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Method development and validation for the estimation of Empagliflozin in bulk form and marketed tablet dosage form by RP-HPLC

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Abstract
Objective: The aim of the research work is to develop and validate a novel, sensitive, specific, rapid, accurate, and precise, isocratic reverse phase high-performance liquid chromatography (RP-HPLC) method for the quantitative determination of Empagliflozin drug substance in pure form and Marketed Pharmaceutical Dosage form.
Methods: Liquid chromatographic method used for the analysis of the anti-Diabetic drug substance like Empagliflozin method was developed and validated by using efficient chromatographic separation method and was achieved with the use of HPLC WATERS with Empower2 Software with Isocratic with UV-Visible Detector.
Results: The separation was achieved using Symmetry C18, 250 mm x 4.6 mm i.d.5µm particle size analytical column at ambient employing an isocratic elution. Empower software was used for data acquisition. During method validation all the parameters were evaluated as per ICH guidelines, which remained well within acceptable limits.
Conclusion: The results of linearity, precision accuracy and specificity were proved to be within the limits. This method can be employed in routine analysis for estimation of Empagliflozin drug substance in quality formulations.

Keywords: Empagliflozin, ICH Guidelines, Method Development, Validation

Introduction

Empagliflozin is an inhibitor of sodium-glucose co-transporter-2 (SGLT2), the transporters primarily responsible for the reabsorption of glucose in the kidney. It is used clinically as an adjunct to diet and exercise, often in combination with other drug therapies, for the management of type 2 diabetes mellitus. Empagliflozin [1] is an orally available competitive inhibitor of sodium-glucose co-transporter 2 (SGLT2; SLC5A2) with antihyperglycemic activity. Upon oral administration, Empagliflozin selectively and potently inhibits SGLT2 in the kidneys, thereby suppressing the reabsorption of glucose in the proximal tubule. Inhibition of SGLT2 increases urinary glucose excretion by the kidneys, resulting in a reduction of plasma glucose levels in an insulin-independent manner. SGLT2, a transport protein exclusively expressed in the proximal renal tubules, mediates approximately 90% of renal glucose reabsorption from tubular fluid. Empagliflozin [2] is indicated as an adjunct to diet and exercise to improve glycemic control in adult patients with type 2 diabetes. It is also indicated to reduce the risk of cardiovascular death in adult patients with both type 2 diabetes mellitus and established cardiovascular disease. Empagliflozin lowers blood glucose levels by preventing glucose reabsorption in the kidneys, thereby increasing the amount of glucose excreted in the urine. It has a relatively long duration of action requiring only once-daily dosing. Patients should be monitored closely for signs and symptoms of ketoacidosis regardless of blood glucose level as Empagliflozin [3] may precipitate diabetic ketoacidosis in the absence of hyperglycemia. As its mechanism of action is contingent on the renal excretion of glucose, Empagliflozin may be held in cases of acute kidney injury and/or discontinued in patients who develop chronic renal disease.

The IUPAC Name of Empagliflozin is (2S, 3R, 4R, 5S, 6R)-2-[4-chloro-3-[[4-[(3S)-oxolan-3-yl] oxy phenyl] methyl] phenyl]-6-(hydroxy methyl) oxane-3, 4, 5-triol. The Chemical Structure of Empagliflozin is as following.

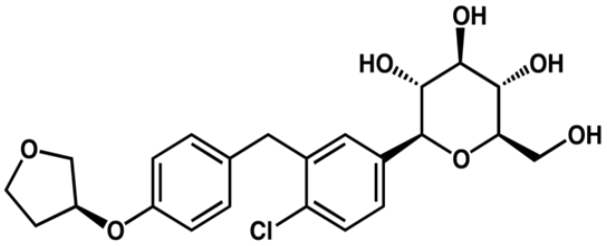

Fig 1: Chemical Structure of Empagliflozin

Materials and Methods

Materials and Instruments

The following are the list of instruments/Equipments, chemicals/reagents and standards to perform the HPLC Analysis [4] of the drug Empagliflozin.

Equipments

Table 1: List of Equipments

S. No.	Instruments/Equipments/Apparatus
1.	HPLC WATERS with Empower2 Software with Isocratic with UV-Visible Detector.
2.	T60-LABINDIA UV – Vis spectrophotometer
3.	High Precision Electronic Balance
4.	Ultra Sonicator (Wensar wuc-2L)
5.	Thermal Oven
6.	Symmetry C_{18} Column, 250 mm x 4.6 mm and 5µm particle size
7.	P^H Analyser (ELICO)
8.	Vaccum Filtration Kit (Labindia)

Chemicals and Reagents

Table 2: List of Chemicals used

S. No.	Name	Grade	Manufacturer/Supplier
1.	HPLC grade water	HPLC	Sd fine-Chem ltd; Mumbai
2.	Methanol	HPLC	Loba Chem; Mumbai.
3.	Ethanol	A.R.	Sd fine-Chem ltd; Mumbai
4.	Acetonitrile	HPLC	Loba Chem; Mumbai.
5.	DMSO	A.R.	Sd fine-Chem ltd; Mumbai
6.	DMF	A.R.	Sd fine-Chem ltd; Mumbai

Working Standard: Working Standard of Empagliflozin: 10ppm

HPLC Instrumentation & Conditions: The HPLC system[5] employed was **HPLC WATERS** with Empower2 Software with Isocratic with UV-Visible Detector.

Standard preparation for UV-spectrophotometer analysis

The standard stock solutions: 10 mg of Empagliflozin standard was transferred into 10 ml volumetric flask, dissolved & make up to volume with Methanol. Further dilutions were done by transferring 1 ml of the above solution into a 10ml volumetric flask and make up to volume with methanol to get 10ppm concentration.

It scanned in the UV spectrum in the range of 200 to 400nm. This has been performed to know the maxima of Empagliflozin, so that the same wave number can be utilized in HPLC UV detector for estimating the Empagliflozin.

Preparation of Mobile Phase

The mobile phase [6] used in this analysis containing of a mixture of Methanol and Acetonitrile in the ratio of 70:30 v/v was prepared in the volume of 1000ml in which 700ml of Acetonitrile was mixed with 300ml of Methanol.

Preparation of Standard Solution

Accurately weigh and transfer 10 mg of Empagliflozin working standard into a 10ml of clean dry volumetric flasks add about 7ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution).

Further pipette out 0.1ml of Empagliflozin from the above stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

Method Validation

The analytical procedure refers to the way of performing the analysis. It should describe in detail the steps necessary to perform each analytical test. This may include but is not limited to: the sample, the reference standard and the reagents preparations, use of the apparatus, generation of the calibration curve, use of the formulae for the calculation, etc.

1. Specificity

Specificity [7] is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc.

2. Accuracy

The accuracy [8] of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. This is sometimes termed trueness.

3. Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision[9] may be considered at three levels: repeatability, intermediate precision and reproducibility.

3.1. Repeatability

Repeatability [10] expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra-assay precision[11].

3.2. Intermediate precision

Intermediate precision [12] expresses within-laboratories variations: different days, different analysts, different equipment, etc.

3.3. Reproducibility

Reproducibility [13] expresses the precision between laboratories (collaborative studies, usually applied to standardization of methodology).

4. Detection Limit

The detection limit [14] of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value.

5. Quantitation Limit

The quantitation limit [15] of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy [16]. The quantitation limit is a parameter of quantitative assays for low levels of compounds in sample matrices, and is used particularly for the determination of impurities and/or degradation products.

6. Linearity

The linearity [17] of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample.

7. Range

The range [18] of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity [19].

8. Robustness

The robustness [20] of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

Results and Discussion

Method Development

Detection of Wavelength

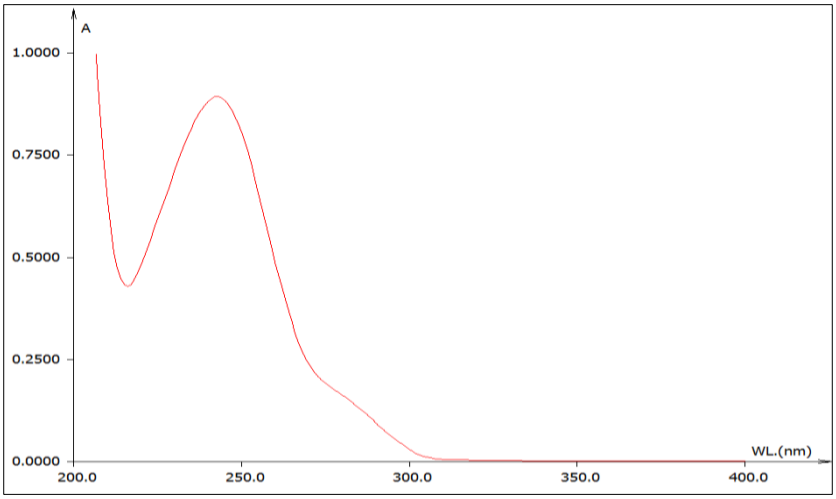

Fig 2: UV-Spectrum for Empagliflozin

Observation

While scanning the Empagliflozin solution we observed the maxima at 245nm.

Optimized Chromatographic Conditions

Column : Symmetry C18, 250 mm x 4.6 mm i.d.5µm particle size

Mobile Phase : Methanol: Acetonitrile (70: 30% v/v)
Flow Rate : 1.0ml/minute
Wave length : 245 nm
Injection volume : 10 µl
Run time : 7 minutes
Column temperature : Ambient

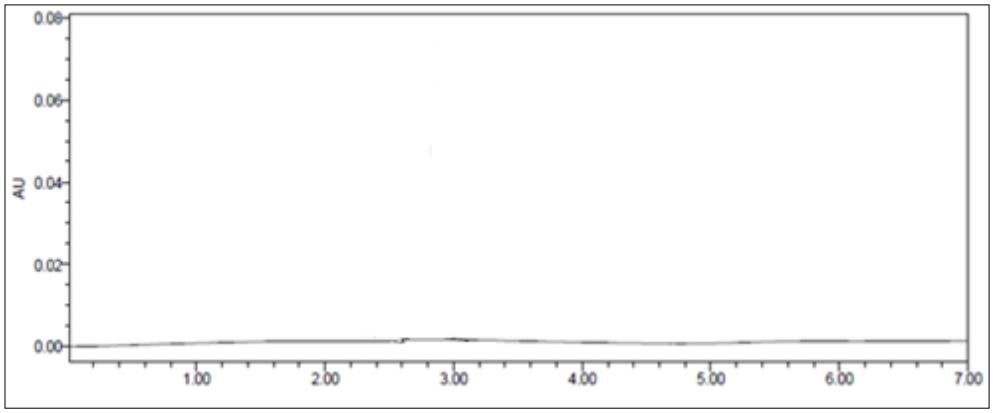

Fig 3: Chromatogram for Blank Solution

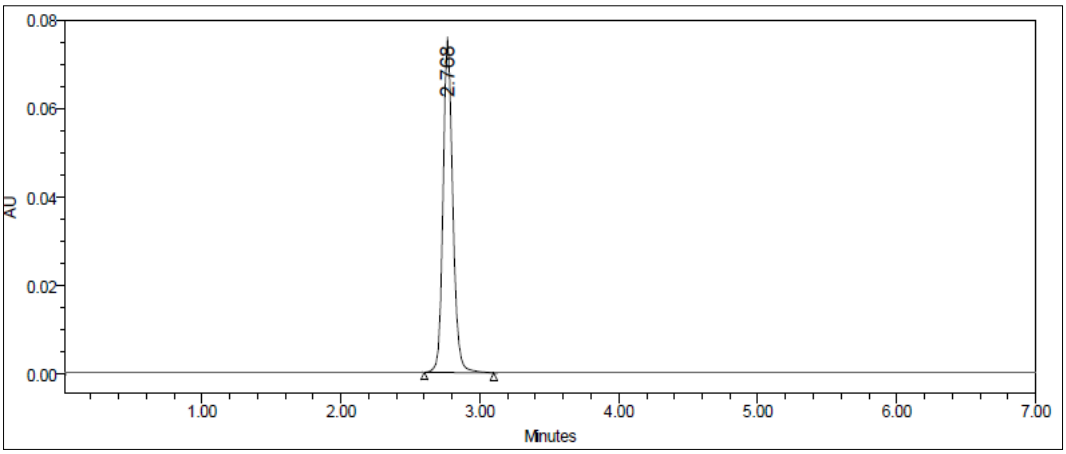

Fig 4: Optimized Chromatogram for Empagliflozin

Result

The selected and optimized mobile phase was Methanol: Acetonitrile (70: 30% v/v) and conditions optimized were flow rate (1.0 ml/minute), wavelength (245nm), Run time was 07 mins. Here the peak has shown better theoretical plate count and symmetry [21]. The proposed chromatographic conditions [22] were found appropriate for the quantitative determination of the drug.

Analytical Method Validation

System Suitability Test

System suitability testing is an integral part of many analytical procedures. The tests are based on the concept that the equipment, electronics, analytical operations and samples to be analysed constitute an integral system that can be evaluated as such. Following system suitability [23] test parameters were established. The data are shown in Table-3.

Table 3: Data of System Suitability Test

S. No.	Injection No.	RT	Area	Height	USP Plate Count	USP Tailing
1	Injection 1	2.786	715268	47844	5857	1.36
2	Injection 2	2.784	716584	46985	5986	1.38
3	Injection 3	2.768	715364	47258	5784	1.35
4	Injection 4	2.789	714895	47152	5896	1.34
5	Injection 5	2.784	716587	47258	5749	1.36
6	Injection 6	2.781	718549	47985	5657	1.39
Mean			**716207.8**		**5821.5**	**1.36**
S.D			**1347.976**			
%RSD			**0.18821**			

Table 4: Acceptance Criteria and Result

S. No.	Parameter	Limit	Result
1	Tailing factor	T ≤ 2	1.36
2	Theoretical plate	N > 2000	5821.5

Accuracy

Recovery Study

To determine the accuracy of the proposed method, recovery studies were carried out by adding different amounts (80%, 100%, and 120%) of pure drug of Empagliflozin were taken and 3 replications of each has been injected to HPLC system. From that percentage recovery values were calculated from the linearity equation [24] y = 74143x + 7294.9. The results were shown in table-5.

Table 5: Accuracy Readings

Sample ID	Concentration (µg/ml)		Peak Area	% Recovery of Pure drug	Mean % Recovery	
	Amount Injected	Amount Recovered				
S_1 : 80 %	8	8.013	601425	100.162	Mean = 100.195%	% Mean Recovery = 100.364%
S_2 : 80 %	8	8.012	601396	100.150		
S_3 : 80 %	8	8.022	602123	100.275		
S_4 : 100 %	10	10.038	751584	100.380	Mean = 100.356	
S_5 : 100 %	10	10.039	751642	100.390		
S_6 : 100 %	10	10.030	750969	100.300		
S_7 : 120 %	12	12.057	901253	100.475	Mean = 100.541	
S_8 : 120 %	12	12.073	902431	100.608		
S_9 : 120 %	12	12.065	901864	100.541		

Observation: From the Accuracy Method, we observed that the mean %Recovery of the drug is 99.686 which are within the range of 98-102%.

Precision

Repeatability

The precision of each method was ascertained separately from the peak areas & retention times obtained by actual determination of six replicates of a fixed amount of drug Empagliflozin (API). The percent relative standard deviation was calculated for Empagliflozin.

Table 6: Results of Repeatability readings

HPLC Injection Replicates of Empagliflozin	Retention Time	Peak Area	Theoretical Plates	Tailing Factor
Replicate – 1	2.777	716984	5986	1.36
Replicate – 2	2.795	715698	5897	1.37
Replicate – 3	2.789	716859	5869	1.39
Replicate – 4	2.797	718548	5967	1.37
Replicate – 5	2.797	714895	5984	1.35
Replicate – 6	2.799	715986	5879	1.38
Average		**716495**	**5930.333**	**1.37**
Standard Deviation		**1268.126**		
% RSD		**0.17699**		

Observation: From the Precision method, we observed that the %RSD of the Peak Area is 0.176 which are within the acceptable range as per ICH guidelines [25].

Intermediate Precision

The Intermediate Precision consists of two methods:

Intra Day: In Intra Day process, the 80%, 100% and 120% concentration are injected at different intervals of time in same day.

Inter Day: In Inter Day process, the 80%, 100% and 120% concentration are injected at same intervals of time in different days.

Table 7: Peak results for Intra-Day Precision

S.No.	Name	RT	Area	Height	USP Tailing	USP Plate Count	Injection
1	Empagliflozin	2.784	716587	48685	1.38	5954	1
2	Empagliflozin	2.768	717845	48698	1.39	5935	2
3	Empagliflozin	2.786	716857	46989	1.36	5798	3
4	**Average**		**717096.3**	**48124**	**1.376**	**5895.66**	
5	**S.D**		**662.2698**				
6	**% RSD**		**0.092354**				

Table 8: Peak results for Inter-Day Precision

S. No.	Name	RT	Area	Height	USP Tailing	USP Plate Count	Injection
1	Empagliflozin	2.780	716987	49867	1.34	5968	1
2	Empagliflozin	2.794	718695	48574	1.33	5998	2
3	Empagliflozin	2.775	718542	48569	1.39	5859	3
4	**Average**		**718074.7**	**49003.33**	**1.353333**	**5941.667**	
5	**S.D**		**945.0483**				
6	**% RSD**		**0.131609**				

Observations

The intra & inter day variation of the method was carried out for standard deviation & % RSD (% RSD < 2%) within a day & day to day variations for Empagliflozin revealed that the proposed method is precise.

Linearity & Range

To evaluate the linearity, serial dilution of analyte were prepared from the stock solution was diluted with mobile phase to get a series of concentration ranging from 6-14µg/ml. The prepared solutions were sonicated. From these solutions, 10µl injections of each concentration were injected into the HPLC system and chromatographed under the optimized conditions. Calibration curve [26] was constructed by plotting the mean peak area (Y-axis) against the concentration (X-axis).

Table 9: Linearity Concentrations of Empagliflozin

S. No.	Concentration (in ppm)	Peak Area
1	0	0
2	6	457896
3	8	607574
4	10	752268
5	12	896587
6	14	1036579

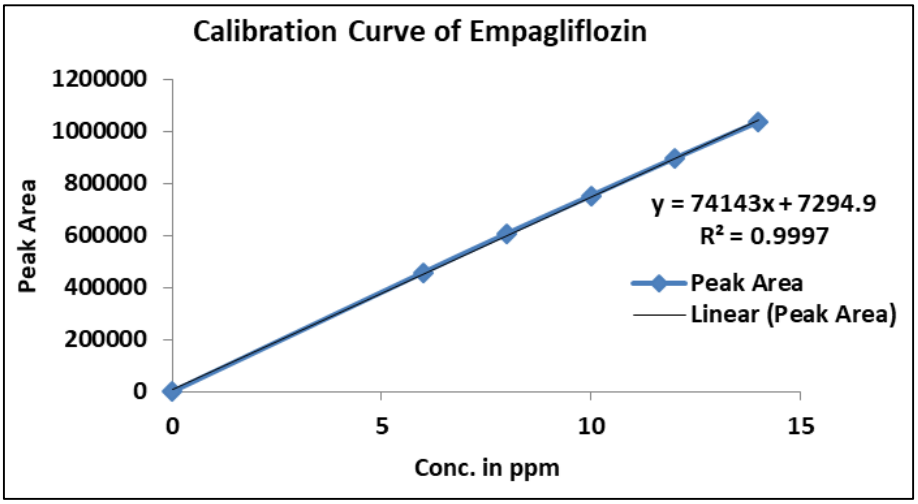

Fig 5: Calibration Curve of Empagliflozin

Observation

We observed that the calibration curve showed good linearity in the range of 6-14 µg/ml, for Empagliflozin with correlation coefficient (R^2) of 0.9997. A typical calibration curve has the regression equation of y = 74143x + 7294.9 for Empagliflozin.

Method Robustness

Influence of small changes in chromatographic conditions such as change in flow rate 1ml (± 0.1ml/min), Wavelength of detection 245nm (±2nm) & organic phase content in mobile phase 60 (±5%) studied to determine the robustness[27] of the method are also in favour of (Table-10, % RSD <2%) the developed RP-HPLC method for the analysis of Empagliflozin (API).

Table 10: Results of Method Robustness Test

Change in Parameter	Theoretical Plates	Tailing Factors
Flow (1.1 ml/min)	5954	1.35
Flow (0.8 ml/min)	6188	1.39
More Organic (70+5)	5748	1.41
Less Organic (70-5)	6185	1.48
Wavelength of Detection (250 nm)	6184	1.69
Wavelength of detection (240nm)	6247	1.47

LOD & LOQ: The detection limit (LOD) and quantization limit (LOQ) may be expressed as:

L.O.D. = 3.3(SD/S)

L.O.Q. = 10(SD/S)

Where, SD = Standard deviation of the response

S = Slope of the calibration curve

The slope S may be estimated from the calibration curve of the analyte.

The Minimum concentration level at which the analyte can be reliable detected (LOD) & quantified (LOQ) were found to be 0.507 & 1.539 µg/ml respectively.

Estimation of Empagliflozin in Pharmaceutical Dosage Form

Twenty pharmaceutical dosage forms were taken and the I.P. strategy was taken after to decide the normal weight. Above measured tablets were at last powdered and triturated well. An amount of powder proportionate to 25 mg of medications were exchanged to 25 ml volumetric flagon, make and arrangement was sonicated for 15 minutes, there after volume was made up to 25 ml with same dissolvable. At that point 10 ml of the above arrangement was weakened to 100 ml with versatile stage. The arrangement was separated through a layer channel (0.45 m) and sonicated to degas. The arrangement arranged was infused in five reproduces into the HPLC framework and the perceptions were recorded.

A copy infusion of the standard arrangement was additionally infused into the HPLC framework and the peak regions were recorded. The information is appeared in Table-11.

$$\text{Assay \%} = \frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{WT} \times \frac{P}{100} \times \text{Avg. Wt} = \text{mg/tab}$$

Where:

AT = Peak Area of medication acquired with test arrangement

AS = Peak Area of medication acquired with standard arrangement

WS = Weight of working standard taken in mg

WT = Weight of test taken in mg

DS = Dilution of Standard arrangement

DT = Dilution of test arrangement

P = Percentage virtue of working standard

Table 11: Recovery Data for estimation Empagliflozin in Empaone 10

Brand Name of Empagliflozin	Labelled amount of Drug (mg)	Mean (± SD) amount (mg) found by the proposed method (n=6)	Assay % (± SD)
Empaone 10 (MSN)	10mg	9.896 (±0.627)	99.79 (±0.277)

Result & Discussion

The amount of drug in Empaone 10 was found to be 9.896 (±0.627) mg/tab for Empagliflozin & % assay was 99.79 %.

Summary and Conclusion

The analytical method was developed by studying different parameters.

First of all, maximum absorbance was found to be at 245nm and the peak purity was excellent.

Injection volume was selected to be 10µl which gave a good peak area.
The column used for study was Symmetry C_{18}, 250 mm x 4.6 mm i.d.5µm particle size because it was giving good peak.
Ambient temperatures were found to be suitable for the nature of drug solution. The flow rate was fixed at 1.0ml/min because of good peak area and satisfactory retention time.
Mobile phase is Methanol: Acetonitrile (70:30 v/v) was fixed due to good symmetrical peak. So this mobile phase was used for the proposed study.
Methanol: water was selected because of maximum extraction sonication time was fixed to be 10min at which all the drug particles were completely soluble and showed good recovery.
Run time was selected to be 7min because analyze gave peak around 2.768min and also to reduce the total run time.
The percent recovery was found to be 98.0-102 was linear and precise over the same range. Both system and method precision was found to be accurate and well within range.
The analytical method was found linearity over the range of 6-14ppm of the Empagliflozin target concentration.
The analytical passed both robustness and ruggedness tests. On both cases, relative standard deviation was well satisfactory.

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