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ORIGINAL ARTICLE **OPEN ACCESS**

Development and Validation of a Novel Stability Indicating RP-HPLC Method for the Determination of Empagliflozin in Bulk and Pharmaceutical Film-Coated Tablet Dosage Form

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ABSTRACT
A novel stability indicating RP-HPLC method was developed and validated for the quantitative determination of Empagliflozin in bulk drug and pharmaceutical film-coated tablet dosage form. An isocratic separation was done by using analytical Thermo-Hypersil keystone ODS 1 column (250 x 4.6 mm i.d, 5 µm particles) using mobile phase composition of Acetonitrile, Methanol and water in the ratio of (30:40:30 v/v/v) that was set at injection volume 10µl/min and flow rate of 1.0 ml/min with UV detection at 233 nm. The method was found to be linear over the concentration range of 50–150 µg/mL with a correlation coefficient (r2) of 0.999.The high correlation coefficient (r^2) value indicated clear correlation and their peak area within the LOQ (Limit of quantification) to 150% level.The retention time of Empagliflozin was found to be 4.52 minutes (+/-0.5). The proposed method was validated by determining system suitability, specificity, linearity, precision, accuracy, robustness, ruggedness, LOD, LOQ, stability studies, degradation studies. The developed method was effectively applied to film-coated tablet of Empagliflozin and the % recovery of Empagliflozin from film-coated tablet formulation was found to be 99.6 %. The method was found to be simple, accurate, and precise and hence can be applied for routine quality control analysis of Empagliflozin in pure and film-coated tablet dosage form.
Keywords: *RP-HPLC, Empagliflozin, Method Development and Validation, ICH Guidelines, Stability Studies*

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INTRODUCTION

A Stability-indicating RP-HPLC assay method can be defined as "Validated quantitative analytical method that can detect the change with time in the chemical, physical or microbiological properties of the drug substance and drug products are specific so that the content of active ingredients and degradation products can be accurately measured without interference." [1]

High Performance Liquid Chromatography:

HPLC is advanced form of liquid chromatography used for separation of compounds that are dissolved in solution. The instruments of HPLC consist of a reservoir of mobile phase, a pump, an injector, a separation column, and a detector [2].

HPLC is basically a highly improved form of column liquid chromatography. Instead of a solvent being allowed to drip through a column under gravity, it is forced through under high pressure of up to 6000 psi, which makes the separation process much faster.

All chromatographic separation techniques, including HPLC operates under the same basic principle where molecules in mixture applied onto the surface or into the solid, and fluid stationary phase (stable phase) is separating from each other while moving together with mobile phase.

Compounds are separated by injecting a sample mixture onto the column. The different components in the mixture pass through the column at differentiate due to differences in their partition behavior between the mobile phase and the stationary phase.

Empagliflozin:

Empagliflozin is an anti-diabetic medication (sold under the brand name "Jardiance" 10mg, 25mg among others), sodium glucose co-transporter-2 (SGLT-2) inhibitor indicated as an adjunct to diet and exercise to improve glycemic control in adult patients with type-II diabetes [3-6]. Inhibition of SGLT2-mediated glucose transport in the kidney decreases the threshold at which urinary glucose excretion (UGE) occurs,

which results in loss of glucose in the urine and a reduction in hyperglycemia [7-9]. Chemically, it is known as (2S, 3R, 4R, 5S, 6R)-2-[4-chloro-3-({4-[(3S)-oxolan-3-yloxy] phenyl}methyl) phenyl]-6-(hydroxymethyls) oxane-3,4,5-triol. The structural formula of Empagliflozin was given in figure 1. Empagliflozin is a white to yellowish, non-hygroscopic crystalline solid, which is commercially marketed under the name 'Jardiance' and marketed by "BoehringerIngelheim".

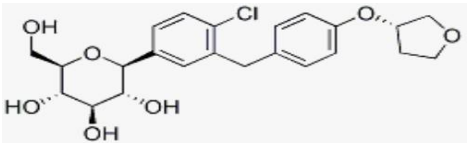

Fig.1: Structure of Empagliflozin

Empagliflozin is not official in any pharmacopoeia. A literature survey on Empagliflozin discloses that, until now only few analytical methods were reported for estimation of Empagliflozin such as UV-Visible Spectroscopy[10], HPLC method[11-13] [14-15] in bulk and API form. However, the reported RP-HPLC method uses complex mobile composition so that it is essential to develop an RP-HPLC method having simple composition of mobile phase. Hence, a trial has been taken to develop and validate a novel, simple and sensitive RP-HPLC method according to ICH guidelines for the determination of Empagliflozin in its film-coated tablet formulation.

MATERIAL AND METHODS:

Instrumentation:

Chromatographic separation was performed on a Shimadzu LC-20AD HPLC system equipped with aThermo-Hypersil Keystone ODS 1 column (250 x 4.6 mm i.d, 5 µm particles), binary pumps, degasser, variable wavelength detector andauto sampler with 10µl loop volume. 'LC solution' software was used to collect and process the data [1-3].

Chemicals and Reagents:

Pharmaceutically active ingredient (Empagliflozin) was kindly obtained from 'Lupin Research Park'. Acetonitrile, methanol of HPLC grade were used. Acetonitrile, Methanol and Water were obtained from Research Lab Fine Chemical Industries, Mumbai'. Water used was freshly prepared by Double Distillation Assembly and further used in analysis after filtering through 0.45µ membrane filter papers.

Preparation of Mobile Phase:

The mobile phase Acetonitrile: Methanol: Water were mixed in the ratio of 30:40:30 v/v/v and filtered through membrane filter (Millipore Nylon disc filter of 0.45 µ). This filtered mobile phase was then sonicated for 15 min in ultrasonic bath before use.

Preparation of Standard Stock Solution:

Accurately weighed quantity of 10 mg of Empagliflozin was transferred into 100 ml volumetric flask, dissolved and diluted up to mark by using methanol. This was a stock solution having strength of 1000 µg/mL of Empagliflozin. From this solution, 10 mL of solution was withdrawn and then diluted up to 100 mL to get 100 ppm solution. From this, 15 mL of solution was withdrawn and then diluted up to 25 mL to get 10 µg/mL of Empagliflozin. Mix well and filtered through 0.45 µm filter. The obtained UV Spectra and chromatogram for standard Empagliflozin was shown in figure 2& 3.

Preparation of Sample Solution:

The assay method was performed on two commercial brands purchased from local pharmacists. Average of 10 film-coated Empagliflozin tablets were weighed and finely crushed in a mortar by using pestle. The equivalent content of each tablet (25 mg) was transferred into 100 mL volumetric flask and make up the volume up to mark with mobile phase. Sonicate for about 10 minutes, cool to room temperature, dilute the volume with water and mixed properly.

Transferred 5 mL of above solution into 25 mL volumetric flask and volume made with mobile phase and mixed. Pipetted 2.0 mL from the above solution into a 25 mL volumetric flask diluted the volume with mobile phase and mixed. A portion of the clear solution obtained was filtered through 0.45 µ Nylon disc filter [11-13].

Method Validation:

The method was validated for parameters like system suitability, linearity, specificity, precision, accuracy, robustness, ruggedness, LOD (Limit of Detection), LOQ (Limit of Quantitation), stability studies, Degradation Studies (acid degradation, alkali degradation, oxidative degradation, thermal degradation and photolytic degradation), application of proposed method [1,11].

System Suitability Test:

System suitability test was conducted to verify that the analytical system is working properly and can give accurate and precise results. The overall system suitability was evaluated for the system suitability of the

proposed method. Data obtained from 5 injections (10µg/mL) were used for calculating the system suitability parameters like theoretical plates (N), Resolution, Tailing factor, and % RSD of 5 injections [8].

•Specificity:

Specificity is the ability to measure unequivocally the desired analyte in the presence of components such as excipients and impurities. Specificity study ensures the identity of the analyte of interest. Specificity is used when the method's ability responding to one single analyte only, while selectivity is used when the method is able to respond to several different analytes in the sample. Here, dextrose was used as excipient. Three solutions of 10µg/mL were injected and one excipient solution as a blank injected and compared the chromatogram with the standard solution of Empagliflozin was shown in Figure 4 & 5 [1,13].

•Linearity:

Linearity is the ability of the method to elicit test results that are proportional to concentration of the analyte in the sample. [13, 17-18]To determine the linearity of the method, calibration solutions were prepared from the stock solution at five concentration levels from 20-100% concentration of analyte concentration. The correlation coefficient (r^2), Y-intercept and slope of the calibration curve were calculated. This was well explained by plotting a graph of peak area vs. concentration.

•Precision:

The precision of an analytical procedure expresses the closeness of agreement between individual results obtained from a repeatedly applied procedure in a homogeneous sample comprising repeatability and IP under the prescribed conditions. Intra-day precision was determined by analyzing Empagliflozin for 3 times in the same day (intra-day). Inter-day precision was determined by analyzing Empagliflozin daily for3 days and % RSD for intra-day and inter-day precision was calculated.

•Accuracy:

The accuracy of the assay method was evaluated in triplicate at 3 concentration levels i.e., 40, 60, and 80 µg/mL (50,100, 150 % of the normal assay concentration) for bulk drug sample. The %recoveries were calculated. The study was carried out in triplicate (n=3). The solutions were injected into HPLC system and the mean peak area of analyte (Empagliflozin) peak was calculated for assays. Assay (%w/w) of test solution was determined against 3 injections (n=3) of qualified Empagliflozin reference or working standard.

•Robustness:

The robustness is a measure of its capacity to remain unaffected by small but deliberate changes in parameters given in procedure documentation because the described method was designed for future application in the routine drug analysis by pharmaceutical laboratories and quality control laboratories and to provide an indication of suitability during normal usage. In all the varied conditions like flow rate (0.8 ml/min & 1.2ml/min), mobile phase composition (20:45:35 v/v/v&25:50:25 v/v/v) and wavelength (224 nm & 243 nm), the components of the mobile phase (A: M: W) were held constant.

•Ruggedness:

The ruggedness of an analytical method is the ability to reproduce an analytical method in different laboratories or in different circumstances without the occurrence of unexpected differences in the obtained results.

•Limit of Detection (LOD):

It is the lowest amount of analyte present in a sample which can be detected but not necessarily quantitised as the exact value. It can be calculated by using formula, **LOD= 3.3 x SD/Slope**, where SD= standard deviation.

•Limit of Quantitation (LOQ):

It is the lowest amount of analyte present in the sample which can be quantitatively determined with suitable precision and accuracy and variability (exact value).It can be calculated by using formula, **LOQ = 10 x SD/Slope**, where SD= standard deviation.

•Stability Studies:[16]

Short term stability study was performed on standard and working drug solution using 3 different concentration ranges (0.05, 15, 30 µg/mL), exposed at ambient temperature (25^0C) and refrigeration (4^0C) for one day.

•Degradation Studies:

Degradation studies were carried out on Empagliflozin under various conditions explained in ICH guideline Q1 A (R_2), namely acid, alkali, oxidative, thermal and photolytic conditions were given in Table 9.

i) Acid Degradation:

The degradation study in acidic medium was performed by separately taking 1 mL stock solution of Empagliflozin; into 10 mL volumetric flask, and then this flask was kept at 60^0C for 48 hrs after addition of 1 mL of 1 M HCL, then the solution was neutralized with 1 N NaOH and diluted up to the mark with the help of methanol and made to get concentration of 10µg/mL.

ii) Alkali Degradation:
The degradation study in alkali medium was performed by separately taking 1 mL stock solution of Empagliflozin; into 10 mL volumetric flask, and then this flask was kept at 60^0C for 24hrsafter addition of 1 mL of 1 N NaOH, then the solution was neutralized with 1 M HCL and diluted up to the mark with the help of methanol and made to get concentration of 10µg/mL.

iii) Oxidative Degradation:
The degradation study in oxidative medium was performed by separately taking 1 mL stock solution of Empagliflozin; into 10 mL volumetric flask, and then this flask was kept at 60^0C for 48hrs after adding 1 mL of 30% v/v H_2O_2. The solution was diluted up to the mark with the help of methanol and made to get concentration of 10µg/mL.

iv) Thermal Degradation:
10 mg of Empagliflozin was weighed accurately and was exposed to 60^0C for 10 days. After this exposure, the drug powder was mixed and transferred into 10 mL volumetric flask, dissolve in methanol and diluted up to mark by using methanol and made to get concentration of 10µg/mL.

v) Photolytic Degradation:
10 mg of Empagliflozin was weighed accurately and was exposed to sunlight for 10-12 days. After this exposure, the drug powder was mixed and transferred into 10 mL volumetric flask, dissolve in methanol and diluted up to mark by using methanol and made to get concentration of 10µg/mL. The absorbance of this prepared solution was measured at 233 nm.

•Application of Proposed Method:
The percentage purity of tablet dosage form was determined by injecting the 10µg/mL standard solution and sample solution equivalent to 10µg/mL of Empagliflozin [1-13].

RESULTS AND DISCUSSION

Initially, the solubility of Empagliflozin was checked in various solvents. The drug was found to be slightly soluble in methanol, freely soluble in Acetonitrile and water.

Method Development and Optimization of Chromatographic Conditions:
Stock solution of 1000 µg/mL of Empagliflozin was prepared and further diluted to get the concentration of 10µg/ml of Empagliflozin by using methanol. The wavelength was selected by scanning the above standard drug solution in between 200-400 nm. The scanned result shows that reasonable maximum absorbance was recorded at 233 nm. Therefore, 233 nm was selected as the detection wavelength for the RP-HPLC investigation.

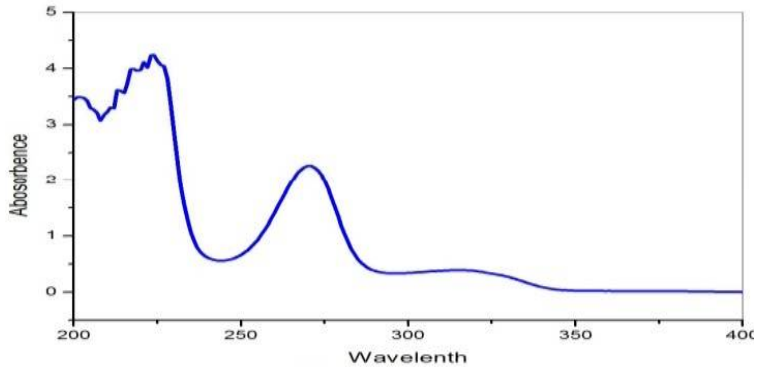

Fig. 2: UV Spectra of Empagliflozin

Optimized Method:
In this work, simple, accurate and stability indicating RP-HPLC method was developed and validated for the quantitative determination of Empagliflozin in bulk drugs and pharmaceutical film-coated tablet dosage form using a ThermoHypersil Keystone ODS 1analytical column (250 x 4.6 mm i.d, 5 µm particles). The mobile phase was selected after several trials to match the optimum stationary/mobile phase. The present method contains mobile phase composition of Acetonitrile, Methanol and Water in the ratio of (30:40:30 v/v/v), which was found to be most suitable, as the chromatographic peaks obtained were better defined, well resolved and almost free from tailing as compared with previous paper (Nagappan et al.). The flow rate is 1.0 ml/min with detection wavelength of 233 nm. The average retention times under the optimized conditions were 4.52min for Empagliflozin. The total runtime is 20 minutes during which all the system suitability parameters were ideal for the mixture of standard solutions. Figure 3 represents the mixture of standard solutions respectively Table 1 [1-13].

Table 1: Optimized Chromatographic Conditions

Optimized	Chromatographic Conditions
Mode of Separation	Isocratic
Mobile Phase	Acetonitrile: Methanol: Water (30: 40:30 v/v/v)
Column	Thermo-Hypersil Keystone ODS 1 column (250 x 4.6 mmi.d, 5 µm particles)
UV Detection	233 nm
Runtime	20 min
Injection Volume	10µl/mL
Flow Rate	1.0 ml/min
Temperature	Ambient

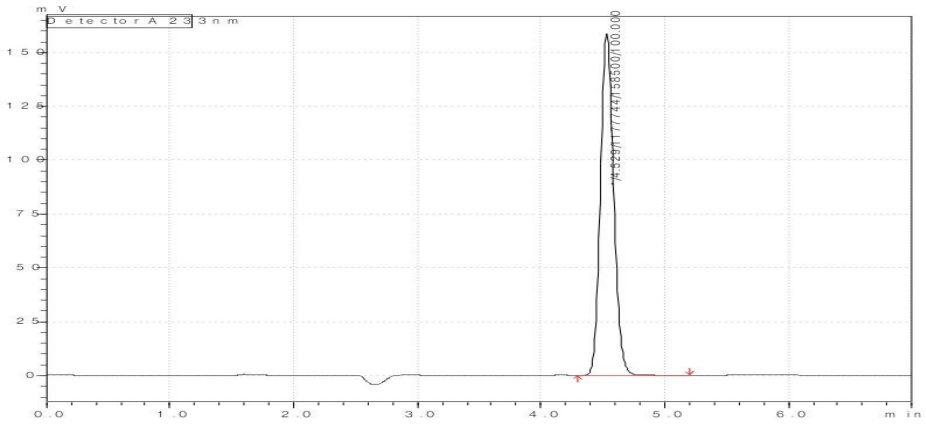

Fig. 3: Chromatogram of Empagliflozin under optimized chromatographic conditions

Method Validation:

•System Suitability:

A RP-HPLC method was developed by monitoring the system suitability parameters i.e. tailing factor (T), number of theoretical plates (N), runtime and the cost effectiveness. System suitability method acceptance criteria set in each validation run were: tailing factor ≤ 2.0 and theoretical plates > 2000. In all cases, the relative standard deviation (R.S.D) for the analytic peak area for five consecutive injections was < 2.0%. A chromatogram obtained from reference substance solution was presented. Typical system suitability parameter includes % RSD, tailing factor (T), and theoretical plates (N) and peak areas from replicate injections are within range and results were shown in Table 2[19].

Table 2: Result of System Suitability Parameters Data for Empagliflozin 10 µg/mL

Parameters	Results
% RSD for peak areas	0.2
No. of Theoretical plates	7707
Tailing Factor	1.12
Retention Time	4.52 min

•Specificity:

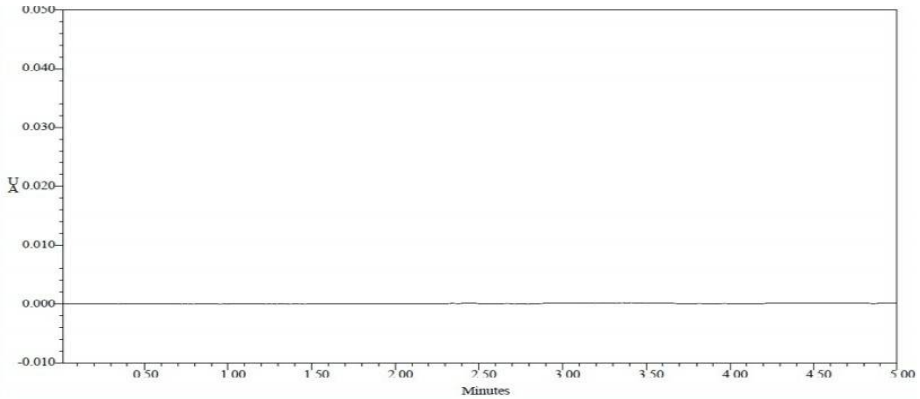

Fig. 4: Chromatogram of blank run (mobile phase)

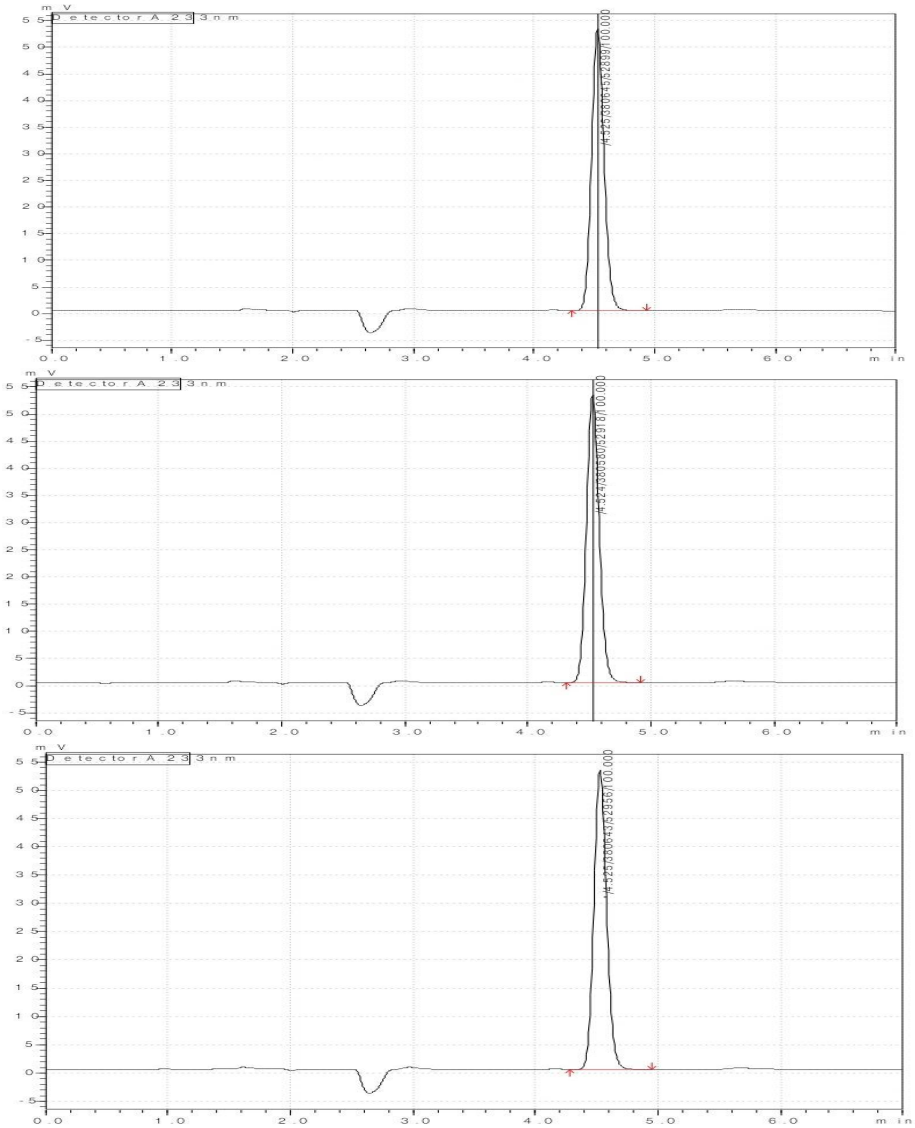

Fig. 5: Representative Chromatograms of Specificity

•Linearity:

The linearity of drug was determined by plotting the calibration curve with concentration on X-axis and peak area on Y-axis over a concentration range of 20-100 µg/mL. Linearity was found to be in the range of 20-100 µg/mL. The calibration graph was plotted, the slope and Y-intercept of calibration curve were 17380 and 7484 and the drug was found to be linear with a correlation coefficient (r^2) of 0.999 were shown in Figure 6.

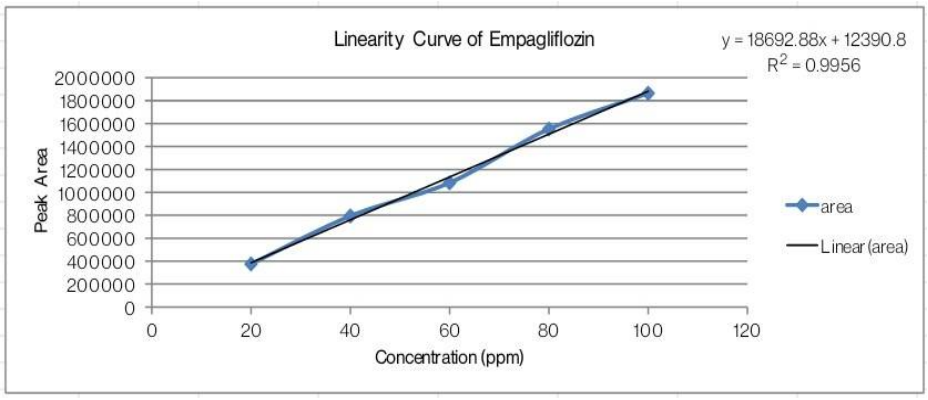

Fig. 6: Calibration Curve of Empagliflozin

Table 3: Linearity of Empagliflozin

Concentrations (µg/mL)	Mean Peak Area
20	375045
40	793951
60	1083255
80	1552517
100	1865050
Correlation Coefficient	0.999
Slope	18693
Y- Intercept	7484

•Precision:

Six solutions of same concentrations were prepared and their peak area was noted. The obtained results were shown in terms of % RSD. The inter-day and intra-day precision results of Empagliflozin indicate that % RSD less than 1, which implies that the method was precise. The obtained results of inter-day and intra-day precision were shown in Table 4.

Table 4: Results of Precision

Precision	Intraday	Inter Day		
		Day 1	Day 2	Day 3
Mean	380682	376242.6	376248.6	3765990
Std. Deviation	873.0406634	811.2972945	814.3127543	810.4013965
% RSD	0.2	0.19	0.18	0.18

•Accuracy:

Accuracy (% recovery) of the method was obtained by spiking 50, 100, and 150 % of Empagliflozin working standard concentrations, in which the marketed formulation was kept constant and the amount of pure drug was varied. Solutions were prepared in triplicates and accuracy was indicated by % recovery. The recovery was within the limit. Hence, the method was accurate. The obtained results of accuracy were shown in Table 5.

Table 5: Accuracy (% recovery) Results of Empagliflozin

% Spike Level	Sample (µg/mL)	Amount added (Std.)	Amount found (µg/mL)	% Recovery	% RSD
50	50	40	39.34	98.35	1.77
	50	40	39.85	99.62	
	50	40	40.74	101.85	
100	50	50	49.17	98.34	0.69
	50	50	49.83	99.66	
	50	50	49.67	99.34	
150	50	60	59.82	99.70	0.11
	50	60	59.74	99.56	
	50	60	59.87	99.78	

•Robustness:

In all the varied chromatographic conditions (flow rate, mobile phase composition and detection wavelength), there was no significant change in assay values was observed, which confirms that the developed method was found to be robust. The results of robustness were given in Table 6.

Table 6: Results of Robustness of Empagliflozin

Conditions	% Assay	System Suitability Parameters	
		Theoretical Plates	Tailing Factor
Flow rate 0.8 ml/min	99.66	7789	1.21
Flow rate 1.2 ml/min	99.48	7798	1.21
Mobile Phase ACN:M:W (20:45:35)	99.72	7768	1.120
Mobile Phase ACN:M:W (25:50:25)	99.87	7789	1.120
Wavelength 224 nm	99.81	7793	1.119
Wavelength 243 nm	99.63	7792	1.119

•Ruggedness:

The ruggedness was studied by evaluating the sample by different analysts but in the same chromatographic conditions. The results of ruggedness of developed method were given in Table 7.

The results were obtained from different analysts but in the same chromatographic conditions of the test solutions were not affected by applied variable parameters and were in the accordance with the actual. The suitability parameters were shown to be good; hence this method was concluded as rugged.

Table 7: Ruggedness results of Empagliflozin

ID Precision	No. of Injections	Empagliflozin	
		Peak Area	Retention Time
	1	257874	4.523
ID Precision 1	2	259537	4.522
	3	257124	4.523
	1	254589	4.521
ID Precision 2	2	256298	4.523
	3	258327	4.521
Mean		256624.83	
Std. Deviation		4421.45	
% RSD		1.0	

•Limit of Detection (LOD) and Limit of Quantitation (LOQ):

The LOD and LOQ were calculated by obtaining linear curve using above formulae and the results were shown in Table 8. The low values of LOD and LOQ indicatesthat the method was highly sensitive.

Table 8: LOD and LOQ of Empagliflozin

Drug	LOD (µg/mL)	LOQ (µg/mL)
Empagliflozin	0.13	0.40

•Degradation Studies:

Table 9: Degradation Conditions

Sr. No.	Type of Degradation	Degradation Conditions
1	Acid Degradation	Drug solution prepared in 1 m HCL, 60^0C for 48 hrs.
2	Alkali Degradation	Drug solution prepared in 1 N NaOH, 60^0C for 24 hrs.
3	Oxidative Degradation	Drug solution prepared in 30% v/v H_2O_2, 60^0C for 48 hrs.
4	Thermal Degradation	Drug substance was subjected to dry heat at 60^0C for 10 days.
5	Photolytic Degradation	Drug substance in UV energy of NLT 200 W/h for 10-11 days.

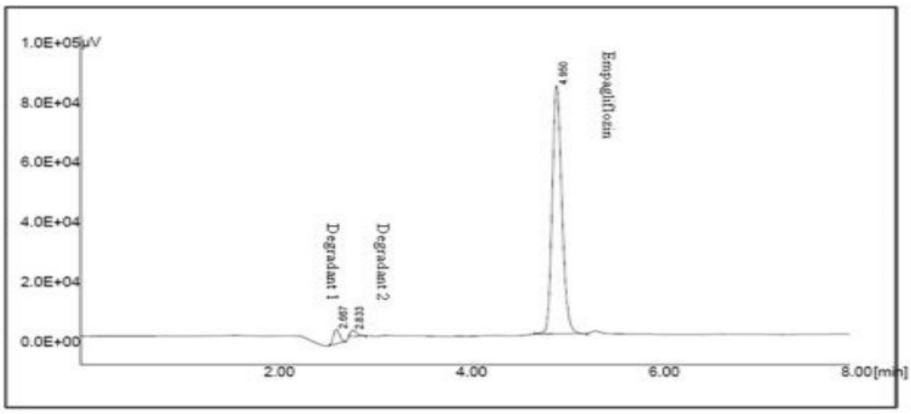

Fig. 7: Acid Degradation study of Empagliflozin

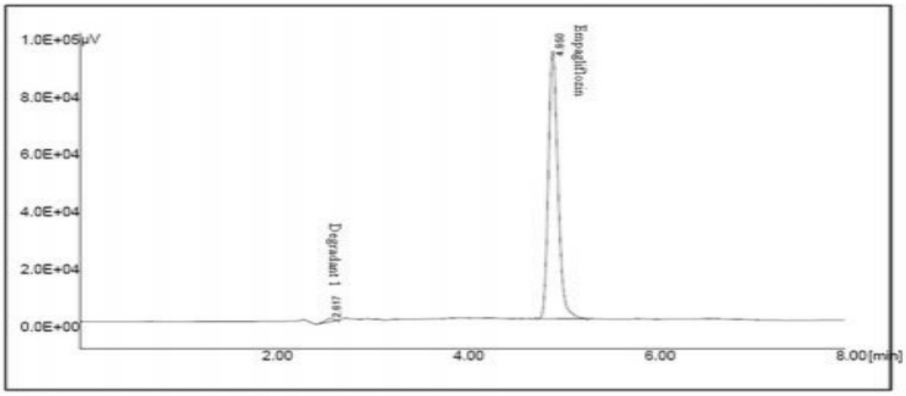

Fig. 8: Alkali Degradation study of Empagliflozin

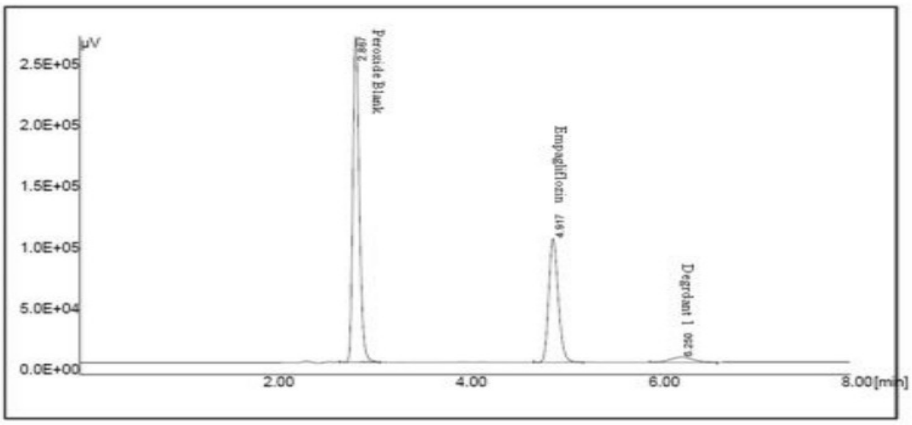

Fig. 9: Oxidative Degradation study of Empagliflozin

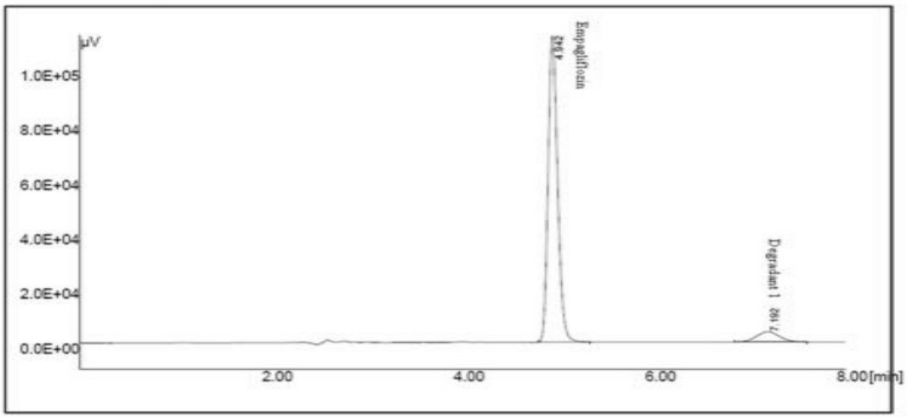

Fig. 10: Thermal Degradation of Empagliflozin

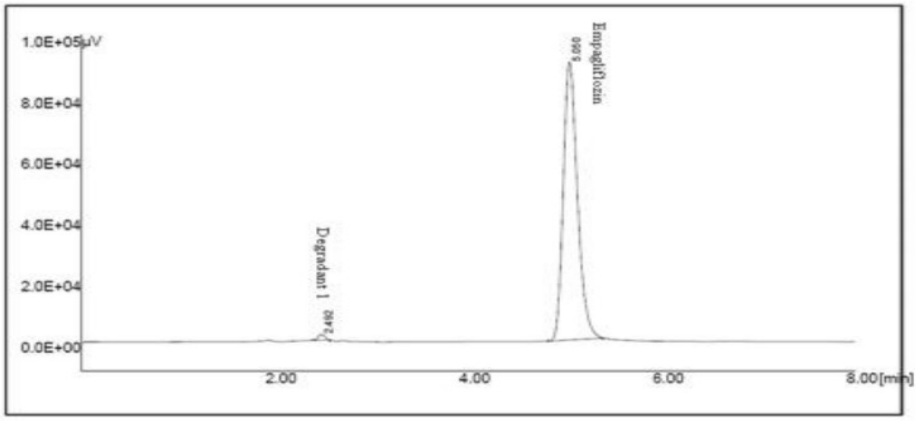

Fig. 11: Photolytic Degradation study of Empagliflozin

Degradation of the Empagliflozin drug was observed under acid, alkali, oxidative, and thermal degradation and the results were given in table 10.

Table 10: Results of Degradation Studies of Empagliflozin

Type of Degradation	Assay (%w/w)	% Of Degradation
Acid Degradation	92.03	14.72 %
Alkali Degradation	93.09	16.83 %
Oxidative Degradation	92.03	12.05 %
Thermal Degradation	92.58	13.39%
Photolytic Degradation	93.44	Photo Stable

•Application of Proposed Method:

Based on the data (Table 11), the percentage purity of Empagliflozin in film-coated tablet dosage form was found to be 99.6%. The proposed analytical method can be successfully applied for the routine analysis of tablet dosage forms.

Table 11: System Suitability Parameters of Empagliflozin (Standard and Test) in Assay Method

Name	Retention Time	% RSD for Peak Area	% Area	USP Tailing	USP Plate Count
Empagliflozin (Std)	4.522 min	0.52	100%	1.121	12876
Empagliflozin (Test)	4.521min	0.48	100%	1.118	12913

CONCLUSION:

In conclusion, a simple, selective, sensitive and accurate stability indicating RP-HPLC method was developed and validated for the analysis of Empagliflozin. The drug shows good results of system suitability parameters as compared with previous paper [9] in which tailing of peaks observed. By using Acetonitrile, Methanol and Water for HPLC in appropriate ratio (30:40:30v/v/v) in mobile phase, this problem has been resolved. Statistical results and low % RSD of drug indicates that the method was found to be linear, precise, accurate and robust, specific and can be used fora wide range of concentrations. [17] The degradation studies indicate the stability of the drug. [15] Hence the proposed method can be used for the determination of Empagliflozin in routine analysis.

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