Development and Validation of a Bioanalytical Method to Determine Empagliflozin in Human Plasma Using UPLC-MS/MS

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ABSTRACT

Aim: The study we are conducting aims to establish a validated method for measuring empagliflozin in plasma using UPLC MS/MS. Materials and Methods: Sodium-Glucose co-Transporter inhibitors (SGLTi), a novel class of diabetes medication. Non-insulin dependent diabetes and adult-onset diabetes were the prior names for type 2 diabetes. Empagliflozin was the first SGLT2-inhibitor a drug used that reduced the risk of cardiovascular risk those with type 2 diabetes and pre-existing cardiovascular disease. The empagliflozin D4 as Internal Solution (IS). Liquid-liquid extraction was used to achieve separation on Synergi 2.5µ Fusion-Reverse phase 100A (100 mm×2.0 mm), 2.5 µm column. Mixture of Methanol: As a mobile phase, 0.2% formic acid in a 75:25 volumetric ratio is utilised. The flow rate is set at 0.3µl/min. The calibration curve is linear and plotted. Results: The developed method is accurate, precise, sensitive method for determination of empagliflozin from human plasma solution. Ethyl acetate and water in HPLC grade are preferred solvent for empagliflozin. Performing LLE extraction with centrifuge and LV Nitrogen Evaporator it with Ethyl acetate and water. Ethyl acetate is used as non-polar solvent and water is used as polar solvent for liquid-liquid extraction. LLE is a low cost extraction process compare to solid liquid extraction. The reported method is suitable for bioequivalence and pharmacokinetic studies.

Keywords: Type 2 diabetes (T2D), Empagliflozin, Human Plasma, UPLC-MS/MS.

INTRODUCTION

The micro- and macrovascular systems are both impacted by the chronic condition known as diabetes mellitus. T2DM, formerly 6.4% of people worldwide already have adult-onset diabetes that is not dependent on insulin, generally known as the disease. and is projected to reach 7.7% by 2030–2022. Patients with type 2 diabetes are most commonly unwell and suffer from cardiovascular disease. Glucose and cholesterol levels, and also pulse rate, must be constantly maintained to limit the risk of complications. Elevated insulin sensitivity, insulin resistance, and pancreatic cell failure are all associated with T2D, which can cause up to 50% tissue loss at diagnosis. Teenagers lost -cells at a faster pace that might explain why those diagnosed at an early age have a greater likelihood of therapeutic failure. SGLTi is a new type of diabetic medication and its analytical approach Glucose was reabsorbed from the glomeruli via the sodium-dependent glucose co-transporter inhibitors 1 and 2. An SGLT2 inhibitor called empagliflozin has been shown to authorized for the purpose of those with type 2 diabetes in Europe, US, and Japan, among others places. In individuals with T2D and CVD, empagliflozin showed cardio protective and Reno protective effects that were essentially independent of glycemic management. A modern technique called UPLC has given liquid chromatography a fresh start of life. It may be used on particles with a diameter of less than 2mm to improve resolution, speed, and sensitivity. In the meanwhile, Analytical labs are no exception to the trend that UPLC analysis enhances the quality of their products. High pressure (up to 100M Pa) is used in UPLC for separation and quantification. Time and money are saved, solvent consumption is minimized, and more goods are evaluated using current resources. Liquid-Liquid Extraction (LLE) is a process that was created and used to process the method. LLE is a traditional process that involves dispersing appropriate extraction and dispersant solvents into aqueous solutions, primarily from water and biological fluid samples. For HPLC-grade empagliflozin, the ideal solvents are ethyl acetate and water. Adjustments to the aqueous phase's pH and ionic strength have an impact on how well substances are extracted. The LLE extraction process with
centrifuge and LV Nitrogen Evaporator it with Ethyl acetate and water. Ethyl acetate is used as nonpolar solvent and water is used as polar solvent for liquid-liquid extraction. LLE is a low cost extraction process compared to solid liquid extraction.

**MATERIALS AND METHODS**

The raw materials of empagliflozin and empagliflozin D4 are procured from MTR lab, Chennai. Melting and solubility investigations are used to verify the samples. UPLC method that has given liquid chromatography a new lease of life. It may be used on particles with a diameter of less than 2 mm to improve resolution, speed, and sensitivity. The human plasma collected from human volunteers. The Ethyl acetate and water used in HPLC grade. This method helps to reduce the cost and time consumption. The structure of empagliflozin as shows the Figure 1.

Empagliflozin is used as Working Standard (WS) and it is soluble in Ethanol, DMSO, and dimethyl formamide. Appearance of empagliflozin is White powder and the colour is white to yellowish non-hygroscopic crystalline solid. Empagliflozin D4 is used as Internal Standard (IS) and it is soluble in methanol and DMSO. Appearance of empagliflozin D4 is White powder and the colour is white to yellowish non-hygroscopic crystalline solid.

**Chromatography condition**

The liquid chromatography contains Synergi 2.5µ Fusion-Reverse Phase 100A (100 mm×2.0 mm), 2.5 µm and maintained column temperature 50°C. The flow rate is 0.3 µL/min, and the injection volume is 10 µL. Auto sampler Temperature is 10°C. Empagliflozin parent and daughter ion condition set as 451.17 and 355.11. And for Empagliflozin D4 parent and daughter ion condition set as 455.15 and 358.21. Dwell (sec), Cone (Volts), Collision energy (ev) condition for WS and IS is 0.200, 20.00, 12.00.

Retention Time: Empagliflozin: 1.0 ± 0.35 duration
Empagliflozin D4: 1.0 ± 0.35 duration

**Mass chromatography parameter**

Waters Acquity UPLC-MS, model-Quattro Micro Mass, Software-Mass Lynx V 4.1 it contains ESI-Positive mode and the desolvation temperature is 500°C. The desolvation gas flow is 800L/hr, Capillary voltage is 4.00 kV and the cone gas flow: 50 L/hr.

**Preparation of Reagents**

*Preparation of Buffer-1: [Water with 0.2% formic acid]*

Add 250 mL of Buffer-1 and well blend after adding 750 mL of Methanol to a 1000 mL reagent bottle. Through a 0.2 m nylon membrane filter, filter. For a few minutes, sonicate in an ultrasonic bath. Fill out the “Solution Preparation Form” with a batch number and a document. Within four days of the preparation date, use this solution.

*Preparation of Mobile Phase: [Methanol: Buffer: 1 (75:25, v/v)]*

Add 250 mL of Buffer-1 and well blend after adding 750 mL of Methanol to a 1000 mL reagent bottle. Through a 0.2 m nylon membrane filter, filter. For a few minutes, sonicate in an ultrasonic bath. Fill out the “Solution Preparation Form” with a batch number and a document. Within four days of the preparation date, use this solution.

*Preparation of Diluent – [H₂O: Methanol (50:50, v/v)]*

Transfer 500 mL of methanol into a 1 L beaker that already has 500 mL of water in it. Add thoroughly and sonicate for a few minutes in an ultrasonic bath. Fill out the “Solution Preparation Form” with a batch number and a document. Use this solution for four days from the date of preparation.

*Preparation of Analytical Solution (Empagliflozin stock solution) for CC*

Accurately measure the weight of 2 mg of Empagliflozin into a 2 mL volumetric flask, and transfer. Include 1 mL of Methanol to dissolving and adding volume with same. Determine the final concentration of Empagliflozin µg/mL as follows:

\[
\text{Empagliflozin’s weight (2 mg) } \times \frac{\text{Strength (as basis)}}{100} \times \frac{1000}{2 \text{ mL}} = \frac{\text{Empagliflozin’s weight (2 mg) } \times \text{Strength (as basis)}}{2 \text{ mL} \times 1000} \times M2
\]

**Figure 1**: Chemical structure of Empagliflozin.
Figure 2: Representative chromatogram of standard A for empagliflozin.

Figure 3: Representative chromatogram of standard H for empagliflozin.

Figure 4: Calibration curve for empagliflozin.
Sumithra, et al.: Bio Analytical method Development for Empagliflozin by UPLC-MS/MS

Provide Equations in MS Equation

Where, $M_1$ & $M_2$ are the molecular weight.

Preparation of Empagliflozin D4 stock solution

Empagliflozin D4 should be accurately weighed at around 2 mg before being poured into a 2 mL volumetric flask. To dissolve, include 1 mL of Methanol (Table 3). Use it to fill the remaining space. Calculate the final Empagliflozin D4 concentration in g/ml as follows:

$$\text{Empagliflozin's weight D4 (mg)} \times \text{Strength (as is basis)} \times \frac{1000}{M_1}$$

$$\text{Empagliflozin's weight D4 (mg)} \times \text{Strength (as is basis)} \times \frac{1000}{M_1}$$

Provide Equations in MS Equation

Internal standard Dilution

Develop the internal standard solution with diluent and empagliflozin D4 at a concentration of approximately 5 g/ml as instructed in (Table 1).

Spiked Calibration Curves for Standards

Add 0.200 mL of the stock aliquot of matching concentrations of the aforementioned stock dilutions of empagliflozin into a 10 mL volumetric flask. The remaining volume should subsequently be filled with pooled screened K2EDTA plasma. To obtain the concentrations indicated by the subsequent calibration curve in (Table 2).

Developing an empagliflozin stock solution for quality control

Empagliflozin WS should be accurately weighed at around 2 mg before being poured into a 2 mL volumetric flask. To dissolve, include 1 mL of methanol. Use it to fill the remaining space. Determine the empagliflozin g/mL final concentration using the formula below:

$$\text{Empagliflozin's weight (mg)} \times \text{Strength (as is basis)} \times \frac{1000}{M_1}$$

$$\text{Empagliflozin's weight (mg)} \times \text{Strength (as is basis)} \times \frac{1000}{M_1}$$

Provide equations in MS Equation

Where, $M_1$ & $M_2$ are the molecular weight of Empagliflozin (Free). Quality Control (QC) Samples.

RESULTS AND DISCUSSION

Empagliflozin was quantified for the duration of human plasma’s concentration range of 10.2172 to 3075.213 ng/ml using a sensitive and specific UPLC-MS/MS technology that was devised and validated. Our research aims to create a validated method for UPLC MS/MS-based empagliflozin plasma measurement. The Internal Solution (IS) of empagliflozin D4. Liquid-liquid extraction was used to separate the samples on a Synergi 2.5 Fusion-RP 100A (100 mm2.0 mm), 2.5 m column. The ideal solvents for empagliflozin are methanol and water at HPLC grade. Performing LLE extraction with centrifuge and LV Nitrogen Evaporator it with Ethyl acetate and water. Ethyl acetate is used as nonpolar solvent and water is used as polar

<table>
<thead>
<tr>
<th>Stock CID</th>
<th>Stock Concentration (µg/mL)</th>
<th>Stock Aliquot (mL)</th>
<th>Diluent (mL)</th>
<th>Final Volume (mL)</th>
<th>Final Concentration (µg/mL)</th>
<th>Spiked SCCID</th>
</tr>
</thead>
<tbody>
<tr>
<td>STD-A</td>
<td>10.188</td>
<td>0.200</td>
<td>9.800</td>
<td>10.000</td>
<td>10.2172</td>
<td>STD-A</td>
</tr>
<tr>
<td>STD-B</td>
<td>28.942</td>
<td>0.200</td>
<td>9.800</td>
<td>10.000</td>
<td>28.7211</td>
<td>STD-B</td>
</tr>
<tr>
<td>STD-C</td>
<td>115.306</td>
<td>0.200</td>
<td>9.800</td>
<td>10.000</td>
<td>114.7023</td>
<td>STD-C</td>
</tr>
<tr>
<td>STD-D</td>
<td>288.266</td>
<td>0.200</td>
<td>9.800</td>
<td>10.000</td>
<td>290.6332</td>
<td>STD-D</td>
</tr>
<tr>
<td>STD-E</td>
<td>576.532</td>
<td>0.200</td>
<td>9.800</td>
<td>10.000</td>
<td>574.5374</td>
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<tr>
<td>STD-F</td>
<td>1153.062</td>
<td>0.200</td>
<td>9.800</td>
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<td>1162.021</td>
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<tr>
<td>STD-G</td>
<td>2306.124</td>
<td>0.200</td>
<td>9.800</td>
<td>10.000</td>
<td>2284.641</td>
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<tr>
<td>STD-H</td>
<td>3054.468</td>
<td>0.200</td>
<td>9.800</td>
<td>10.000</td>
<td>3075.213</td>
<td>STD-H</td>
</tr>
</tbody>
</table>

Table 1: Preparation of Internal Standard solution for Empagliflozin D4.

Table 2: Preparation of Empagliflozin spiked calibration curve standards:
Table 3: Preparation of stock dilution Empagliflozin of QC.

<table>
<thead>
<tr>
<th>Stock Concentration (µg/mL)</th>
<th>Stock Aliquot (mL)</th>
<th>Diluents Added (mL)</th>
<th>Final Volume (mL)</th>
<th>Final Concentration (µg/mL)</th>
<th>Stock QCID</th>
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</thead>
<tbody>
<tr>
<td>2296.855</td>
<td>0.225</td>
<td>1.775</td>
<td>2.000</td>
<td>2221.6796</td>
<td>AQ-HQC</td>
</tr>
<tr>
<td>1148.428</td>
<td>1.000</td>
<td>1.000</td>
<td>2.000</td>
<td>1143.1551</td>
<td>AQ-MQC</td>
</tr>
<tr>
<td>287.107</td>
<td>1.000</td>
<td>1.000</td>
<td>2.000</td>
<td>284.1430</td>
<td>AQ-INTQC</td>
</tr>
<tr>
<td>28.711</td>
<td>0.100</td>
<td>1.900</td>
<td>2.000</td>
<td>30.5438</td>
<td>AQ-LQC</td>
</tr>
<tr>
<td>10.214</td>
<td>0.705</td>
<td>1.295</td>
<td>2.000</td>
<td>12.4002</td>
<td>AQ-LOQQC</td>
</tr>
</tbody>
</table>

Table 4: K₂EDTA plasma screening for empagliflozin and internal standard.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Analyte peak area</th>
<th>IS peak area</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extracted blank</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>Extracted LLOQ+IS</td>
<td>420</td>
<td>14862</td>
</tr>
<tr>
<td>Extracted ULOQ+IS</td>
<td>132634</td>
<td>12250</td>
</tr>
<tr>
<td>Extracted blank-I</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Extracted blank-II</td>
<td>5</td>
<td>18</td>
</tr>
<tr>
<td>% Carry Over from Blank I</td>
<td>-0.71</td>
<td>-0.01</td>
</tr>
<tr>
<td>% Carry Over from Blank II</td>
<td>-0.24</td>
<td>0.12</td>
</tr>
<tr>
<td>Result for blank - I</td>
<td>Pass</td>
<td>Pass</td>
</tr>
<tr>
<td>Result for blank - II</td>
<td>Pass</td>
<td>Pass</td>
</tr>
</tbody>
</table>

Table 5: Carry over test for empagliflozin and empagliflozin D4.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Analyte peak area</th>
<th>IS peak area</th>
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</tr>
<tr>
<td>Extracted ULOQ+IS</td>
<td>132634</td>
<td>12250</td>
</tr>
<tr>
<td>Extracted blank-I</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Extracted blank-II</td>
<td>5</td>
<td>18</td>
</tr>
<tr>
<td>% Carry Over from Blank I</td>
<td>-0.71</td>
<td>-0.01</td>
</tr>
<tr>
<td>% Carry Over from Blank II</td>
<td>-0.24</td>
<td>0.12</td>
</tr>
<tr>
<td>Result for blank - I</td>
<td>Pass</td>
<td>Pass</td>
</tr>
<tr>
<td>Result for blank - II</td>
<td>Pass</td>
<td>Pass</td>
</tr>
</tbody>
</table>
solvent for liquid-liquid extraction. LLE is a low cost extraction process compared to solid liquid extraction. The developed method is accurate, precise, sensitive, and specific for the determination of empagliflozin from human plasma. The reported technique is suitable for bioequivalence and pharmacokinetic studies.

**Biological Matrix**

The screening was conducted on the blank K2EDTA lots of human plasma. Micro Therapeutics Research Labs Pvt Limited in Chennai provided a plasma sample.

**Standard calibration curve and quality assurance samples**

Standards and quality control for curve calibration were all produced in accordance with ICH recommendations and kept between -70° and 15° and -20° and 5°C.

**Validation and Characteristics of Method**

**Specificity and selectivity**

In order to test the specificity and selectivity, one lot of heparin plasma, one lot of lipemic plasma, and one lot of hemolyzed plasma were used, totaling six lots of blank plasma, confirming that the acceptance requirements were fulfilled and the sample passed the test (Table 4). Selectivity and Specificity. The matrix lots under study showed acceptable S/N intensities, with Signal-to-Noise ratios ranging from 24.604 to 207.594.

**Carry Over Test**

Carryover is determined as the proportion of peak area that is seen in a processed blank plasma that is injected in triplicate following a processing ULOQ calibration standard that was created from a PA batch sample. Internal standard and empagliflozin did not exhibit any discernible carryover (Table 5).

**Linearity**

To calculate the linearity concentration, an 8 point standardised calibration curve is prepared in human plasma. Using empagliflozin D4 as an internal standard and a CC curve, the concentration of empagliflozin varied from 10.2172 to 3075.213 µg/mL using empagliflozin D4 as IS and for CC to the curve in (Figure 4).
Accuracy and precision

Six samples of the LLOQC, LOC, MQC, and HQC samples were examined to evaluate the assay’s precision and accuracy (Table 6). Refer to Figure 2 for information regarding chromatogram A. Hare references in (Figure 3).

Empagliflozin recovery studies

The recovery of empagliflozin was used to evaluate the contrasting detector responses from un-extracted aqueous samples at three various levels with detector responses from low, moderate, and extremely high quality control samples of analyte extracted from extracted CC and QC samples from the PA batch. The average recovery rate for empagliflozin was 85.30 percent. The empagliflozin percent CV was 6.52 percent at 3 distinct QC levels. An average of 87.05 percent of the IS was recovered for empagliflozin D4.

CONCLUSION

A selective and sensitive UPLC MS/MS is recommended by ICH guidelines, technique for determining the amount of empagliflozin in human blood at concentrations between 10.2172 to 3075.213 ng/ml was developed. It was successfully validated using the experimental parameters, and Table 7 displays the results. The mobile phase and multiple liquid extractions are used in this single compound approach. In contrast to solid liquid extraction, LLE is a low-cost extraction method. For the detection of empagliflozin in a solution of human plasma, a sensitive, accurate, and exact approach has been established. Bioequivalence and pharmacokinetic investigations can be conducted using the reported approach.

ACKNOWLEDGEMENT

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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