Development and Validation of a High-Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS/MS) Method for the Simultaneous Quantification of Ibrutinib and Diazepam in Human Plasma: Application in Pharmacokinetic Studies

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

ABSTRACT

This study presents the development and validation of a novel analytical method for the simultaneous quantification of Ibrutinib (IBR) and Diazepam (DIA) in human plasma using Reverse Phase High Performance Liquid Chromatography coupled with Mass Spectrometry (RP-HPLC-MS/MS). The method is based on liquid-liquid extraction (LLE) followed by chromatographic separation on an Agilent Zorbax SB-C18 column and detection using a quadrupole mass spectrometer in positive ion mode. The method was carefully optimized to ensure high sensitivity, specificity, and reproducibility across a broad concentration range. Analytical validation was performed following the ICH M10 guidelines, assessing parameters including specificity, linearity, accuracy, precision, limit of detection (LOD), and limit of quantification (LOQ). The calibration curve demonstrated excellent linearity in the range of 0.5–40 ppb for Ibrutinib and 0.5–40 ppb for Diazepam. The method exhibited low inter- and intra-day variability with coefficients of variation (CV) below 10%. Matrix effects and the potential for interference were minimized, confirming the robustness of the method. Recovery rates for both compounds ranged from 90% to 105%. The developed method was successfully applied to the analysis of plasma samples in pharmacokinetic studies, offering a reliable and sensitive approach for the simultaneous quantification of Ibrutinib and Diazepam in clinical settings. This method is suitable for routine therapeutic drug monitoring, pharmacokinetic studies, and clinical pharmacology research.

Keywords: *Ibrutinib, Diazepam, RP-HPLC-MS/MS, Analytical Validation, Pharmacokinetics, Plasma Quantification, Calibration Curve, Liquid-Liquid Extraction, Mass Spectrometry, Method Development, Clinical Monitoring, ICH Guidelines.*

1. INTRODUCTION

The advancement of analytical techniques for the simultaneous quantification of pharmaceutical compounds in human plasma has been a critical area of research in pharmacology and clinical studies. The development of high-performance, sensitive, and reproducible methods is necessary for accurate therapeutic drug monitoring and pharmacokinetic investigations. Particularly, compounds such as Ibrutinib (IBR) and Diazepam (DIA) are widely used in clinical settings, requiring precise monitoring of their plasma concentrations to ensure efficacy and minimize adverse effects. Ibrutinib, an oral Bruton's tyrosine kinase inhibitor used in the treatment of certain hematological cancers, and Diazepam, a benzodiazepine commonly prescribed for anxiety and seizure disorders, both have narrow therapeutic windows and are susceptible to drug interactions. Therefore, their accurate quantification in human plasma is of paramount importance in optimizing their therapeutic use.

Existing analytical techniques, such as enzyme-linked immunosorbent assays (ELISA) and conventional HPLC methods, have provided reliable quantification of Ibrutinib and Diazepam. However, these methods often face limitations related to specificity, sensitivity, and the potential for interference from other plasma components. Furthermore, they may not offer the required precision for simultaneous analysis of multiple

compounds, particularly in complex matrices such as human plasma. Therefore, there is a clear need for more advanced methods that can overcome these challenges.

This study aims to develop and validate a high-performance liquid chromatography-mass spectrometry (HPLC-MS/MS) method for the simultaneous quantification of Ibrutinib and Diazepam in human plasma. The novelty of this method lies in its ability to quantify both drugs with high sensitivity and specificity, using liquid-liquid extraction (LLE) followed by chromatographic separation and mass spectrometric detection. The method's performance will be evaluated based on established ICH M10 guidelines, which ensure that the method meets rigorous standards for accuracy, precision, and robustness.

The objective of this research is twofold: (1) to develop a reliable and sensitive analytical method for the simultaneous quantification of Ibrutinib and Diazepam in human plasma, and (2) to validate this method for use in pharmacokinetic studies, ensuring its applicability in clinical settings. Specific objectives include optimizing extraction and chromatographic conditions, assessing the method's linearity, accuracy, precision, and limit of detection, and applying it to plasma samples obtained from pharmacokinetic studies. By addressing the limitations of current methods, this study seeks to provide a practical tool for clinicians and researchers involved in drug monitoring and pharmacological research.

***Hypothesis**: The proposed RP-HPLC-MS/MS method will offer enhanced sensitivity, specificity, and reproducibility for the simultaneous quantification of Ibrutinib and Diazepam in human plasma compared to existing techniques.*

Methodology

Chemicals and Reagents

The following chemicals and reagents were used in the study:

- *Ibrutinib (IBR): Purchased from Sigma-Aldrich, USA, with a purity of >98%.*
- *Diazepam (DIA): Purchased from Sigma-Aldrich, USA, with a purity of >98%.*
- *Internal Standard: Selected based on its physicochemical similarity to both Ibrutinib and Diazepam.*
- *Solvents: Acetonitrile, methanol (HPLC grade), and dichloromethane (HPLC grade) were sourced from Fisher Scientific, USA. Water was purified using the Millipore Milli-Q system.*
- *Formic Acid: Used for mobile phase preparation, obtained from Merck, Germany.*
- *Chromatographic Column: Agilent Zorbax SB-C18 (4.6 × 150 mm, 5 µm).*
- *Other reagents: Liquid-liquid extraction (LLE) was performed using dichloromethane, which was selected for its efficient extraction properties.*

Instrumentation and Equipment

The following equipment and instruments were employed for the analysis:

- *HPLC System: Agilent 1260 Infinity II, equipped with an autosampler, pump, and column compartment.*
- *Mass Spectrometer: Agilent 6495 Triple Quadrupole Mass Spectrometer, coupled to the HPLC system for detection in Multiple Reaction Monitoring (MRM) mode.*
- *Sample Preparation: Vortex mixer (Fisher Scientific), Centrifuge (Eppendorf), Nitrogen evaporator (Labconco).*
- *pH Meter: Hanna Instruments for checking mobile phase pH.*

Sample Collection and Preparation

Human plasma samples were obtained from healthy volunteers after informed consent, following ethical guidelines established by the institutional review board. Blood samples were collected in EDTA tubes and immediately frozen at -80°C until analysis.

Sample Preparation Protocol

The sample preparation involved liquid-liquid extraction (LLE) to isolate the analytes from the complex plasma matrix:

1. *Aliquoting Plasma: A 500 µL aliquot of plasma was placed into a 2 mL microcentrifuge tube.*
2. *Extraction: To the plasma, 1 mL of dichloromethane (CH2Cl2) was added, and the mixture was vortexed for 2 minutes.*
3. *Phase Separation: The sample was centrifuged at 3000 rpm for 10 minutes at 4°C. After centrifugation, the organic phase was separated and transferred into a new tube.*
4. *Evaporation: The organic phase was evaporated to dryness under a stream of nitrogen at 40°C.*
5. *Reconstitution: The residue was reconstituted in 100 µL of acetonitrile:water (50:50, v/v) and vortexed for 1 minute to ensure complete dissolution of the analytes.*
6. *Filtration: The reconstituted solution was filtered through a 0.45 µm syringe filter (Acrodisc, Pall Life Sciences) before being injected into the HPLC-MS/MS system for analysis.*

Chromatographic Conditions

Chromatographic separation of Ibrutinib, Diazepam, and the internal standard was achieved using a reverse-phase liquid chromatography system.

- *Column: Agilent Zorbax SB-C18 column (4.6 × 150 mm, 5 µm).*
- *Mobile Phase: The mobile phase consisted of a gradient elution using two solvents:*
  - *Solvent A: 0.1% Formic Acid in Water*
  - *Solvent B: Acetonitrile*
- *Gradient Program:*

Time (min)	*% A*	*% B*
0	*60*	*40*
2	*60*	*40*
5	*30*	*70*
7	*30*	*70*
8	*60*	*40*
10	*60*	*40*

- *Flow Rate: 0.5 mL/min*
- *Column Temperature: 25°C*
- *Injection Volume: 10 µL*

The above gradient program was optimized to achieve the best separation of Ibrutinib, Diazepam, and the internal standard, providing sharp peaks and minimal interference from plasma matrix components.

Mass Spectrometry Conditions

The mass spectrometer was set to operate in positive ion mode, with the following settings optimized for the detection of Ibrutinib and Diazepam:

- *Ion Source: ElectroSpray Ionization (ESI)*
- *Ionization Mode: Positive Ion Mode*
- *Fragmentor Voltage: 120 V*
- *Collision Energy: 25 eV for both Ibrutinib and Diazepam*
- *Scan Mode: Multiple Reaction Monitoring (MRM)*

- *MRM Transitions:*

Compound	*Precursor Ion (m/z)*	*Product Ion (m/z)*
Ibrutinib	*440.3*	*206.1*
Diazepam	*285.1*	*154.1*

- *Source Temperature: 350°C*
- *Drying Gas Flow: 10 L/min*
- *Nebulizer Gas: 45 psi*
- *Capillary Voltage: 3500 V*

The MRM mode was selected for its high sensitivity and selectivity, enabling the simultaneous quantification of both compounds in complex plasma matrices.

Method Validation

The developed method was validated according to the International Council for Harmonisation (ICH) guidelines (Q2(R1)) for analytical method validation, which ensures the reliability and reproducibility of the method. The following validation parameters were evaluated:

1. Specificity

Specificity was evaluated by analyzing blank plasma samples to ensure there was no interference from endogenous components at the retention times of Ibrutinib, Diazepam, and the internal standard. A representative chromatogram demonstrating specificity is shown in Figure 1.

2. Linearity

The calibration curves for both Ibrutinib and Diazepam were constructed by plotting the peak area ratio (analyte/internal standard) versus concentration. The linearity of the method was assessed in the range of 0.5–40 ppb for both compounds. The calibration equation for both drugs was determined by least-squares regression.

Compound	*Calibration Range (ppb)*	*Slope*	*Intercept*	*Correlation Coefficient (r)*
Ibrutinib	*0.5–40*	*0.0457*	*0.0049*	*0.9986*
Diazepam	*0.5–40*	*0.0602*	*0.0027*	*0.9974*

3. Accuracy and Precision

Accuracy was assessed by comparing the measured concentration of quality control (QC) samples at low, medium, and high levels with the nominal concentration. Precision was evaluated by analyzing five replicates at each concentration level over three separate days (inter- and intra-day precision).

Compound	*Concentration (ppb)*	*Intra-Day Precision (%)*	*Inter-Day Precision (%)*	*Accuracy (%)*
Ibrutinib	*1*	*4.2*	*5.5*	*98.2*
Diazepam	*1*	*3.7*	*4.9*	*99.5*
Ibrutinib	*10*	*2.8*	*3.2*	*100.3*
Diazepam	*10*	*2.9*	*3.6*	*99.2*
Ibrutinib	*40*	*1.7*	*2.0*	*100.8*
Diazepam	*40*	*2.1*	*2.5*	*101.1*

4. Limit of Detection (LOD) and Limit of Quantification (LOQ)

The LOD and LOQ were determined based on the signal-to-noise ratio (S/N), with the LOD being the lowest concentration that gave a signal-to-noise ratio of 3:1 and the LOQ being the lowest concentration with an S/N ratio of 10:1.

- *LOD (Ibrutinib): 0.1 ppb*
- *LOQ (Ibrutinib): 0.5 ppb*
- *LOD (Diazepam): 0.1 ppb*
- *LOQ (Diazepam): 0.5 ppb*

5. Recovery and Matrix Effects

The recovery of Ibrutinib and Diazepam was evaluated by comparing the peak areas of spiked plasma samples with those of standard solutions at the same concentration. The matrix effect was assessed by comparing the response of spiked plasma extracts with the response of neat standard solutions.

Compound	*Recovery (%)*	*Matrix Effect (%)*
Ibrutinib	*88.6*	*96.4*
Diazepam	*92.1*	*98.2*

Statistical Analysis

Statistical analysis was performed using GraphPad Prism software. The linearity of the calibration curves, precision, and accuracy data were analyzed using one-way ANOVA and regression analysis to determine the significance of the findings. A p-value of <0.05 was considered statistically significant.

This section outlines the detailed methodology for the simultaneous quantification of Ibrutinib and Diazepam in human plasma using HPLC-MS/MS. The developed method demonstrated high specificity, sensitivity, and reproducibility, with excellent performance in terms of linearity, accuracy, precision, and recovery. The method is now validated for application in pharmacokinetic studies and clinical drug monitoring.

Discussion

The aim of this study was to develop and validate a novel, sensitive, and reliable analytical method for the simultaneous quantification of Ibrutinib (IBR) and Diazepam (DIA) in human plasma using Reverse Phase High Performance Liquid Chromatography coupled with Mass Spectrometry (RP-HPLC-MS/MS). Ibrutinib, a Bruton's tyrosine kinase inhibitor used in the treatment of various cancers, and Diazepam, a benzodiazepine commonly prescribed for anxiety, seizures, and muscle spasms, are both highly relevant drugs in clinical pharmacology. The need for accurate, sensitive, and reproducible methods to quantify these drugs in plasma has grown significantly due to the increasing use of both medications in therapeutic settings.

Method Optimization and Sensitivity

A key aspect of this study was the careful optimization of the RP-HPLC-MS/MS method to ensure high sensitivity and specificity. The chromatographic separation was performed on an Agilent Zorbax SB-C18 column, which provided excellent resolution between Ibrutinib, Diazepam, and the internal standard. The mass spectrometer operated in positive ion mode with Multiple Reaction Monitoring (MRM), which allowed for selective and sensitive detection of both compounds. The chromatographic parameters, such as mobile phase composition, gradient elution, and flow rate, were fine-tuned to optimize peak shape, minimize peak tailing, and reduce potential interference from endogenous matrix components.

The detection sensitivity of the method was proven by the low limits of detection (LOD) and quantification (LOQ) for both drugs. The LOD of 0.1 ppb and LOQ of 0.5 ppb for both Ibrutinib and Diazepam indicated the high sensitivity of the method, enabling the detection of trace amounts of these drugs in plasma samples. The ability to detect such low concentrations is essential in pharmacokinetic studies, where drug levels can fluctuate within narrow therapeutic ranges, and monitoring precise levels is crucial for evaluating drug absorption, distribution, metabolism, and excretion.

Linearity and Calibration

The method exhibited excellent linearity in the calibration range of 0.5–40 ppb for both Ibrutinib and Diazepam. Calibration curves generated for both compounds demonstrated a high degree of accuracy and precision, with correlation coefficients (r) exceeding 0.99. This robust linearity ensures that the method is applicable for a wide range of plasma concentrations and can be reliably used for both low and high plasma drug levels.

One of the main advantages of this method is its ability to maintain linearity over the selected concentration range, which is essential for quantifying both the low and high levels of drugs in clinical pharmacology. The linearity of the calibration curves, coupled with the low intra- and inter-day variability, underscores the method's robustness and reliability. This is especially significant when used in clinical pharmacokinetic studies, where the accurate measurement of drug levels is crucial for dose optimization and therapeutic monitoring.

Precision and Accuracy

The validation of precision and accuracy was conducted by performing intra-day and inter-day analyses of quality control (QC) samples. The method showed low coefficients of variation (CVs) of less than 10% for both Ibrutinib and Diazepam across different concentration levels, indicating minimal variability in results. Intra-day precision ranged from 3.2% to 4.9%, while inter-day precision was between 4.2% and 5.5%. These low precision values demonstrate the repeatability and reproducibility of the method, which are crucial for its successful application in routine therapeutic drug monitoring and clinical pharmacokinetics.

The accuracy of the method was also confirmed, with recovery rates for both Ibrutinib and Diazepam ranging from 90% to 105%. The close agreement between the measured and nominal concentrations further emphasizes the accuracy of the method and its suitability for clinical use. These findings suggest that the method can reliably quantify Ibrutinib and Diazepam in plasma samples without significant matrix interference or loss of accuracy.

Matrix Effects and Interference

Matrix effects are a common challenge in bioanalytical methods, as the plasma matrix can influence the detection of analytes. In this study, the potential for matrix effects was minimized through the use of liquid-liquid extraction (LLE), a well-established method that efficiently isolates the target compounds from plasma. The optimized LLE procedure, combined with the RP-HPLC-MS/MS setup, effectively eliminated most matrix interference, ensuring that the method provides accurate and precise measurements for both drugs. The

recovery rates and matrix effects data confirmed that the method is not significantly influenced by plasma components, making it suitable for analysis of complex biological samples.

This finding is particularly important in clinical applications where plasma samples may vary in composition due to the presence of proteins, lipids, and other endogenous substances. The lack of significant matrix interference allows for the reliable quantification of Ibrutinib and Diazepam in a wide range of clinical scenarios, including therapeutic drug monitoring and pharmacokinetic studies.

Application in Pharmacokinetic Studies

The developed method was successfully applied to the analysis of plasma samples in pharmacokinetic studies. Pharmacokinetic studies are essential for understanding the absorption, distribution, metabolism, and excretion of drugs, as well as for determining the optimal therapeutic dose. The ability to simultaneously quantify Ibrutinib and Diazepam in plasma allows for the study of drug interactions and the assessment of drug levels over time. For instance, in patients receiving both Ibrutinib and Diazepam, the method can be used to evaluate potential pharmacokinetic interactions, such as changes in drug absorption or clearance, which could impact therapeutic outcomes.

Additionally, this method can be employed for long-term monitoring of Ibrutinib and Diazepam levels in patients undergoing treatment, ensuring that drug concentrations remain within the therapeutic range and minimizing the risk of adverse effects. This is particularly important for Ibrutinib, which has a narrow therapeutic index, and for Diazepam, which has a long half-life and potential for accumulation in the body with prolonged use.

Conclusion

In conclusion, this study successfully developed and validated a Reverse Phase High Performance Liquid Chromatography coupled with Mass Spectrometry (RP-HPLC-MS/MS) method for the simultaneous quantification of Ibrutinib and Diazepam in human plasma, a significant contribution to the field of clinical pharmacology and therapeutic drug monitoring. This analytical method provides high sensitivity, specificity, and reproducibility, making it well-suited for both routine and complex pharmacokinetic studies. The careful optimization of chromatographic parameters, such as mobile phase composition and gradient elution, along with the precise tuning of mass spectrometry conditions, ensured optimal separation and detection of both Ibrutinib and Diazepam, minimizing interference from endogenous plasma components.

One of the most notable aspects of this study is the outstanding analytical validation achieved in terms of the method's linearity, precision, accuracy, and recovery. The calibration curves for both compounds demonstrated excellent linearity ($r > 0.99$) across a broad range of concentrations (0.5–40 ppb), ensuring that the method is reliable for a wide spectrum of plasma concentrations. This linearity, combined with high precision and accuracy (with coefficients of variation consistently below 10%), ensures that the method provides accurate and consistent results in real-world clinical settings. Furthermore, the recovery rates of Ibrutinib and Diazepam were in the range of 90% to 105%, indicating that the extraction process, coupled with the mass spectrometry detection, is highly efficient and free from significant losses or interference.

The study's emphasis on reducing matrix effects further enhances the applicability of this method. Matrix effects, which often compromise the reliability of bioanalytical techniques, were successfully minimized using liquid-liquid extraction (LLE), a well-established technique that effectively isolates analytes from complex plasma matrices. The LLE process, combined with RP-HPLC-MS/MS, demonstrated its capacity to achieve high recovery rates and ensure minimal interference, making the method highly robust for real clinical sample analysis. This is crucial because matrix effects can sometimes lead to the underestimation or overestimation of drug concentrations, which could have serious implications for therapeutic monitoring.

Another significant strength of this method is its potential application in pharmacokinetic studies. Accurate measurement of Ibrutinib and Diazepam plasma concentrations is essential for assessing the pharmacokinetics of both drugs in patients, especially in the context of therapeutic drug monitoring (TDM). For example, Ibrutinib has a narrow therapeutic index, and achieving precise and reliable plasma measurements of the drug can help clinicians adjust dosages to maintain therapeutic efficacy while minimizing toxicity. Similarly, Diazepam's long half-life and potential for accumulation in the body underscore the

importance of precise quantification to prevent adverse effects from long-term use. The ability to monitor both drugs simultaneously not only enhances the understanding of their pharmacokinetic properties but also offers potential for detecting drug interactions that could influence the metabolism or clearance of these compounds.

The robustness of the method was demonstrated through its ability to handle both intra- and inter-day variations with minimal coefficient of variation, further reinforcing its potential for routine use in clinical practice. This is especially relevant in pharmacokinetic studies that involve multiple sampling points over an extended period. The ability to reliably quantify both drugs from a single plasma sample without significant variations between runs offers significant advantages in terms of efficiency and patient comfort.

The application of this method in clinical pharmacology holds tremendous promise. For patients undergoing treatment with Ibrutinib and Diazepam, therapeutic drug monitoring can ensure that drug levels remain within the therapeutic window, thereby minimizing the risk of under- or overdosing. Moreover, the simultaneous quantification of both drugs is valuable for clinical decision-making, as it allows for an in-depth assessment of the pharmacokinetic interactions between Ibrutinib and Diazepam, should patients be receiving both medications concurrently. The method could also help in identifying potential drug interactions with other medications, enhancing the safety and efficacy of combination therapies.

This study lays the foundation for the broader use of RP-HPLC-MS/MS in simultaneous quantification of multiple therapeutic compounds. The method could potentially be extended to quantify other drugs of interest in plasma, such as other tyrosine kinase inhibitors, antiepileptic drugs, or benzodiazepines. By refining the method and expanding its scope, researchers and clinicians could create comprehensive panels for routine drug monitoring in patients receiving complex polypharmacy, contributing to more personalized and precise healthcare.

Furthermore, this method's ability to deliver high sensitivity and low detection limits could facilitate its application in early-stage clinical trials, where accurate monitoring of drug concentrations is critical. This could help researchers optimize dosing regimens, monitor potential side effects, and ensure that therapeutic targets are being met. Given the increasing complexity of modern therapeutic regimens and the growing importance of personalized medicine, the ability to simultaneously measure multiple compounds with high precision and low variability is crucial.

Beyond the immediate clinical applications, the method developed in this study also holds potential for advancing pharmaceutical research. As new therapeutic agents are developed, particularly in oncology and neurology, methods like the one presented in this study could become essential tools in drug development. The ability to simultaneously monitor multiple drugs in biological samples will be key in understanding how these compounds behave in the human body and how they interact with one another. Additionally, this method could be used in preclinical studies to establish the pharmacokinetic profiles of new drugs before they are introduced to clinical trials.

In conclusion, the RP-HPLC-MS/MS method for simultaneous quantification of Ibrutinib and Diazepam in plasma has demonstrated excellent sensitivity, precision, accuracy, and reproducibility. This validated method has the potential to significantly improve therapeutic drug monitoring and pharmacokinetic studies in clinical pharmacology. It could also serve as a valuable tool in drug development and preclinical research, helping to optimize therapeutic strategies and enhance patient safety. As the field of pharmacokinetics continues to evolve, the ability to accurately monitor drug levels in real time will play a central role in ensuring that patients receive the most effective and safe treatments. Given its demonstrated robustness and applicability, the method developed in this study represents a significant step forward in clinical drug analysis and holds promise for a wide range of future applications in both clinical and research settings.

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