Assay Method Validation Protocol for Empagliflozin In Empagliflozin 10&25 mg FCT

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1 Purpose

This Validation plan details the purpose, scope and experimental methods for the Assay method Validation. The Validation parameters that should be addressed are defined in the section risk analysis.

1.1 Purpose of the Outlined Project

The purpose of this study is to provide documented evidence that HPLC method for determining Assay of Empagliflozin is suitable for its intended purpose in:

- Empagliflozin 25 mg F.C.T
- Empagliflozin 10 mg F.C.T

2 Validation Object

2.1 Test Description

A. Apparatus: High Performance Liquid Chromatography with column heater and UV/PDA Detector.

B. Chromatographic Conditions:

Column	Inertsil C18 (250 x 4.6 mm), 5µm.
Wavelength	225 nm
Flow rate	1.0 mL/min
Injection volume	5µL
Column Temperature	25°C
Run time	10 min
Buffer preparation:	Transfer 2 ml of Ortho phosphoric acid in 1000ml water then filter on 0.45 µm membrane filter.
Mobile phase	A Filtrated and degassed mixture of Buffer and Acetonitrile in Proportion (60%:40%)
Diluent	0.1% orthophosphoric acid: ACN (70:30).
Needle wash	Water: Acetonitrile (50:50)

C. Solution preparations:

Standard solution	- Prepare 0.2mg /ml of Empagliflozin , Recommended preparation: - Accurately weigh about 10 mg of Empagliflozin working standard into a 50 mL volumetric flask, add 30 ml diluent, sonicate for 10 minutes , allow to cool to room temperature then complete the volume with the same diluent. ($C_{Empagliflozin}$: 0.2 mg/ml). - Prepare the standard in a duplicate preparations
Test Sample Solution	- For Empagliflozin 10 mg F.C.T, Recommended preparation: - Transfer 5 whole tablets into 250 ml volumetric flask. Add 150 ml diluent. Shake at 200 rpm for 10 min, Sonicate for about 10 minutes with intermittent shaking. Allow to cool to room temperature then complete the volume with the same diluent. Filter the solution through a 0.45 µ PTFE filter. - For Empagliflozin 25 mg F.C.T, Recommended preparation:

	- Transfer 4 whole tablets into 200 ml volumetric flask. Add 150 ml diluent. Shake at 200 rpm for 10 min, Sonicate for about 10 minutes with intermittent shaking. Allow to cool to room temperature then complete the volume with the same diluent, further dilute 10 ml into 25 ml volumetric flask, dissolve in and dilute to volume with diluent. Filter the solution through a 0.45 µ PTFE - Prepare the Test solution in a duplicate preparations - ($C_{Empagliflozin}$: 0.2mg/ml)

D. Procedure:

Equilibrate the column with mobile phase composition for not less than 15 minutes or until get stable base line at a flow rate of 1.0 mL/minute and run the next sequence:

Step	Injection name	Injection times
1	Standard solution A	6
2	Standard solution B	3
3	Sample solution	2
4	Standard solution A	1

E. Suitability Criteria

F.
- The similarity of 2 standard preparations between 98.0-102%.
- The RSD: NMT 2.0% (Peak area and retention time), Standard solution.
- Tailing factor: NMT 2.0%, Standard solution.
- Number of theoretical Plate Count: NLT 2000, Standard solution.

Calculation:

% Assay "$_{Empagliflozin}$ " = (Au/Astd) x (Wstd /D_{F1}) x (P/100) x (D_{F2}/ LC) x 100

- Au : Area response of Empagliflozin in test solution.
- Astd : Area response of Empagliflozin standard solution (average of two standard preparations).
- Wstd: Weight of Empagliflozin standard solution in mg
- D_{F1}: Dilution factor of standard solution.
- D_{F2}: Dilution factor of sample solution.
- LC: Label claim (10 mg) or (25 mg).
- P: Purity of standard (as is)

2.2 Principle of Analysis

The Assay testing method for determining the Assay of Empagliflozin.

2.3 Applicability of Method

This method is used for the determination of Assay of Empagliflozin in the following matrices:

- Empagliflozin 25 mg F.C.T
- Empagliflozin 10 mg F.C.T

2.4 Responsibility

The executive are responsible for:

- Performing the test and following these guidelines as stated in this protocol.

The department Section head is responsible for:

- Checking the adherence to the protocol by the analyst.

The department manager is responsible for:

- Approving the protocol.

2.5 Site of Execution:

R & D Laboratory, Primacy, Al Obour Cairo, Egypt.

3 Abbreviations Used in this Protocol:

HPLC	: High Performance Liquid Chromatography
RSD	: Relative Standard Deviation
R&D	: Research and Development
USP	: United State Pharmacopeia
CV	: Coefficient of Determination
NLT	: Not Less Than
NMT	: Not More Than
r^2	: Coefficient of Determination

4 Risk Analysis

4.1 Classification of the Type of Analytical Method under Validation

- ☐ Identification
- ☐ Test for contaminants/impurities (☐ Quantitative or ☐ Limit)
- ☒ Assay
- ☐ Dissolution
- ☐ Not classified (if so, then provide rationale why)

4.1.1 Relevance of the Validation Parameters

4.1.1.1 System Suitability

Is the analysis of System Suitability relevant?	☒ yes ☐ not required

4.1.1.2 Accuracy

Is the analysis of accuracy relevant?	☒ yes ☐ not required

4.1.1.3 Repeatability

Is the analysis of repeatability relevant?	☒ yes ☐ Not required

4.1.1.4 Intermediate Precision

Is the analysis of intermediate precision relevant?	☒ yes	☐ not required

4.1.1.5 Specificity

Is the analysis of specificity relevant?	☒ yes	☐ not required

4.1.1.6 Linearity

Is the analysis of linearity relevant?	☒ yes	☐ not required

4.1.1.7 Range

Is the analysis of range relevant?	☒ yes	☐ not required

4.1.1.8 Detection limit

Is the analysis of detection limit relevant?	☐ yes	☒ not required

4.1.1.9 Quantitation limit

Is the analysis of quantitation limit relevant?	☐ yes	☒ not required

4.1.2 Robustness

The following robustness parameters are identified:

4.1.2.1 Change in Flow rate:

Is the influence of change in Flow rate investigated during Validation?	☒ yes	☐ not required

4.1.2.2 Change in organic concentration:

Is the influence of change in organic concentration investigated during Validation?	☒ yes	☐ not required

4.1.2.3 Change in wavelength:

Is the influence of change in wavelength investigated during Validation?	☒ yes	☐ not required

4.1.2.4 Change in Buffer

Is the influence of change in Buffer investigated during Validation?	☒ yes	☐ not required

4.1.3 Solution Stability

Is the stability of solution investigated during validation?	☒ yes	☐ not required

4.1.4 Filter Recovery

Is the Filter recovery investigated during validation?	☒ yes	☐ not required

4.1.5 Control standard

Is establishment of a control standard necessary?	☐ yes	☒ not required

5 Experimental Procedures

5.1 Materials Used

- Orthophosphoric acid 85% (Analytical grade)
- Acetonitrile (HPLC grade)
- Purified water (Analytical grade)

5.2 Test Samples and Reference/ In-house Standards for Use in the Validation Study

- Standard: Empagliflozin
- Batch number: 1-PQY-173-1
- Supplier: TRC

5.3 Equipment and Accessories

- HPLC Column - Inertsil C18, 4.6 x 250mm, 5 µm, Serial No.: 20G0137708
- HPLC system Shimadzu 2050 C with PDA Detector.
- Analytical Balance/Microbalance
- Ultrasonic Bath
- Volumetric pipettes (as mentioned in the preparations)
- Volumetric flasks (as mentioned in the preparations)
- 0.45µm PTFE Syringe Filter

Note: Other volumetric flask may be used to achieve the same concentrations.

5.4 Experimental Design:

Item	Day 1	Day 2	Day 3
System Suitability	√		
Accuracy	√		
Repeatability	√		
Intermediate Precision	√	√	
Specificity	√		
Linearity and Range	√		
Robustness	√		
Filter recovery	√		
Solution Stability	√	√	√

5.5 System suitability:

Experimental Plan

System suitability test should be conducted and checked before running the rest of validation items
For the conditions, and preparations, Refer to Section 2.1 Test Description

Procedure:

Equilibrate the column with mobile phase composition for not less than 15 minutes or until get stable base line at a flow rate of 1.0 mL/minute and run the next sequence:

steps	Injection name	Injection times
1	Standard solution A	6

Calculation

The % RSD is calculated from the average of the results.

$$RSD\ [\%] = \frac{SD}{MV_n} * 100$$

Where

$$SD = \sqrt{\frac{1}{n-1} * \sum_{i=1}^{n} \left(x_i - MV_n \right)^2}$$

n = No. of values
Xi = Value of single value in series
MV = Mean value

Acceptance Criteria

The RSD of peak area of six standard injections, NMT 2.0%.
Tailing factor: NMT 2.0%, Number of theoretical Plate Count: NLT 2000.

5.6 Accuracy

Experimental Plan

Accuracy for the Assay test should be prepared within the working concentration range of 50%, 100%, and 160%.
For the conditions, and standard preparations, Refer to Section 2.1 Test Description.
Stock Standard Solution:
Accurately weigh 200 mg of Empagliflozin working standard, into 200 ml volumetric flask, add 150 ml of diluent, sonicate for 10 min, cool to room temperature, mix well and complete to volume with diluent.
Spiking Solution Preparations:

Concentration%	Stock solution	Placebo	Dilution volume	Final Concentration mg/ml
50 %	5 ml	200 mg	50 ml	0.10
100%	10 ml	200 mg	50 ml	0.20
160%	8 ml	100 mg	25 ml	0.32

Procedure:
Perform every concentration in 3 preparations and equilibrate the column for about 15 minutes with the mobile phase or until a steady base line is obtained at a flow rate of 1.0 mL/minute and run the next sequence:

Injection name	Injection times
Standard solution	6 injections
Spiking Solution (50% Concentration)	1 injection of each Preparation
Spiking Solution (100% Concentration)	1 injection of each Preparation
Spiking Solution (160% Concentration)	1 injection of each Preparation
Standard solution	2 injections

Calculation/ Documentation

Calculate the amount of active using the following formula:

$$Amount\ Spiked = C_{ST} \times \frac{V_1}{V_2}$$

- C_{ST} : concentration of Empagliflozin in stock solution.
- V_1 : Volume Taken
- V_2 : Dilution Volume

$$Amount\ Found = \frac{rU}{rS} \times CS$$

- rU = peak response of Empagliflozin from the Sample solution
- rS = peak response of Empagliflozin from the Standard solution
- CS = Concentration of Empagliflozin WS in the Standard solution (mg/mL).

$$\%\ Recovery = \frac{Amount\ Found}{Amount\ Spiked} \times 100$$

Confidence Interval

Confidence interval (CI) is a type of interval estimate, computed from the statistics of the observed percent Accuracy data that might contain the true value of an unknown population parameter. The interval has an associated confidence level that quantifies the level of confidence that the parameter lies in the interval

$$CI = \bar{x} \pm z\frac{s}{\sqrt{n}}$$

- $\bar{x}$ = Average of % Recovery
- S = Standard Deviation of % Recovery
- n = Sample Size
- 1.96 = the Z value at 95% Confidence level

$$Margin\ of\ Error = \frac{1.96 \times Standard\ Deviation\ of\ \%\ Recovery}{Sample\ ¿\ 0.5}$$

- Upper bound = Average of % Recovery + Margin of Error
- Lower bound = Average of % Recovery - Margin of Error

Acceptance Criteria

-Recovery%: 98.0% - 102.0% for each selected level.

5.7 Repeatability (Precision)

Experimental Plan

For the conditions, and preparations, Refer to Section 2.1 Test Description and 5.6 Accuracy.

Procedure:

From the accuracy data using the nine determinations (3 Concentrations and 3 replicate of each concentration) and determine the RSD.

Calculation

The % RSD is calculated from the average of the Accuracy results.

Acceptance Criteria

%RSD NMT 2.0%.

5.8 Intermediate Precision

Experimental Plan

Perform on the Another Day by the same Analyst; perform on the same Day, by Another Analyst, and following the procedure as stated in the Procedure experimental plan.

For the conditions, and preparations, Refer to Section 2.1 Test Description.

Procedure:

Equilibrate the column with mobile phase composition for not less than 15 minutes or until get stable base line at a flow rate of 1.0 mL/minute and inject 6 Sample preparations for each condition as mentioned in section 2.1 Test Description.
NB: Intermediate precision shall be performed on the highest strength which is:

- Empagliflozin 25 mg FCT.

Calculation

% Assay "$_{Empagliflozin}$ " = (Au/Astd) x (Wstd /D_{F1}) x (P/100) x (D_{F2}/ LC) x 100
- Au : Area response of Empagliflozin in test solution. - Astd : Area response of Empagliflozin standard solution (average of two standard preparations). - Wstd: Weight of Empagliflozin standard solution in mg - D_{F1}: Dilution factor of standard solution. - D_{F2}: Dilution factor of sample solution. - LC: Label claim (25 mg). - P: Purity of standard (as is)

Acceptance Criteria

- The pooled %RSD between each condition NMT 3.0%.

5.9 Specificity

Experimental Plan

For the conditions, and preparations, Refer to Section 2.1 Test Description

- **Placebo:** weigh 100 mg of Placebo into 50 ml volumetric flask, add 30 ml of diluent, sonicate for 10 min, cool to room temperature, mix well and complete to volume with diluent.
- **Forced degradation:**

  Stock solution preparation: Crush 20 tablets to fine powder, weigh about 1020mg from fine powder of tablets (equivalent to 100 mg from Empagliflozin) into 50 ml volumetric flask. Add 30 ml of diluent, sonicate for 10 min, allow to cool to room temperature then complete the volume with the same diluent.

  Acid degradation: Transfer 5 ml from stock solution into 20 ml volumetric flask, add 4 ml from 5N Methanolic HCl, then place the sample at 50°C in water bath for 5 days, after specified time, allow the sample to cool down, then add 4 ml from 5 N Methanolic NaOH, and complete the volume with diluent. Transfer 4 ml from the previous solution into 10 ml volumetric flask, and complete the volume with diluent.

  Base degradation: Transfer 5 ml from stock solution into 20 ml volumetric flask, add 4 ml from 5N Methanolic NaOH, then place the sample at 50°C in water bath for 5 days, after specified

time, allow the sample to cool down, then add 4 ml from 5 N Methanolic HCl, and complete the volume with diluent. Transfer 4 ml from the previous solution into 10 ml volumetric flask, and complete the volume with diluent.

Oxidation degradation: Transfer 5 ml from stock solution into 20 ml volumetric flask, add 0.5ml from 10% H_2O_2, then place the sample at 50°C in water bath for 5 days, after specified time, allow the sample to cool down, and complete the volume with diluent. Transfer 4 ml from the previous solution into 10 ml volumetric flask, and complete the volume with diluent.

Heat degradation: Transfer 5 ml from stock solution into 20 ml volumetric flask, then place the sample at 50°C in water bath for 5 days, after specified time, allow the sample to cool down, and complete the volume with diluent. Transfer 4 ml from the previous solution into 10 ml volumetric flask, and complete the volume with diluent.

Procedure:

Equilibrate the column with mobile phase composition for not less than 15 minutes or until get stable base line at a flow rate of 1.0 mL/minute and run the next sequence:

steps	Injection name	Injection times
1	Diluent	2
2	Placebo	2
3	Standard solution	2
4	Sample solution	2
5	Acid degradation of sample	2
6	Base degradation of sample	2
7	Oxidation degradation of sample	2
8	Heat degradation of sample	2

Acceptance Criteria

There should not be any interference from diluent, placebo and forced degradation prepared solutions at the retention time and detection wavelength of active peak.

5.10 Linearity /Range

Experimental Plan

For the conditions, Refer to Section 2.1 Test Description

Standard Stock Solution Preparation:

Accurately weigh 100 mg of Empagliflozin working standard into 100 ml volumetric flask, add 70 ml of diluent, sonicate for 10 min, cool to room temperature, mix well and complete to volume with diluent.

Spiking Solution Preparation:

Concentration%	Stock solution	Dilution volume	Final Concentration mg/ml
50 %	5 ml	50 ml	0.10
80%	8 ml	50 ml	0.16
100%	10 ml	50 ml	0.20

120%	6 ml	25 ml	0.24
160%	8 ml	25 ml	0.32

Procedure:

Equilibrate the column with mobile phase composition for not less than 15 minutes or until get stable base line at a flow rate of 1.0 mL/minute and run the next sequence:

Sample ID	Injection times
Prepared Solutions	3 injections/Level

Calculation/ Documentation

Active peak area response is used to calculate linearity. Plot the linearity curve of the peak area of Active against its respective concentrations. Determine the linearity regression coefficient (coefficient of determination), y-intercept and slope from the linearity curve of active using the formula function of Excel.

Acceptance Criteria

The Linearity Correlation Coefficient (r) is NLT 0.999

5.11 Robustness

NB: Robustness shall be performed on the highest strength which is:

- Empagliflozin 25 mg FCT.

5.11.1 **Robustness Parameter-1 (Change in Flow rate)**

Experimental Plan

For the conditions, and preparations, Refer to Section 2.1 Test Description

Procedure:

Run the prepared standard and sample solution using the stated chromatographic conditions with a change in flow rate from 0.9 to 1.1 ml /min.

5.11.2. **Robustness Parameter-2 (Change in Organic concentration)**

Experimental Plan

For the conditions, and preparations, Refer to Section 2.1 Test Description

Procedure:

Run the prepared standard and sample solution using the stated chromatographic conditions with a change in Acetonitrile % in mobile phase from 37 to 43%.

5.11.3. **Robustness Parameter-3 (Change in wavelength)**

Experimental Plan

For the conditions, and preparations, Refer to Section 2.1 Test Description

Procedure:

Run the prepared standard and sample solution using the stated chromatographic conditions with a change in wavelength from 223 to 227 nm.

5.11.4. **Robustness Parameter-4 (Change in Buffer Strength)**

Experimental Plan

For the conditions, and preparations, Refer to Section 2.1 Test Description

Procedure:
Run the prepared standard and sample solution using the stated chromatographic conditions with a change in Buffer from 0.2% Orthophosphoric acid to 0.1% Orthophosphoric acid

Calculation

For the calculation of the percentage of active in the portion of sample taken, Refer to Section 5.6 Intermediate Precision

Acceptance Criteria

- The RSD of peak area of six standard injections, NMT 2.0% for each condition.
- The RSD of peak area of six standard injections and two standard injections as bracketing NMT 2.0% for each condition.
- Tailing factor: NMT 2.0%, Standard solution for each condition.
- Number of theoretical Plate Count: NLT 2000, Standard solution for each condition.
- The pooled RSD% between conditions, NMT 3.0% for each parameter.

5.12 Stability of Solution

NB: Stability of solution shall be performed on the highest strength which is:

- Empagliflozin 25 mg FCT.

Experimental Plan

For the conditions, and preparations, Refer to Section 2.1 Test Description
Procedure:
Separately inject equal volumes (5µl) of the standard and sample solution, Stored in room temperature for 72 hours into the liquid chromatograph and record the peak response of active in each injection.

Calculation/ Documentation

For the calculation of the percentage of active in the portion of sample taken, Refer to Section 5.6 Intermediate Precision

Acceptance Criteria

- The RSD of peak area of six standard injections, NMT 2.0%.
- The RSD of peak area of six standard injections and two standard injections as bracketing NMT 2.0%.
- Tailing factor: NMT 2.0%, Standard solution.
- Number of theoretical Plate Count: NLT 2000, Standard solution.
- The recovery of assay value between 98.0-102%

5.13 Filter recovery

NB: Filter recovery shall be performed on the highest strength which is:

- Empagliflozin 25 mg FCT.

Experimental Plan

For the conditions, and preparations, Refer to Section 2.1 Test Description
Procedure:

Separately inject equal volumes (5µl) of the standard solution and sample solution, this time using 0.45 µm (PTFE) syringe filter to filter the standards and sample solutions against un-filtered standard and Centrifuged sample solutions, into the liquid chromatography, record the chromatograms and measure the peak response of the major peak.

Calculation/ Documentation

For the calculation of the percentage of active in the portion of sample taken, Refer to Section 5.6 Intermediate Precision

Acceptance Criteria

- The RSD of peak area of six standard injections, NMT 2.0%.
- The RSD of peak area of six standard injections and two standard injections as bracketing NMT 2.0%.
- Tailing factor: NMT 2.0%, Standard solution.
- Number of theoretical Plate Count: NLT 2000, Standard solution.
- The recovery of assay value between 98.0-102%

6 Time-Plan

Proposed completion date: 1 Month after approval of the protocol.

7 References

ICH guidelines Q2 (R1): Validation of analytical procedures: Text, Methodology and in-house requirements.