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# "DEVELOPMENT AND VALIDATION OF LCMS/MS METHOD FOR SIMULTANEOUS ESTIMATION OF METFORMIN & EMPAGLIFLOZIN IN TABLET AND CHARACTERIZATION OF DEGRADANT BY LC-MS/MS"

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**ABSTRACT**

A simple, rapid, precise and accurate Stability indicating LC-MS/MS method for simultaneous estimation of Metformin and Empagliflozin in their combined dosage form has been developed. The separation was achieved by Agilent, Zorbax, C18, (150mm x 4.6mm), 5µm column and Buffer (Ammonium acetate): Acetonitrile (75:25) as mobile phase, at a flow rate of 1 ml/min. Detection was carried out. Retention time of Empagliflozin and Metformin were found to be 2.6 min and 5.16 min respectively. The method has been validated for linearity, accuracy and precision. Linearity observed for Metformin 5µg/ml and for Empagliflozin 1.25 µg/ml. Developed method was found to be accurate, precise and rapid for simultaneous estimation of Metformin and Empagliflozin their Combined Dosage Form. The drug was subjected to stress condition of hydrolysis, oxidation, photolysis and Thermal degradation, Considerable Degradation was found in Alkali and Oxidation degradation. The proposed method was successfully applied for the simultaneous estimation of both the drugs in commercial combined dosage form.

KEY WORDS: Metformin, Empagliflozin, Stability indicating LC-MS/MS Method, Validation.

## 1 INTRODUCTION

Metformin's mechanisms of action are unique from other classes of oral antihyperglycemic drugs. Metformin decreases blood glucose levels by decreasing hepatic glucose production (also called gluconeogenesis), decreasing the intestinal absorption of glucose, and increasing insulin sensitivity by increasing peripheral glucose uptake and utilization. It is well established that metformin inhibits mitochondrial complex I activity, and it has since been generally postulated that its potent antidiabetic effects occur through this mechanism. . The above processes lead to a decrease in blood glucose, managing type II diabetes and exerting positive effects on glycaemic control.

Empagliflozin is used along with diet and exercise, and sometimes with other medications, to lower blood sugar levels in people with type 2 diabetes (condition in which blood sugar is too high because the body does not produce or use insulin normally). Empagliflozin is also used to reduce the risk of stroke, heart

attack, or death in people who have type 2 diabetes along with heart and blood vessel disease. Empagliflozin is also used in adults with heart failure to reduce the risk of needing to be hospitalized and death due to heart and blood vessel disease. Empagliflozin is in a class of medications called sodium-glucose co-transporter 2 (SGLT2) inhibitors. It lowers blood sugar by causing the kidneys to get rid of more glucose in the urine. Empagliflozin is not used to treat type 1 diabetes (condition in which the body does not produce insulin and, therefore, cannot control the amount of sugar in the blood) or diabetic ketoacidosis (a serious condition that may develop if high blood sugar is not treated).

## 2 Methodology:

### 2.1 Preparation of standard solutions

**Preparation of Diluent**

The diluent containing water and acetonitrile is prepared in the ratio of 50:50.

**Preparation of Standard Stock Solution of Empagliflozin**

Take 12.5 mg of Empagliflozin was weighed and transferred to a 100 ml volumetric flask. Volume was made up to the mark with methanol.

**Preparation of Standard Stock Solution of Metformin**

Take 50.0 mg of metformin was weighed and transferred to a 100 ml volumetric flask. Volume was made up to the mark with methanol.

**Preparation of Working Standard Solution of Empagliflozin**

From above Standard Stock Solution of Empagliflozin, 1 ml was taken in to 100 ml volumetric flask and was made up to the mark with the diluent to get 1.25 µg/ml of Empagliflozin.

**Preparation of Working Standard Solution of Metformin**

From above Standard Stock Solution of Metformin,1ml was taken in to 100 ml volumetric flask and was made up to the mark with the diluent to get 5.0µg/ml of metformin

**Combine Preparation of Working Standard Solution of Empagliflozin and Metformin**

Take 1ml from metformin stock solution and 1ml from Empagliflozin stock solution into 100ml volumetric flask and make up the volume with diluent to get 5.0 µg/ml of Metformin and 1.25 µg/ml of Empagliflozin.

**Preparation of Mobile Phase**

preparation-Prepare 10mM ammonium acetate buffer in water as mobile phase A and Methanol as mobile phase B. 10mM ammonium acetate buffer preparation: Take 77.0mg of ammonium acetate buffer and dissolve in 100ml LCMS grade water. Mix well and degas by sonication. **Preparation of Sample Stock Solution of Empagliflozin and Metformin**

The average weight of 10 tablets was determined and was ground in a mortar. Stock solution was prepared by dissolving tablet powder equivalent to 1.25 mg of Empagliflozin and 500 mg of Metformin was transferred to 100ml volumetric flask. Then 50 ml diluent was added and sonicated for 5 mins to ensure complete solubilization of drug. After sonication, volume was made up to the mark with diluent. Filter the stock solution with 0.45µ Millipore filter and the final filtrate is collected as sample stock solution.

**Preparation of Sample Working Solution of Empagliflozin and Metformin**

From above Sample Stock Solution of Empagliflozin and Metformin, 1 ml was taken in to 100 ml volumetric flask and was made up to the mark with the diluent to get 1.25 µg/ml of Empagliflozin and 5.0

µg/ml Metformin.

**Table 1: Chromatographic Conditions of LC-MS/MS**

| | | | |
|---|---|---|---|
| Instrument | Liquid chromatography Mass spectrometer (API-2000) equipped with auto sample, auto injector, column oven, ion source ESI electron spray ionizer with Q1 and collision energy. | | |
| ***Ion Source setting*** | | ***Scan setting*** | |
| Ion source | ESI | Polarity | Positive ion |
| Curtain Gas | 30psi | Scan type | MRM |
| Ion Spray Voltage | 5500 | Scan time | 1-10 min |
| Temperature | 400°C | Declustering Potential | 90 |
| Ion Source Gas(GS1) | 50psi | Focusing Potential | 500 |
| Ion Source Gas(GS2) | 50psi | Entrance Potential | 10 |
| Scan type | Empagliflozin | MRM:(Q1)786.800 Da and (Q3) 727.500 Da | |
| | Metformin | MRM:(Q1)172.100 Da and (Q3) 126.400 Da | |

- **Chromatographic condition:**

| | | | | | |
|---|---|---|---|---|---|
| Column | : | Agilent, Zorbax, C18, (150mm x 4.6mm), 5µm | | | |
| Flow rate | : | 1.0 mL/min | Injection volume | : | 20 µL |
| Column oven temperature | : | 35 °C | Run time | : | 10 min |
| Column oven compartment | : | Ambient | Mode | : | Isocratic |
| Empagliflozin R.T | : | About 4.9 min | | | |
| Metformin R.T | : | About 2.6 min | | | |

## 3 Stability Indicating

**Method.**

**3.1 Acid degradation**: Acid decomposition studies were performed by transferring 1 ml of stock solution to 100 ml of volumetric flask. 1 ml of 0.1 N HCl solutions was added and mixed well and put for 4 hrs at Room temperature. Then the volume was adjusted with diluent to get 1.25µg/ml for Empagliflozin and 5 µg/ml for Metformin.

**3.2 Base degradation**: Base decomposition studies were performed by transferring one ml of stock solution to 100 ml of volumetric flask. 1 ml of 0.1 N NaOH solutions was added and mixed well and put for 3 hrs at Room temperature. Then the volume was adjusted with diluent to get 1.25µg/ml for Empagliflozin and 5µg/ml for Metformin

**3.3 Oxidative degradation**: Oxidation decomposition studies were performed by transferring 1 ml of stock solution to100 ml of volumetric flask. Two ml of 3% H2O2 solutions was added and mixed well and put for 3 hrs at Room temperature. Then the volume was adjusted with diluent to get 1.25µg/ml for Empagliflozin and 5µg/ml for Metformin.

**3.4 Photo degradation**: Put about 100.0 mg of Empagliflozin and 100 mg of Metformin standard into Petri dish and place the Petri dish into photo stability chamber for 4 hrs. After 4 hrs weigh and transfer about 12.5 mg of Empagliflozin powder and 50.0 mg Metformin into a 100ml volumetric flask and make up volume with diluent. Transfer 1.0ml each solution into a 100ml volumetric flask and make up volume with diluent to get 1.25µg/ml for Empagliflozin and 5µg/ml for Metformin.

**3.5 Thermal Degradation**

Put about 100.0mg of Empagliflozin and 100 mg of Metformin standard into Petri dish and place the Petri dish into hot air oven at $100^0$C for 4 hrs. After 4 hrs weigh and transfer about 12.5mg of Empagliflozin powder into a 100ml volumetric flask and 50.0 mg of Metformin in to 100 ml volumetric flask and make up volume with diluent. Transfer 1.0ml each solution into a 100ml volumetric flask and make up volume with diluent to get 12.5µg/ml for Empagliflozin and 50µg/ml for Metformin.

## 4 VALIDATION OF LC-MS/MS METHOD

### 4.1 Specificity

The blank solution, working standard solution and working sample solution of Empagliflozin and Metformin is injected in to the LC-MS/MS system. The chromatogram of standard and sample has no interference with the chromatogram of blank.

### 4.2 Linearity and Range

The linearity for Empagliflozin and Metformin were assessed by analysis of standard solution in range of 0.625-1.875 µg/ml for Empagliflozin and 2.5-7.5 µg/ml Metformin respectively. 0.5, 0.75, 1.0, 1.25, 1.50 ml solutions were pipette out from the Stock solution of Empagliflozin and Metformin and transfer to 100 ml volumetric flask and make up with diluent to obtain 1.0, 1.5, 2.0, 2.5 and 3.0 µg/ml for Empagliflozin and 2.0, 3.0, 4.0, 5.0 and 6.0 µg/ml for Metformin respectively. In term of slope, intercept and correlation co-efficient value is obtained. The graph of peak area obtained verses respective concentration was plotted.

**Acceptance criteria:** Value of $r^2$ should be more than 0.99 should be less than 1.0.

### 4.3 Precision

#### 4.3.1 Repeatability

Standard solution containing Empagliflozin (1.25 µg/ml) and Metformin (5.0 µg/ml) was injected six times and areas of peaks were measured and % R.S.D. was calculated.

**Acceptance criteria:** % RSD of Area should not be more than 2.0%

#### 4.3.2 Intraday Precision

Standard solution containing (0.625, 1.25, 1.87 µg/ml) of Empagliflozin and (2.5, 5.0, 7.5 µg/ml) Metformin were analysed three times on the same day and % R.S.D was calculated.

**Acceptance criteria:** % RSD of Area should not be more than 2.0%

#### 4.3.3 Interlay Precision

Standard solution containing (0.625, 1.25, 1.87 µg/ml) of Empagliflozin and (2.5, 5.0, 7.5 µg/ml) Metformin were analysed three times on different day and % R.S.D was calculated.

**Acceptance criteria:** % RSD of Area should not be more than 2.0%

**Accuracy**

2.5 µg/ml drug solutions were taken in three different flask label A, B and C. Spiked 80%, 100%, 120% of standard solution in it and diluted up to 100ml. The area of each solution peak was measured. The amount of Empagliflozin and Metformin was calculated at each level and % recoveries were computed.

**Acceptance criteria**

% Recovery (individual) at each level should be between 98.00% and 102.00%

**4.4 Limit of Detection and Limit of Quantitation**

The LOD was estimated from the set of 3 calibration curves used to determination method linearity. The LOD may be calculated as,

LOD = 3.3 × (SD/Slope)

Where, SD = Standard deviation of Y-intercepts of 3 calibration curves.

Slope = Mean slope of the 3 calibration curves.

The LOQ was estimated from the set of 3 calibration curves used to determine method linearity. The LOQ may be calculated as,

LOQ = 10 × (SD/Slope)

Where, SD = Standard deviation of Y-intercepts of 3 calibration curves.

Slope = Mean slope of the 3 calibration curves.

**4.5 Robustness**

Following parameters were changed one by one and their effect was observed on system suitability for standard preparation.

1. Flow rate of mobile phase was changed (± 0.2 ml/min) 0.8 ml/min and 1.2 ml/min.
2. Ratio of Mobile phase was changed (±2) Buffer: acetonitrile and Buffer: Methanol.

**Acceptance criteria**

- % RSD for the analyte peak should not be more than 2.0%

**4.6 Analysis of Market Formulation**

Take tablet powder equivalent to 1.25 mg Empagliflozin and 500.0 mg of Metformin was transferred to a 100 ml volumetric flask, shake for 15 minutes and made up volume up to the mark with diluent. The solution was filtered through 0.45µ Millipore filter and first few drops of filtrate were discarded. 1 ml of this solution was diluted to 100 ml with diluent. The solution was injected 20 µl into the LC-MS/MS system. The areas of resulting peak were measured.

**5 RESULT AND DISCUSSION:**

**5.1 FORCED DEGRADATION STUDY**

Empagliflozin and Metformin standard was injected under various stress conditions. The optimized degradation condition is shown below.

**Table 2: Different Degradation Conditions for Empagliflozin**

| Sr. No. | Stress Type | Stress Condition |
|---|---|---|
| 1 | Acid Degradation | 1 N HCl at RT for 4 hours |
| 2 | Base Degradation | 1 N NaOH at RT for 3 hours |
| 3 | Oxidative Degradation | 30% $H_2O_2$ at RT for 3 hours |
| 4 | Thermal Degradation | 105ºC for 4 hours |
| 5 | Photolytic Degradation | UV for 4 hours |

**Table 3: Different Degradation Conditions for Metformin**

| Sr. No. | Stress Type | Stress Condition |
|---|---|---|
| 1 | Acid Degradation | 1 N HCl at RT for 5 hours |
| 2 | Base Degradation | 1 N NaOH at RT for 4 hours |
| 3 | Oxidative Degradation | 30% $H_2O_2$ at RT for 3 hours. |
| 4 | Thermal Degradation | 100ºC for 72 hours |
| 5 | Photolytic Degradation | UV for 72 hours |

**Selection of Elution Mode:**

Reverse phase chromatography was chosen because of its recommended use for ionic and moderate to non-polar compounds. Reverse phase chromatography is not only simple, convenient but also better performing in terms of efficiency, stability and reproducibility. C18 column is least polar compare to C4 and C8 columns. Here, 150x4.6mm column of 5.0µm particle packing was selected for separation of Empagliflozin and Metformin. Isocratic mode was chosen due to simplicity in application and robustness with respect to longer column stability.

## 5.2 Observation

- The acidic, Thermal and Photolytic degradation solution of Empagliflozin and Metformin is infused into the mass spectrometer to identified and characterized acidic degradation product (i.e DP1).
- From the mass spectra, it is found that, Empagliflozin and Metformin is not degraded under acidic condition.
- Hence, it was found that Empagliflozin and Metformin was stable under thermal and photo degradation conditions.

- The basic degradation solution of Empagliflozin is infused into the mass spectrometer to identified and characterized basic degradation product (i.e DP2) of Empagliflozin.
- From the mass spectra and fragmentation pathway, it is found that, Empagliflozin is degraded under

basic condition.

- The ESI-MS/MS spectra shows basic degradation product DP1 whose m/z ratio is obtained around 451.00 Da and its fragment ion peak is found whose m/z ratio is obtained around 365.800 Da and 202.200 Da.
- For MRM scan, Empagliflozin basic degradation product (DP2) molecular mass (Q1) is 365.800 Da and its fragment mass (Q3) is 202.200 Da is selected

- The acid degradation solution of Empagliflozin is infused into the mass spectrometer to identified and characterized oxidative degradation product (i.e. DP2) of Empagliflozin.
- From the mass spectra and fragmentation pathway, it is found that, Empagliflozin is degraded under oxidative condition.
- The ESI-MS/MS spectra shows oxidative degradation product DP2 whose m/z ratio is obtained around 451.100Da and its fragment ion peak is found whose m/z ratio is obtained around 381.500 Da and 288.400 Da.
- For MRM scan, Empagliflozin acid degradation product (DP2) molecular mass (Q1) is 381.500Da and its fragment mass (Q3) is 288.400 Da is selected

- The acid degradation solution of Metformin is infused into the mass spectrometer to identified and characterized base degradation product (i.e DP1) of Metformin
- From the mass spectra and fragmentation pathway, it is found that, Metformin is degraded under acid condition.
- The ESI-MS/MS spectra shows acid degradation product DP1 whose m/z ratio is obtained around 130.300 Da and its fragment ion peak is found whose m/z ratio is obtained around 131.400 Da and 86.100Da
- For MRM scan, Metformin base degradation product (DP1) molecular mass (Q1) is 131.400 Da and its fragment mass (Q3) is 86.100 Da is selected.

- The oxidative degradation solution of Metformin is infused into the mass spectrometer to identified and characterized oxidative degradation product (i.e DP2) of Metformin
- From the mass spectra and fragmentation pathway, it is found that, Metformin is degraded under oxidative condition.
- The ESI-MS/MS spectra shows oxidative degradation product DP1 whose m/z ratio is obtained around 130.300 Da and its fragment ion peak is found whose m/z ratio is obtained around 102.600 Da and 58.300 Da
- For MRM scan, Metformin oxidative degradation product (DP1) molecular mass (Q1) is 102.600 Da and its fragment mass (Q3) is 58.300 Da is selected.

**Table-4 Chromatographic Conditions of LC-MS/MS for Forced Degradation Studies**

| | | | |
|---|---|---|---|
| Instrument | Liquid chromatography Mass spectrometer (API-2000) equipped with auto sample, auto injector, column oven, ion source ESI electron spray ionizer with Q1 and collision energy. | | |
| ***Ion Source setting*** | | ***Scan setting*** | |
| Ion source | ESI | Polarity | Positive ion |
| Curtain Gas | 20psi | Scan type | MRM |
| Ion Spray Voltage | 5400 | Scan time | 1-10 min |
| Temperature | 400°C | Declustering Potential | 80 |
| Ion Source Gas(GS1) | 60psi | Focusing Potential | 450 |
| Ion Source Gas(GS2) | 60psi | Entrance Potential | 10 |
| Scan type | Empagliflozin | MRM:(Q1)451.100 Da and (Q3) 364.700 Da | |
| | Metformin | MRM:(Q1)130.300 Da and (Q3) 86.400 Da | |
| | Empagliflozin DP1 | MRM:(Q1)744.100 Da and (Q3) 126.400 Da | |
| | Empagliflozin DP2 | MRM:(Q1)804.100 Da and (Q3) 743.700 Da | |
| | Metformin DP1 | MRM:(Q1)381.500 Da and (Q3) 288.400 Da | |
| | Metformin DP2 | MRM:(Q1)365.800 Da and (Q3) 202.200 Da | |

- **Chromatographic condition:**

| | | | | | |
|---|---|---|---|---|---|
| Column | : | Agilent, Zorbax, C18, (150mm x 4.6mm), 5µm | | | |
| Flow rate | : | 1.0 mL/min | Injection volume | : | 20 µL |
| Column oven temperature | : | 35 °C | Run time | : | 10 min |
| Column oven compartment | : | Ambient | Mode | : | Isocratic |
| Empagliflozin R.T | : | About 5.7 min | | | |
| Metformin R.T | : | About 2.8 min | | | |
| Empagliflozin DP1 R.T | : | About 8.7 min | | | |
| Empagliflozin DP2 R.T | : | About 9.6 min | | | |
| Metformin DP1 R.T | : | About 3.2 min | | | |
| Metformin DP2 R.T | : | About 4.3 min | | | |

## 6 METHOD VALIDATION

### 6.1 Specificity

The Chromatograms of Empagliflozin and Metformin standards and Empagliflozin and Metformin sample show no interference with the Chromatogram of Empagliflozin and Metformin Blank, so the Developed method is Specific.

### 6.2 Linearity and Range

The linearity for Empagliflozin and Metformin were assessed by analysis of standard solution in range of 0.625-1.875 µg/ml and of 2.5-7.5 Empagliflozin and Metformin respectively. Correlation coefficient for calibration curve Empagliflozin and Metformin was found to be 0.994 and 0.999 respectively.

The regression line equation for Empagliflozin and Metformin are as following:

For Empagliflozin and Metformin : **y = 4547.1x – 104.47 and y = 80.48x – 19.12**

**Table 5: Linearity Data for Empagliflozin**

| Sr. No | Concentration (µg/ml) | Area |
|---|---|---|
| 1 | 0.625 | 3074.529 |
| 2 | 0.9375 | 4360.791 |
| 3 | 1.25 | 5514.493 |
| 4 | 1.5625 | 7264.408 |
| 5 | 1.875 | 8727.582 |

**Fig. Calibration Curve of Empagliflozin (0.625-1.875 µg/ml)**

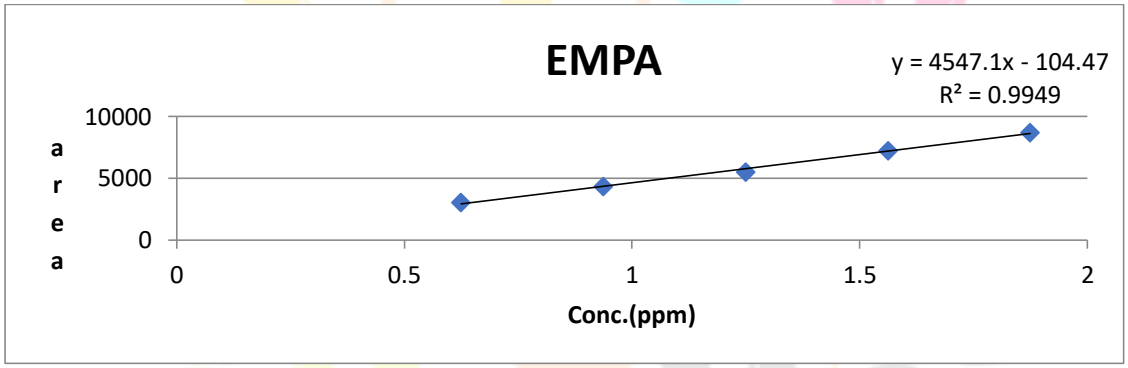


**Table 6: Linearity Data for Metformin**

| Sr. No | Concentration (µg/ml) | Area |
|---|---|---|
| 1 | 2.5 | 1834.531 |
| 2 | 3.75 | 2449.940 |
| 3 | 5 | 3999.487 |
| 4 | 6.25 | 4774.813 |
| 5 | 7.5 | 5790.751 |

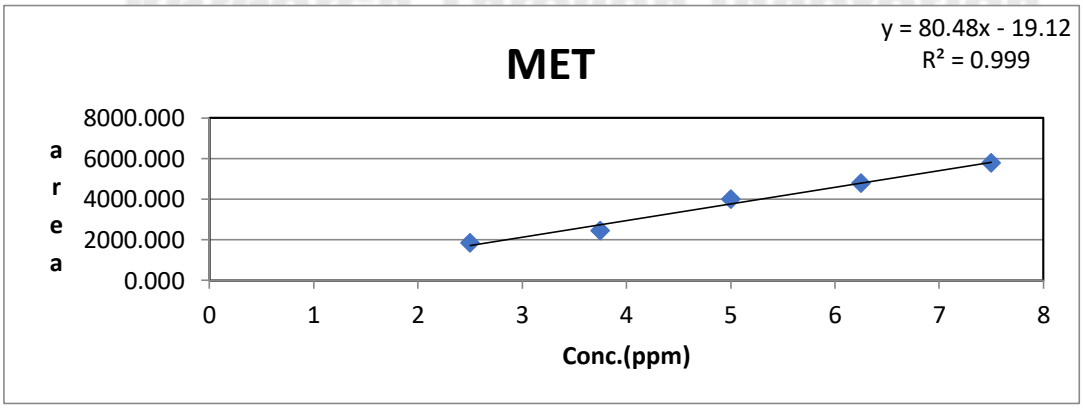


**Fig. Calibration Curve of Metformin (2.5-7.5 µg/ml)**

### 6.3 Precision

#### 6.3.1. Repeatability

The data for repeatability of peak area measurement for Empagliflozin and Metformin , based on six measurements of same solution of Empagliflozin and Metformin are depicted in table 6.12 and 6.13 respectively. The % RSD for Empagliflozin and Metformin was found to be 1.033 and 0.864 respectively.

**Table 7: Repeatability Data for Empagliflozin**

| Empagliflozin | | | | |
|---|---|---|---|---|
| **Sr. No.** | **Conc (µg/ml)** | **Area** | **Mean ± S.D (n=6)** | **% R.S.D** |
| 1. | 1.25 | 4056.238<br>3992.561<br>4044.568<br>4105.023<br>3996.481<br>4046.897 | 4040.295±41.723 | 0.864 |

**Table 8: Repeatability Data for Metformin**

| Metformin | | | | |
|---|---|---|---|---|
| **Sr. No.** | **Conc (µg/ml)** | **Area** | **Mean ± S.D (n=6)** | **% R.S.D** |
| 1. | 5.0 | 5587.942<br>5497.563<br>5510.879<br>5598.782<br>5492.561<br>5506.489 | 5532.369±47.805 | 0.864 |

#### 6.3.2. Intraday precision

The data for intraday precision for Empagliflozin and Metformin is shown in table 6.14 and 6.15 respectively. The % R.S.D. for Intraday precision was found to be 0.948-1.439 for Empagliflozin and 0.890 – 1.838 for Metformin .

**Table 9: Intraday precision data for Estimation of Empagliflozin**

| **Sr. No.** | **Empagliflozin** | | |
|---|---|---|---|
| | **Conc. (µg/ml)** | **Area Mean ± S.D. (n=3)** | **% R.S.D** |
| 1 | 0.625 | 1808.299 ± 13.487 | 0.746 |
| 2 | 1.250 | 4138.324 ± 63.800 | 1.542 |
| 3 | 1.875 | 5778.507 ± 40.570 | 0.702 |

**Table 10 : Intraday precision data for Estimation of Metformin**

| Sr. No. | Metformin | | |
|---|---|---|---|
| | Conc. (µg/ml) | Area Mean ± S.D. (n=3) | % R.S.D |
| 1 | 2.5 | 3139.555 ± 42.090 | 1.341 |
| 2 | 5.0 | 5518.053 ± 43.219 | 0.783 |
| 3 | 7.5 | 8829.368 ± 43.118 | 0.488 |

### 6.3.3. Interday precision

The data for intraday precision for Empagliflozin and Metformin is shown in table 6.16 and 6.17 respectively. The % R.S.D. for interday precision was found to be 0.517-1.583 for Empagliflozin and 1.117-1.331 Metformin .

**Table 11: Interday Precision data for Estimation of Empagliflozin**

| Sr. No. | Empagliflozin | | |
|---|---|---|---|
| | Conc. (µg/ml) | Area Mean ± S.D. (n=3) | % R.S.D |
| 1 | 0.625 | 1821.533 ± 22.579 | 1.240 |
| 2 | 1.250 | 4033.722 ± 49.041 | 1.216 |
| 3 | 1.875 | 5785.388 ± 35.765 | 0.618 |

**Table 12: Interday Precision data for Estimation of Metformin**

| Sr. No. | Metformin | | |
|---|---|---|---|
| | Conc. (µg/ml) | Area Mean ± S.D. (n=3) | % R.S.D |
| 1 | 2.5 | 3099.955 ± 50.765 | 1.638 |
| 2 | 5.0 | 5532.243 ± 40.666 | 0.735 |
| 3 | 7.5 | 8804.500 ± 44.576 | 0.506 |

### 6.4.4. Accuracy

Accuracy of the method was confirmed by recovery study from marketed formulation at three level of standard addition. The results are shown in table 6.18 and 6.19 respectively. Percentage recovery for Empagliflozin and Metformin was 100.179-100.852% and 99.635-100.291 respectively.

**Table 13: Recovery Data for Empagliflozin**

| Sr. No. | Conc. Level (%) | Sample amount (µg/ml) | Amount Added (µg/ml) | Amount recovered (µg/ml) | % Recovery | % Mean Recovery ± S.D |
|---|---|---|---|---|---|---|
| 1 | 80 % | 0.625 | 0.5 | 0.793 | 99.182 | 100.179 ± 0.888 |
| 2 | | 0.625 | 0.5 | 0.807 | 100.887 | |
| 3 | | 0.625 | 0.5 | 0.804 | 100.469 | |
| 4 | 100 % | 0.625 | 0.625 | 0.997 | 99.729 | 100.852 ± 0.972 |
| 5 | | 0.625 | 0.625 | 1.014 | 101.403 | |
| 6 | | 0.625 | 0.625 | 1.014 | 101.423 | |
| 7 | 120 % | 0.625 | 1.2 | 1.190 | 99.144 | 100.607 ± 1.418 |
| 8 | | 0.625 | 1.2 | 1.224 | 101.975 | |
| 9 | | 0.625 | 1.2 | 1.208 | 100.702 | |

**Table 14: Recovery Data for Metformin**

| Sr. No. | Conc. Level (%) | Sample amount (µg/ml) | Amount Added (µg/ml) | Amount recovered (µg/ml) | % Recovery | % Mean Recovery ± S.D |
|---|---|---|---|---|---|---|
| 1 | 80 % | 2.5 | 2 | 1.972 | 98.584 | 100.398 ± 1.646 |
| 2 | | 2.5 | 2 | 2.036 | 101.794 | |
| 3 | | 2.5 | 2 | 2.016 | 100.815 | |
| 4 | 100 % | 2.5 | 2.5 | 2.480 | 99.207 | 100.285 ± 0.964 |
| 5 | | 2.5 | 2.5 | 2.527 | 101.067 | |
| 6 | | 2.5 | 2.5 | 2.514 | 100.580 | |
| 7 | 120 % | 2.5 | 3.0 | 3.018 | 100.586 | 100.379 ± 0.563 |
| 8 | | 2.5 | 3.0 | 3.024 | 100.810 | |
| 9 | | 2.5 | 3.0 | 2.992 | 99.742 | |

### 6.4.5. LOD and LOQ

Calibration curve was repeated for five times and the standard deviation (SD) of the intercepts was calculated. Then LOD and LOQ were calculated as follows:

LOD = 3.3 * SD/slope of calibration curve

LOQ = 10 * SD/slope of calibration curve

**Limit of Detection**

**Table 15: Limit of Detection Data for Empagliflozin and Metformin**

| Empagliflozin | Metformin |
|---|---|
| LOD = 3.3 x (SD / Slope)<br>= 3.3 x (166.125/4035.664)<br>= 0.135 µg/ml | LOD = 3.3 x (SD / Slope)<br>= 3.3 x (1870.385/6758.862)<br>= 0.913 µg/ml |

**Limit of Quantitation**

**Table 16: Limit of Quantitation Data for Empagliflozin and Metformin**

| Empagliflozin | Metformin |
|---|---|
| LOQ = 10 x (SD / Slope)<br>= 10 x (207.385/3493.664)<br>= 0.409 µg/ml | LOQ = 10 x (SD / Slope)<br>= 10 x (2550.556/9228.762)<br>= 2.767 µg/ml |

**6.4.6. Robustness**

The effect of changes was found to be within the acceptance criteria as shown in table 6.22 and 6.23 respectively. The % RSD should be less than 2%.

**Table 17: Robustness data for Empagliflozin**

| Sr No. | Area at Flow rate (- 2.0 ml/min) | Area at Flow rate (+ 2.0 ml/min) | Area at Mobile phase(-2) | Area at Mobile phase(+2) |
|---|---|---|---|---|
| 1 | 5748.672 | 5369.665 | 6073.584 | 5047.692 |
| 2 | 5820.697 | 5469.115 | 6179.64 | 5147.872 |
| 3 | 5790.364 | 5541.006 | 6078.439 | 5036.987 |
| % R.S.D | 0.625 | 1.576 | 0.980 | 1.205 |

**Table 18: Robustness data for Metformin**

| Sr No. | Area at Flow rate (- 2.0 ml/min) | Area at Flow rate (+ 2.0 ml/min) | Area at Mobile phase(-2) | Area at Mobile phase(+2) |
|---|---|---|---|---|
| 1 | 4154.669 | 3841.225 | 4365.879 | 4036.584 |
| 2 | 4069.587 | 3947.005 | 4279.631 | 3987.473 |
| 3 | 4117.983 | 3899.065 | 4408.287 | 4005.879 |
| % R.S.D | 1.037 | 1.360 | 1.507 | 0.619 |

### 6.4.7. Analysis of marketed formulation by developed method.

Applicability of the proposed method was tested by analyzing the commercially available Tablet formulation Jardiance Met. The results are shown in table 6.24.

**Table 19: Analysis of Marketed Formulation**

| Tablet | Label claim | | Assay (% of label claim*) Mean ± S. D. | |
|---|---|---|---|---|
| | Empagliflozin | Metformin | % Empagliflozin | % Metformin |
| Jardiance Met | 1.25mg | 500mg | 99.836± 1.008 | 100.324 ± 0.848 |

The assay results were comparable to labeled value of each drug in combined dosage form. These results indicate that the developed method is accurate, precise, simple and rapid. It can be used in the routine quality control of dosage form in industries.

## 7. METHOD VALIDATION SUMMARY

**Table 20: Summary of Validation Parameters of LC-MS/MS Method for Empagliflozin and Metformin**

| Sr No. | Parameter | | Empagliflozin | Metformin |
|---|---|---|---|---|
| 1 | Specificity | | Specific | specific |
| 2 | Linearity & Range | | 0.625-1.875 | 2.5-7.5 |
| 3 | Regression equation | | y =4547.1x – 104.47 | y = 80.48x – 19.12 |
| 4 | Correlation co-efficient ($r^2$) | | 0.994 | 0.999 |
| 5 | Precision (% RSD) | 1.033 | 0.864 | 1.843 |
| | | 0.618-1.240 | 0.506-1.638 | 1.117-1.331 |
| | | 0.702 – 1.542 | 0.488-1.341 | 0.890 – 1.838 |
| 6 | Accuracy (% recovery) | | 99.954-101.335 | 100.285-100.398% |
| 7 | Limit of Detection(LOD) | | 2.767 µg/ml | 0.913 µg/ml |
| 8 | Limit of Quantification(LOQ) | | 0.409 µg/ml | 0.135 µg/ml |
| 9 | Robustness (% RSD) | | < 2.0 in each parameters | < 2.0 in each parameters |
| 10 | % Assay | | 99.836± 1.008 | 100.324 ± 0.848 |

## 8 SUMMARY AND CONCLUSION

- There is no analytical work has been available regarding LC-MS/MS method for Empagliflozin and Metformin in a literature. Data regarding behavior of drug in chromatographic conditions and other relevant analytical properties are not available.
- Empagliflozin is an antibiotic used in the treatment of several gastrointestinal and liver disease. This activity will review the indications, mechanism of action, administration, safety profile, and contra-indications for Empagliflozin. This activity will highlight the mechanism of action, adverse event profile, and other key factors (e.g., off-label uses, dosing, pharmacodynamics, pharmacokinetics, monitoring, relevant interactions) pertinent for the members of the interprofessional team.
- Metformin is the drug of choice in the chemotherapy of necrotizing ulcerative gingivitis.
- It is also used in high dosage in the chemoprophylaxis and treatment of many strictly anaerobic. Prolonged use has been advocated in the treatment of periodontitis.
- A novel attempt in a field of research has been made to develop and validate stability indicating analytical method via LC-MS/MS
- The objective of this study was to study the degradation behaviour of Empagliflozin and Metformin under acidic, basic, oxidative, photolytic and thermal stress conditions as per prescribed International Conference on Harmonization (ICH) guidelines.
- Empagliflozin was degraded under basic and oxidative stress condition and Metformin was degraded under basic and oxidative stress condition.
- The developed method has been validated as per ICH guideline by applying various validation parameters like specificity, linearity and range, accuracy precision and robustness.
- The LC-MS/MS method developed for the determination of Metformin and Empagliflozin is found to be specific, linear, sensitive, precise, accurate and robust in nature.
- The method was successfully validated in terms of specificity, precision, linearity, accuracy and robustness as per ICH guidelines.
- It can be concluded that the proposed method can be used for routine analysis for estimation of Metformin and Empagliflozin in its Pharmaceutical dosage form by LC-MS/M

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