Development and Validation of a Highly Sensitive LC-MS/MS Method for the Quantification of Empagliflozin and Hydrocortisone in Plasma: Application to Therapeutic Drug Monitoring and Pharmacokinetic Studies

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ABSTRACT

This study presents the development and validation of a highly sensitive and specific Liquid Chromatography-Mass Spectrometry/Mass Spectrometry (LC-MS/MS) method for the simultaneous quantification of Empagliflozin (EMP) and Hydrocortisone (HC) in human plasma. This method is optimized to detect both compounds at low concentrations (from parts per trillion to parts per million), essential for accurate therapeutic drug monitoring (TDM) and pharmacokinetic studies. Standard stock solutions for EMP and HC were prepared following rigorous protocols to ensure precision and minimize errors, with serial dilutions providing a broad range of concentrations. Special attention was given to the preparation of plasma samples, which were spiked with known concentrations of EMP to generate accurate calibration curves. The method was validated according to ICH M10 guidelines, evaluating specificity, linearity, accuracy, precision, and sensitivity. The results demonstrated high specificity with minimal interference from placebo samples, as shown in detailed chromatographic data. Calibration curves exhibited excellent linearity, with weighted linear regression applied to enhance accuracy. The robustness of the method was confirmed across multiple testing phases, ensuring its reliability under varied sample conditions. This optimized LC-MS/MS method has demonstrated its potential for routine clinical applications, providing a reliable tool for assessing drug levels in plasma. Its application in drug monitoring and pharmacokinetic profiling ensures precise management of Empagliflozin and Hydrocortisone therapies, thereby improving patient safety and therapeutic outcomes. The methodology is suitable for clinical laboratories, offering a critical resource for optimizing individualized treatment regimens in diverse patient populations.

Keywords: LC-MS/MS, Empagliflozin, Hydrocortisone, plasma quantification, therapeutic drug monitoring, pharmacokinetics, method validation, specificity, accuracy, linearity, internal standard, bioanalysis.

1. INTRODUCTION

The accurate quantification of therapeutic drugs in plasma is critical for ensuring their effective and safe use, particularly for drugs with narrow therapeutic windows or those used in long-term therapy. Empagliflozin (EMP) and Hydrocortisone (HC) are two widely prescribed medications, each with specific pharmacokinetic properties that require precise monitoring for optimal clinical outcomes. Empagliflozin, a sodium-glucose cotransporter-2 (SGLT2) inhibitor, is commonly used for managing Type 2 diabetes and heart failure, while Hydrocortisone is a corticosteroid used in treating a variety of inflammatory and autoimmune disorders. Both drugs can lead to significant adverse effects if not appropriately monitored, making their quantification in biological samples essential for patient safety (Borges et al., 2021; Zhang et al., 2022).

The problem of accurate and reliable drug quantification in plasma has been widely recognized in clinical pharmacology. For both Empagliflozin and Hydrocortisone, current methods such as High-

Performance Liquid Chromatography (HPLC), Enzyme-Linked Immunosorbent Assay (ELISA), and Radioimmunoassay (RIA) have demonstrated limitations in sensitivity, specificity, and the ability to handle complex biological matrices like plasma (Sharma et al., 2021; Kumar et al., 2022). In particular, these methods often require cumbersome sample preparation procedures and are prone to interference from other plasma constituents, which can lead to inaccurate drug level measurements. Therefore, a highly sensitive, specific, and robust analytical method is necessary to overcome these challenges and provide reliable quantification of these drugs, especially at low concentrations.

Objectives

The general objective of this study is to develop and validate a highly sensitive and reproducible LC-MS/MS method for the simultaneous quantification of Empagliflozin and Hydrocortisone in human plasma. This method will be optimized to address the challenges posed by plasma matrix interference, sensitivity limitations, and reproducibility across various clinical conditions.

Specific objectives include:

1. To optimize the chromatographic separation and mass spectrometric conditions for the simultaneous analysis of Empagliflozin and Hydrocortisone.
2. To validate the method according to ICH M10 guidelines, ensuring its specificity, accuracy, precision, and sensitivity across a wide concentration range.
3. To evaluate the method's applicability to plasma samples from clinical settings, ensuring its suitability for therapeutic drug monitoring (TDM) and pharmacokinetic studies.

Research Problem

The central issue addressed by this study is the need for a highly sensitive and specific LC-MS/MS method for quantifying Empagliflozin and Hydrocortisone in plasma, overcoming the limitations of existing techniques. The research focuses on developing a method that can accurately measure low concentrations of these drugs, ensuring proper dosage adjustments to optimize patient safety and therapeutic outcomes. The problem is particularly relevant in clinical pharmacology, where drug monitoring is critical for minimizing adverse effects and ensuring efficacy, especially in patients with varying metabolic rates or in those receiving polypharmacy treatments.

Importance and Necessity of Research

From a theoretical perspective, the development of a reliable LC-MS/MS method for the quantification of Empagliflozin and Hydrocortisone will contribute significantly to the advancement of drug monitoring technologies. LC-MS/MS has become the gold standard in bioanalytical methods due to its exceptional sensitivity and specificity. By addressing the challenges posed by matrix effects and the need for low detection limits, this method will enhance the precision of pharmacokinetic studies, therapeutic monitoring, and clinical dosing regimens.

From a practical perspective, accurate quantification of Empagliflozin and Hydrocortisone is essential for individualized patient care, especially in conditions like diabetes, heart failure, and autoimmune diseases, where proper dosing can significantly impact treatment outcomes. For instance, inaccurate dosing of Hydrocortisone can lead to adverse effects such as Cushing's syndrome or adrenal suppression, while inadequate Empagliflozin levels could lead to poor control of blood glucose in diabetic patients (Borges et al., 2021). Therefore, the proposed method has the potential to improve clinical decision-making and patient safety, ultimately enhancing therapeutic efficacy and reducing side effects.

Research Background and Theoretical Framework

The need for sensitive and reliable quantification methods in therapeutic drug monitoring has been well documented. Traditional methods, such as HPLC and ELISA, although widely used, are often limited by factors such as poor sensitivity for low concentrations, susceptibility to matrix effects, and extended analysis times (Kumar et al., 2022). Liquid Chromatography coupled with Mass Spectrometry (LC-MS/MS) has been increasingly utilized in bioanalytical applications due to its superior sensitivity, specificity, and ability to analyze complex biological matrices with minimal interference (Sharma et al., 2021). Recent studies have highlighted the growing importance of LC-MS/MS in drug quantification, particularly for drugs like Empagliflozin and Hydrocortisone, where precise plasma concentrations are crucial for ensuring therapeutic success (Zhang et al., 2022).

The theoretical framework for this study is based on the principle that LC-MS/MS, combined with electrospray ionization (ESI) and tandem mass spectrometry (MS/MS), offers the sensitivity and specificity required for accurate quantification of low-concentration drugs in plasma. The use of internal standards, such as Hydrocortisone for Empagliflozin quantification, further minimizes matrix effects and improves precision (Patel et al., 2020).

Hypotheses

1. The LC-MS/MS method developed for the simultaneous quantification of Empagliflozin and Hydrocortisone will show higher sensitivity and accuracy compared to existing techniques.
2. The method will demonstrate minimal matrix effects, ensuring reproducibility and precision in plasma samples from different patients and clinical settings.

Brief Overview of Related Research

Existing studies have explored LC-MS/MS for the quantification of Empagliflozin and other SGLT2 inhibitors in plasma, with promising results regarding their sensitivity and specificity (Jin et al., 2020). Similarly, LC-MS/MS has been effectively applied to measure corticosteroids like Hydrocortisone, ensuring reliable monitoring of cortisol replacement therapies (Kumar et al., 2021). However, the simultaneous quantification of both Empagliflozin and Hydrocortisone in plasma remains underexplored, with very few studies directly addressing the challenges of matrix interference and the need for a single, integrated method for these two distinct drug classes. This research aims to bridge this gap by providing a validated, high-sensitivity LC-MS/MS method for both drugs in a single analytical run.

Methodology

Materials and Consumables

Item	Supplier	Catalog Number
Empagliflozin (Standard)	Sigma-Aldrich	268201-80-2
Hydrocortisone (Internal Standard)	Chemos GmbH	50-23-7
Acetonitrile (HPLC grade)	Merck	100014-1
Methanol (HPLC grade)	Fisher Scientific	A456-4
Formic Acid (Reagent grade)	Sigma-Aldrich	64-18-6
LC-MS/MS Grade Water	Fisher Scientific	7787-62-8
Agilent Zorbax SB-C18 Column	Agilent Technologies	883975-902
Quattro Micro Mass Spectrometer	Waters-Micromass	70000420 12

Instrumentation

The analysis was conducted using a Waters Alliance HT 2795 system coupled to a Waters Quattro Micro Quadrupole Mass Spectrometer equipped with electrospray ionization (ESI). The Agilent Zorbax SB-C18 column (4.6 × 150 mm, 5 µm) was employed for chromatographic separation. Data acquisition and analysis were performed using MassLynx software (version 4.1).

Chromatographic Conditions

Time (min)	Mobile Phase A (%)	Mobile Phase B (%)	Flow Rate (mL/min)
0	60	40	0.5
2	60	40	0.5
6	40	60	0.5
10	60	40	0.5

- **Column Temperature**: 40°C
- **Injection Volume**: 20 µL

Mass Spectrometry Conditions

Parameter	Value
Ionization Mode	Positive ESI
Capillary Voltage	4 kV
Cone Voltage	25 V
Extractor Voltage	1 V
RF Lens Voltage	0.3 V

Source Temperature	C	120°
Desolvation Temperature	C	400°
Desolvation Gas Flow	L/h	1200

The transitions for Empagliflozin and Hydrocortisone were optimized and monitored as follows:

Compound	**Precursor Ion (m/z)**	**Product Ion (m/z)**	**Collision Energy (V)**
Empagliflozin	451.3	70.6	15
Hydrocortisone	363	120.8	20

Sample Preparation

For plasma analysis, samples were spiked with standard solutions of

Empagliflozin and Hydrocortisone. Solid-phase extraction (SPE) was employed to remove plasma proteins and concentrate the drugs before analysis. The resulting plasma extracts were then injected into the LC-MS/MS system for quantification.

Method Validation

The LC-MS/MS method was validated according to the International Council for Harmonisation (ICH M10) guidelines. Key validation parameters included specificity, linearity, accuracy, precision, sensitivity, and robustness.

Discussion

The development and validation of the highly sensitive LC-MS/MS method for simultaneous quantification of Empagliflozin (EMP) and Hydrocortisone (HC) in human plasma represent significant advancements in analytical pharmacology. This study successfully optimized and validated a methodology capable of quantifying both drugs with high precision, sensitivity, and specificity, meeting the critical demands of therapeutic drug monitoring (TDM) and pharmacokinetic studies. Given the narrow therapeutic windows of both drugs, accurate quantification of their plasma concentrations is essential for managing patient treatment effectively, minimizing adverse effects, and optimizing therapeutic outcomes.

Comparison with Existing Methods

Empagliflozin and Hydrocortisone are both widely used drugs, but their quantification in plasma has traditionally been challenging due to their low concentrations in patient samples and the complex nature of biological matrices. Previous methods such as High-Performance Liquid Chromatography (HPLC) and enzyme-linked immunosorbent assays (ELISA) have been used to measure drug levels; however, these methods have certain limitations in terms of sensitivity, specificity, and the need for time-consuming sample preparation (Sharma et al., 2021; Patel et al., 2020). For example, HPLC, while effective, is less sensitive than LC-MS/MS when detecting low plasma concentrations, which is especially critical in drugs like Empagliflozin that are often administered at low doses in chronic disease management (Zhang et al., 2022).

LC-MS/MS, on the other hand, offers superior sensitivity and specificity, especially in complex matrices like plasma, where various endogenous compounds may interfere with the analytes of interest. This method allows for the simultaneous quantification of multiple compounds, which is a significant advantage over traditional single-target methods. Our study's findings confirm that LC-MS/MS is the ideal technique for drug monitoring in clinical pharmacology, providing both high sensitivity and the ability to process complex biological samples with minimal interference (Jin et al., 2020). This ability to handle complex plasma matrices was particularly important for our study, as the method showed excellent specificity, with minimal interference from placebo samples, which is crucial for ensuring the reliability of therapeutic monitoring.

Optimization and Performance of the Method

A crucial aspect of this study was the optimization of chromatographic conditions for the simultaneous analysis of Empagliflozin and Hydrocortisone. The selection of the Agilent Zorbax SB-C18 column and the use of a two-phase gradient mobile phase, including methanol and formic acid, enabled effective separation of both analytes, even in plasma samples with complex composition. This choice of column and mobile phase composition was confirmed by detailed chromatographic data, which showed distinct, sharp peaks for both drugs, with high resolution and minimal baseline interference. These optimized

conditions are in line with previous studies that highlight the importance of chromatographic optimization for improving the sensitivity and specificity of LC-MS/MS methods for plasma analysis (Patel et al., 2020).

The mass spectrometry parameters, including the choice of ionization mode and collision energy, were optimized for both Empagliflozin and Hydrocortisone. Using positive electrospray ionization (ESI) and selecting the appropriate precursor and product ions for both drugs further improved sensitivity, allowing for the detection of these drugs at concentrations as low as parts per trillion (ppt) for Empagliflozin and parts per billion (ppb) for Hydrocortisone. These sensitivity levels are well within the requirements for therapeutic drug monitoring and pharmacokinetic studies, ensuring the method's applicability in both clinical and research settings.

Internal Standardization and Matrix Effects

The use of Hydrocortisone as an internal standard was a critical component of the method's success. Internal standards are commonly used in LC-MS/MS to compensate for matrix effects and ensure consistency in sample preparation and analysis (Jin et al., 2020). The internal standard provided excellent performance in terms of minimizing matrix interference and improving the accuracy of drug quantification. This was evident from the validation results, where minimal matrix effects were observed even in complex plasma samples. The matrix effects observed in many biological matrices, including plasma, can significantly affect the quantification of target analytes by reducing sensitivity and accuracy (Kumar et al., 2021). However, in our study, the use of the internal standard not only minimized these effects but also contributed to the reliability and reproducibility of the results, with relative standard deviations (RSDs) consistently under 10% for both intra- and inter-day precision.

Method Validation

The validation of the LC-MS/MS method followed the rigorous guidelines set by the International Council for Harmonisation (ICH M10), ensuring that all necessary parameters were thoroughly evaluated. The calibration curves for both Empagliflozin and Hydrocortisone demonstrated excellent linearity over a broad concentration range (5 ppb to 2000 ppb), with weighted linear regression applied to enhance the accuracy of quantification. This level of precision and linearity is consistent with what has been reported in other high-sensitivity LC-MS/MS applications (Zhang et al., 2020).

The method also demonstrated excellent specificity, with no significant interference from endogenous plasma compounds, even at the lower limits of quantification. The robustness of the method was confirmed across different testing phases and conditions, further ensuring its reliability in diverse clinical scenarios. This is particularly important for clinical applications, where sample conditions and the plasma matrix may vary between patients, affecting the consistency of drug measurements (Sharma et al., 2021).

Clinical and Research Applications

The implications of this study for clinical practice are significant. Empagliflozin is increasingly used in the management of Type 2 diabetes and heart failure, while Hydrocortisone plays a vital role in treating a wide range of conditions such as adrenal insufficiency, inflammatory disorders, and autoimmune diseases. Both drugs require careful monitoring to avoid adverse effects such as dehydration, hyperkalemia, and immunosuppression. For instance, patients receiving Hydrocortisone therapy are at risk of developing Cushing's syndrome or experiencing adrenal suppression if not properly dosed, while incorrect dosing of Empagliflozin can lead to poor control of blood glucose levels, contributing to cardiovascular risk (Borges et al., 2021).

The ability to monitor the plasma concentrations of both drugs simultaneously, using a single, highly sensitive and specific method, offers clear advantages in clinical settings. This method provides a valuable tool for optimizing individualized treatment regimens, enabling clinicians to adjust doses based on real-time drug concentration data, thereby improving patient safety and therapeutic outcomes.

Additionally, the method's ability to quantify both Empagliflozin and Hydrocortisone in the same analysis opens the door for broader applications in clinical pharmacokinetic studies. Researchers can use this method to investigate drug interactions, absorption, distribution, metabolism, and excretion (ADME) profiles of these drugs when used in combination with other therapies. This is particularly important for polypharmacy patients, who may be prescribed multiple drugs that interact with each other, affecting their pharmacokinetics.

Conclusion

In conclusion, this study successfully developed and validated a highly sensitive and specific LC-MS/MS method for the simultaneous quantification of Empagliflozin and Hydrocortisone in human plasma. The method demonstrated excellent specificity, accuracy, and precision, making it suitable for therapeutic drug monitoring and pharmacokinetic studies. The optimization of chromatographic and mass spectrometric

conditions ensured the reliable detection of both drugs, even at low concentrations, while the use of an internal standard minimized matrix effects and improved the reproducibility of the results.

The validated method meets the critical requirements of clinical pharmacology, offering a powerful tool for optimizing patient treatment and improving therapeutic outcomes. It provides a reliable, high-throughput solution for quantifying Empagliflozin and Hydrocortisone in plasma, contributing to the advancement of personalized medicine and drug safety monitoring. Future studies should explore the potential of this method for high-throughput clinical applications, including point-of-care testing, and its integration into broader pharmacokinetic studies of polypharmacy patients. The successful application of this LC-MS/MS method in clinical and research settings will not only enhance the precision of drug monitoring but also play a key role in ensuring safer and more effective therapies for patients worldwide.

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