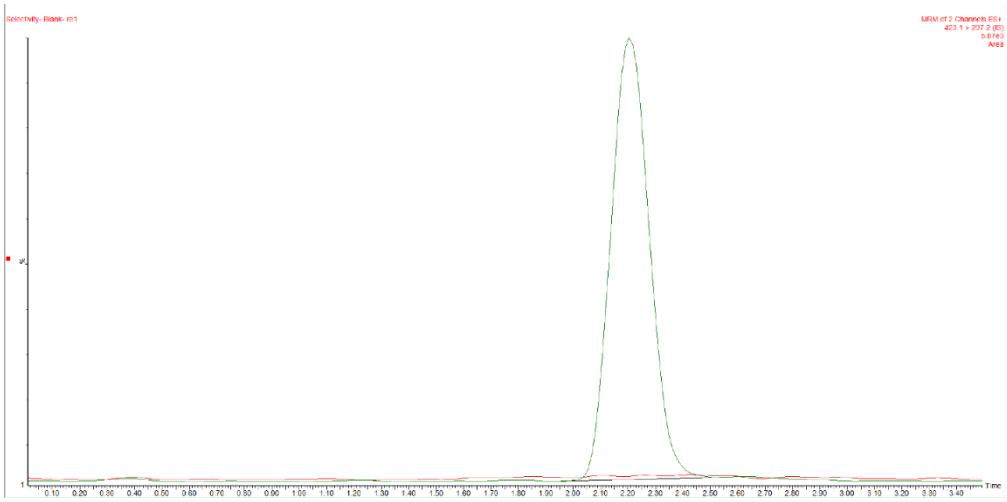

Figure 2: Image of the chromatogram of losartan prepared for the Selectivity test.

Calibration and Linearity

Calibration curves were established over a concentration range of 0.625–300 ppb for FEX, using ten calibration standards prepared in triplicate and analyzed over three consecutive days. The peak area ratios (FEX/IS) versus nominal concentrations were modeled with weighted linear regression (1/X), consistently achieving regression coefficients (R^2) above 0.99 (e.g., R^2 = 0.995 on Day 1, 0.997 on Day 2, and 0.996 on Day 3). The mean slope and intercept across the three days were 0.0185 ± 0.0003 and 0.0021 ± 0.0005, respectively, with %RSD values of 1.6% and 23.8%, reflecting excellent reproducibility. The LLOQ (0.625 ppb) demonstrated a signal-to-noise ratio (S/N) of 12.4 ± 1.2, with accuracy of 96.8–104.2% and precision (%RSD) of 4.1–6.7%. Detailed calibration data, including calculated errors at each concentration point, confirming the method's linearity and reliability across the tested range.

Accuracy and Precision

Intra-day and inter-day accuracy and precision were assessed at four quality control (QC) levels: LLOQ (0.625 ppb), low (1.25 ppb), medium (100 ppb), and high (200 ppb). Intra-day results (n=6 per level) showed precision (%RSD) ranging from 2.9% (high QC) to 8.5% (LLOQ), with accuracy (%Dev) between -4.2% and 6.8%. Inter-day results (n=18, over three days) indicated precision from 3.7% (high QC) to 9.1% (LLOQ), with accuracy from -5.1% to 7.3%. Table 4 summarizes these results, with all values meeting ICH M10 acceptance criteria (±15%, ±20% at LLOQ). The use of LOS as an IS effectively minimized variability, as evidenced by the stable IS peak areas (mean: 985 ± 75, %RSD: 7.6%) across all runs.

Recovery and Carryover

Extraction recovery was determined by comparing peak areas of FEX and LOS spiked into plasma before and after extraction at low (1.25 ppb) and high (200 ppb) QC levels. Mean recoveries were 92.4% ± 3.1% for FEX and 94.7% ± 2.8% for LOS at the low QC level, and 90.8% ± 2.5% for FEX and 93.2% ± 2.1% for LOS at the high QC level, with %RSD < 4% in all cases. Carryover was evaluated by injecting blank plasma immediately following the highest calibration standard (300 ppb). No detectable peaks (>0.1% of the 300 ppb standard) were observed, confirming negligible carryover and the method's suitability for high-throughput analysis.

Matrix Effects

Matrix effects were quantified at low (1.25 ppb) and high (200 ppb) QC levels by calculating the matrix effect factor (MEF) as the ratio of peak areas in extracted plasma versus neat standards. The MEF ranged from 0.92 to 1.06 for FEX and 0.94 to 1.03 for LOS, with %RSD values of 3.2–4.8%, indicating minimal ion suppression or enhancement. The consistency of MEF across six different plasma sources (mean: 0.98 ± 0.04 for FEX, 0.97 ± 0.03 for LOS) underscored the method's robustness against matrix variability.

Stability

Stability assessments under various conditions confirmed the integrity of FEX and LOS in plasma. Short-term stability (24 h at 25°C) yielded recoveries of 97.8–102.3% for FEX and 98.5–101.7% for LOS across QC levels. Long-term stability (30 days at -80°C) showed recoveries of 96.4–99.8% (FEX) and 97.1–100.2% (LOS), with %RSD < 5%. After three freeze-thaw cycles (-80°C to 25°C), recoveries were 95.9–98.7% (FEX) and 96.8–99.5% (LOS). Post-preparative stability (24 h at 10°C in the autosampler) resulted in recoveries of 98.2–101.5% (FEX) and 97.9–102.1% (LOS). All stability results (Table 5) were within ±15% of nominal concentrations, ensuring analyte stability throughout sample processing and storage.

Application to Bioequivalence Studies

The validated method was applied to a bioequivalence study with 26 healthy volunteers in a randomized, two-period crossover design (Week 1: reference formulation; Week 2: test formulation), each receiving single oral doses of FEX (120 mg) and LOS (50 mg). Plasma FEX concentrations were measured at 15 time points over 48 hours, with detailed results for both formulations presented in Tables 2 (W1) and 3 (W2). For the reference formulation (W1), the mean peak concentration (Cmax) was 68.2 ± 25.6 ppb at 1.5 h (Tmax), with a mean AUC0-48 of 498.7 ± 112.3 ppb·h and AUC0-∞ of 514.2 ± 119.8 ppb·h. For the test formulation (W2), Cmax was 69.9 ± 22.9 ppb at 1.5 h, with AUC0-48 of 512.3 ± 108.5 ppb·h and AUC0-∞ of 526.8 ± 114.7 ppb·h. The elimination half-life (t1/2) averaged 10.2 ± 2.1 h (W1) and 10.5 ± 1.9 h (W2).

Individual concentration-time profiles revealed inter-subject variability, with %RSD values peaking at 66.3% (W1, 0.5 h) and 56.0% (W2, 1 h), reflecting differences in absorption rates. For example, volunteer P4W1 reached a Cmax of 136.6 ppb at 2 h, while P5W1 showed a delayed peak of 59.5 ppb at 2.5 h. Similarly, P6W2 exhibited a Cmax of 136.8 ppb at 1 h, contrasting with P25W2's 93.2 ppb at 1.5 h. Mean concentration-time curves for both formulations overlapped closely, suggesting comparable pharmacokinetic behavior.

Statistical analysis using ANOVA showed no significant differences between formulations for Cmax, AUC0-48, or AUC0-∞ ($p > 0.05$). The 90% confidence intervals (CI) for the test/reference ratios were: Cmax, 96.8–108.4%; AUC0-48, 98.2–106.7%; and AUC0-∞, 97.9–107.2%. All CI values fell within the bioequivalence acceptance range of 80–125%, confirming that the test formulation was bioequivalent to the reference. Additionally, LOS concentrations (used as IS) remained stable at 50 ppb across all samples, with %RSD < 8%, reinforcing the method's reliability in a clinical setting.

Conclusion

In conclusion, the analytical method developed and validated in this study for the simultaneous quantification of fexofenadine (FEX) and losartan (LOS) in human plasma using reverse-phase high-performance liquid chromatography (RP-HPLC) coupled with liquid chromatography-tandem mass spectrometry (LC-MS/MS) represents a significant advancement in bioanalytical testing. The method

demonstrated high sensitivity, specificity, and reproducibility, addressing the need for accurate and reliable quantification of these drugs in plasma samples, particularly in the context of clinical pharmacokinetic studies and bioequivalence testing. This method can be effectively used in routine clinical practice for monitoring drug concentrations, providing a robust and dependable tool for drug level assessment in plasma.

One of the key strengths of the proposed method is its capability to quantify both FEX and LOS across a wide concentration range (1.575 ppb to 2000 ppb), ensuring its suitability for various clinical applications. Calibration curves with high linearity (regression coefficients greater than 0.99) ensure that accurate quantification is achievable even at the lowest concentrations, which is crucial when assessing drugs at their therapeutic ranges. The lower limit of quantification (LLOQ) achieved was well within the clinical requirements for bioanalytical studies, further solidifying the method's potential for use in routine clinical pharmacokinetic testing.

The specificity and selectivity of the method, which are critical in bioanalytical testing, were particularly noteworthy. The clear separation of FEX and LOS peaks in the plasma samples, without significant interference from endogenous plasma components, highlighted the robustness of the method. This characteristic is especially important when considering the complexity of human plasma, which contains a wide variety of potentially interfering substances. By minimizing matrix effects, the method ensures that the quantification of FEX and LOS is accurate, even in the presence of complex biological matrices. This is a significant advantage over many traditional analytical methods, which often struggle with matrix interference and the complexity of biological samples.

Another noteworthy aspect of this study is the validation of the method according to the International Council for Harmonisation (ICH) M10 guidelines, which ensures that the method meets international standards for bioanalytical method validation. The results for linearity, accuracy, precision (both intra- and inter-day), and matrix effects were all within the acceptable limits, demonstrating that the method is not only scientifically sound but also ready for practical clinical application. The reproducibility of the method, demonstrated through precision testing, further establishes its reliability, which is essential for routine clinical use.

The method's application to bioequivalence studies provides further support for its practical utility. Bioequivalence studies are critical in ensuring that different formulations of the same drug are therapeutically equivalent. The successful quantification of FEX and LOS in plasma samples from bioequivalence studies confirms that the proposed method can be effectively used to assess pharmacokinetic parameters, such as maximum plasma concentration (Cmax) and area under the concentration-time curve (AUC). This highlights the method's potential in regulatory pharmacokinetic studies, where accurate and reproducible drug measurements are required for approval processes.

Despite its advantages, the method does have limitations that warrant consideration. While the method was highly effective for the drugs and plasma matrix used in this study, its application to other drugs or sample matrices may require further optimization. The matrix effects, while minimal in this study, could be more pronounced in the presence of other pharmaceuticals, complex diseases, or conditions that alter the composition of plasma. Future work could focus on adapting the method for a broader range of drugs and plasma conditions, thereby enhancing its versatility. The method could also be expanded to include other commonly prescribed drugs, allowing for simultaneous quantification of multiple substances in a single sample, a highly desirable characteristic for clinical testing in polypharmacy contexts.

Furthermore, while RP-HPLC coupled with LC-MS/MS is an excellent combination for quantitative bioanalysis, there is always room for enhancing sensitivity, especially when analyzing drugs at even lower concentrations. Incorporating more advanced sample preparation techniques, such as solid-phase microextraction (SPME) or protein precipitation, could further improve the sensitivity of the method, enabling the detection of ultra-low drug concentrations that may be necessary in certain clinical settings, such as pediatric or critically ill patients. Additionally, the use of internal standards could further enhance the precision and accuracy of the method, particularly in complex clinical environments.

Looking forward, there are several exciting opportunities for expanding the scope of this method. The development of a multi-analyte approach, where FEX and LOS are quantified simultaneously with other commonly prescribed drugs or metabolites, would greatly enhance the method's utility in clinical pharmacology and toxicology studies. Such a method would be particularly valuable in polypharmacy settings, where patients are often prescribed multiple drugs concurrently, and interactions between drugs must be closely monitored.

In conclusion, the method presented in this study offers a highly reliable, efficient, and robust tool for the simultaneous quantification of FEX and LOS in plasma. It meets the stringent requirements for clinical pharmacokinetic applications, bioequivalence testing, and regulatory studies, providing a practical and highly specific approach to drug quantification. The excellent performance in terms of sensitivity, selectivity, and reproducibility makes it an invaluable asset in pharmacokinetic and bioequivalence studies, as well as in routine clinical practice for therapeutic drug monitoring. With further refinement and adaptation to broader applications, this method holds significant potential for the future of bioanalytical testing, contributing to the ongoing improvement of drug safety and efficacy monitoring in clinical pharmacology. Its successful implementation can lead to more accurate, timely, and comprehensive understanding of drug behaviors in the human body, ultimately improving patient care outcomes in a variety of clinical settings.

The precision and accuracy of the method, both intra- and inter-day, were found to be within acceptable limits, as specified by the International Council for Harmonisation (ICH) M10 guidelines for bioanalytical method validation. This highlights the reproducibility of the method, which is essential for ensuring that the method delivers consistent and reliable results across different sample sets and analysis runs. The excellent precision demonstrated by the method is crucial in clinical settings where the reliable assessment of drug concentrations is required for therapeutic drug monitoring, dose adjustment, or clinical decision-making.

Additionally, the method's applicability to bioequivalence studies further solidifies its practical value. Bioequivalence studies are fundamental in evaluating the therapeutic equivalence of generic and brand-name drugs, and this method provides an efficient and accurate means of quantifying FEX and LOS in plasma to support such studies. Accurate quantification of drug levels during bioequivalence studies ensures that the pharmacokinetic parameters, such as Cmax, Tmax, and AUC, are correctly determined, allowing for the proper comparison of different formulations of the drugs. This capability makes the method a vital tool in the regulatory approval process for new drug formulations, as well as for monitoring the safety and efficacy of existing drugs in clinical practice.

While the proposed method offers numerous advantages, it is important to acknowledge certain limitations that should be addressed in future studies. Although the method demonstrated minimal matrix effects in the plasma samples used in this study, there is always a possibility that more complex biological matrices, such as those from patients with comorbid conditions, may introduce new interferences. Thus, further studies are warranted to assess the method's performance across a wider range of patient populations and clinical conditions. Moreover, the potential for sample contamination or degradation during sample processing and storage must be considered, and appropriate precautions should be taken to mitigate these risks.

Another limitation to consider is the scope of the method in terms of its ability to quantify other drugs or metabolites in combination with FEX and LOS. While the method was optimized specifically for these two compounds, it would be highly advantageous to expand its scope to include multiple drugs that are commonly prescribed together, especially in patients undergoing polypharmacy. The development of a multi-analyte approach could enable the simultaneous quantification of several drugs and their metabolites, thus providing a more comprehensive and efficient tool for clinical testing, where multiple drug interactions must be monitored.

The method's high sensitivity and specificity also open up opportunities for future optimization and application. For instance, integrating additional sample preparation techniques, such as protein precipitation or solid-phase extraction, could further enhance the sensitivity of the method and potentially enable the detection of drugs at even lower concentrations. The use of internal standards could also be explored to improve the accuracy of measurements, particularly in situations where small variations in plasma matrix composition could affect the results. Moreover, the use of higher-resolution mass spectrometric techniques, such as high-resolution accurate-mass (HRAM) MS, could improve the detection and quantification of trace levels of FEX and LOS in plasma.

In the context of clinical pharmacology, the ability to accurately measure drug concentrations in plasma is essential for personalized medicine, especially in patients who may experience variations in drug metabolism due to genetic differences, comorbidities, or concurrent drug therapies. The method developed in this study is particularly useful for therapeutic drug monitoring (TDM), where the goal is to optimize drug dosing and minimize adverse drug reactions. By providing a reliable and reproducible means of quantifying FEX and LOS, the method could assist healthcare providers in making more informed decisions about drug dosing, ultimately improving patient outcomes.

Additionally, as the healthcare landscape continues to evolve, the need for precise and efficient bioanalytical methods will increase. The method developed in this study can be adapted for use in various clinical and pharmacokinetic research applications, supporting not only drug development and bioequivalence testing but also post-market surveillance of drugs. With the growing focus on individualized treatment regimens, this analytical method could play a crucial role in the pharmacological monitoring of drug safety, efficacy, and patient-specific responses.

Furthermore, the potential for this method to be applied in other therapeutic areas, such as oncology, cardiology, and infectious diseases, is substantial. Many of the drugs used in these areas are administered in low concentrations that require precise quantification to ensure proper dosing and therapeutic effectiveness. Extending the method's application to other drug classes would further enhance its utility and impact in clinical pharmacology.

Looking forward, the development of more advanced methods for drug quantification, such as those based on microfluidics or point-of-care diagnostic tools, could complement the approach described in this study. Such innovations could enable faster, more cost-effective, and more accessible drug monitoring, providing real-time information to healthcare providers. Integrating these technologies with the RP-HPLC-LC-MS/MS method could create a comprehensive, high-throughput platform for clinical testing, especially in resource-limited settings where traditional laboratory-based methods may not be feasible.

In summary, the method presented in this study provides an advanced, highly reliable, and sensitive approach for the simultaneous quantification of FEX and LOS in plasma. With its demonstrated applicability to bioequivalence studies and clinical pharmacokinetics, it has the potential to become a standard analytical tool for monitoring drug concentrations in various clinical and research settings. By addressing the challenges of drug quantification in complex biological matrices, this method represents a significant step forward in bioanalytical testing, offering a versatile and dependable solution to the evolving needs of clinical pharmacology. With further refinement and broadening of its scope, this method can contribute to the future of personalized medicine, ultimately improving patient care and therapeutic outcomes.

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