RESEARCH PAPER

Development Of Stability Indicating Assay Method and Forced Degradation Study of Empagliflozin – The Drug Acting on Metabolic Disorders By RP-HPLC Method

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ABSTRACT
It is a faster, precise and accurate method of analysis for Empagliflozin developed by using ICH Q2(R2) guidelines with the help of RP-HPLC. This method includes Thermo scientific BDS C18 Hypersil (150cm × 4.6 mm, 5 µ) column, Mobile phase solution A: 0.1% OPA in Water (100%), Mobile phase solution B: ACN: Methanol (40:60 %V/V) at wavelength of 224 nm. The linearity, accuracy, precision, and robustness of this method were all confirmed. The range of linearity was discovered 20-60µg/mL & The correlation coefficient value was 0.999. The precision and robustness of this method was proven hence percent RSD was found less than 2%. The percentage recovery was in the range of 99.35-101.54%. LOD and LOQ were 11.75 S/N and 20.81 S/N.
To evaluate the stability indicating properties degradation study by exposed with Acid (3 N HCL in a temperature range of 80°C) 3.97%, Base (3 N HCL in a temperature range of 80°C) 4.02%, A solution containing hydrogen peroxide (6% H2O2) 3.90%, Neutral for 1 hour 4.08%, UV light exposure at 294nm for 24 hours 3.02%, thermal degradation at 80oC for 1hr 3.90%..

Keywords: Development and validation of analytical method, HPLC, Empagliflozin, Stability, Forced degradation.
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INTRODUCTION

In Present study analytical method was developed for analysis of Empagliflozin [1]. Linearity, precision, accuracy, robustness, linearity range parameters of validation for developed analytical method done [1,2]. Stability of substance and specificity of the method were determined by degradation study [3]. Acidic, basic, oxidation, photolytic, thermal degradation study done by performed by exposing of working standard solution of Empagliflozin [3].

MATERIALS AND METHODS:

Empagliflozin:
Empagliflozin is a white to yellowish powder.
Structure:
Structure of Empagliflozin shown in Figure no.1 as below.

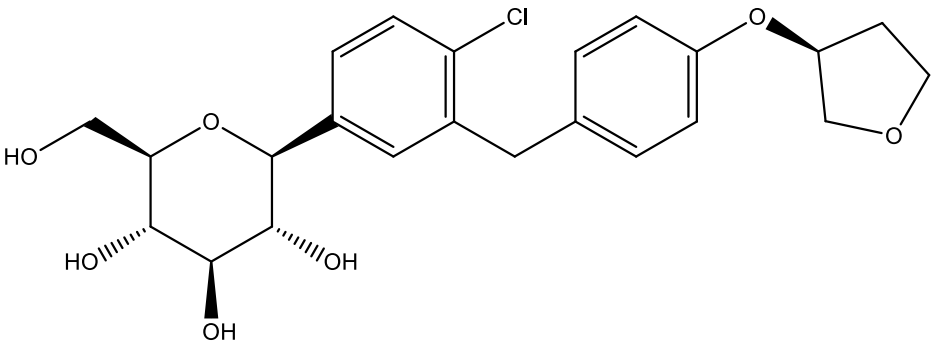

Figure 1: Structure of Empagliflozin
Molecular Formula of Empagliflozin : $C_{23}H_{27}ClO_7$
Molecular Weight of Empagliflozin : 450.9 g/moL

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Solubility:
Empagliflozin was slightly soluble in water.
Empagliflozin was slightly soluble in Acetonitrile.
Empagliflozin was sparingly soluble in Methanol.
Empagliflozin was practically insoluble in Toluene.

The drug Empagliflozin sample was gifted from pharmaceutical industry. Instrumentation system includes HPLC instrument Waters 2695 equipped with UV/ Visible detector, coming volumetric flasks and pipettes of borosilicate glass were used in the study. Methanol, Acetonitrile (HPLC Grade), Orthophosphoric acid, was procured from S.D. Fine Chemicals Ltd., Mumbai, India. Whatman filter paper No. 41 was also used in the study. As per IP 1996 method the solution of 3 N NaOH was prepared in distill water. And as per BP 1999, USP 24 method the solution of 3 N HCL was prepared in distill water.

2.1. Preparation of standard stock solution and selection of parameters for development of chromatographic parameters:

2.1.1 Standard stock solution:

In 25 ml of volumetric flask, weighed about 25.9 mg of Empagliflozin. Added 10 ml of Methanol (HPLC Grade), sonicated and dissolved completely, make up to the mark with methanol. (Concentration: 1000 ppm).

Final standard solution: Further in 50 ml volumetric flask 2 mL of stock solution taken and make up to the mark with methanol (Concentration: 40 ppm). UV spectrum obtained by using UV-Visible Spectrometer in the range of 400-200 nm, standard was scanned using 1 cm cell in double beam spectrophotometer. The wavelength was 224.0 nm estimated form spectrum. UV Spectra of Empagliflozin shown in Figure no.2 as below.

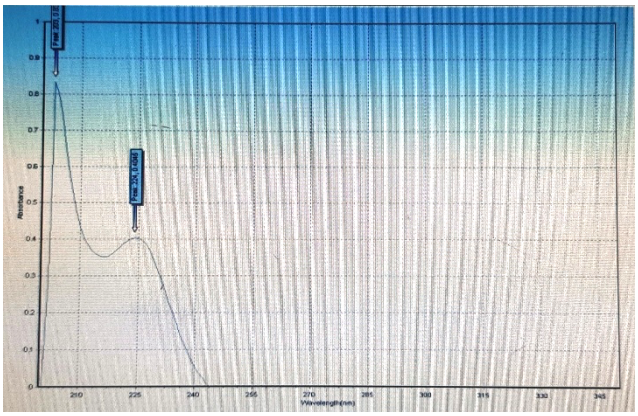

Figure 2: Empagliflozin show the maximum absorbance at 224 nm

2.1.2 Selection of mobile phase

The pure drug of Empagliflozin was injected into the HPLC system and run in different solvent systems. Normal baseline was obtained by saturating HPLC column using each mobile phase. Various types of organic and inorganic combination of solvents proportions were used and trials taken for each. After various trials a sharp peak was obtained. By using 0.45µm nylon membrane filter each buffer was filtered and combination of solvents sonicated using ultrasonic bath [4,5]. Development of mobile phase for Empagliflozin shown in Table no.1 as below.

Table 1: Development of mobile phase

Sr. No	Solvents / Combinations	Concentration	Comment
1.	Water	100%	System suitability parameters not satisfactory
2.	ACN:Water	50:50	System suitability parameters not satisfactory
3.	ACN:Water	80:20	System suitability parameters not satisfactory
4.	Methanol:Water	50:50	System suitability parameters not satisfactory
5.	Methanol:Water	80:20	System suitability parameters not satisfactory
6.	ACN:Methanol	50:50	System suitability parameters not satisfactory
7.	ACN:Water	40:60	System suitability parameters not satisfactory
8.	A: 0.1% OPA in Water B: ACN:Methanol	A: 100% B: 50:50	System suitability parameters not satisfactory
9.	A: 0.1% OPA in Water B: ACN:Methanol	A: 100% B: 40:60	System suitability parameters found satisfactory

After several trials sharp peak obtained with combination of buffer 0.1% OPA in Water (100% Port A) and combination of organic solvents Acetonitrile: Methanol (40:60 Port B). This combination was found to be the most satisfactory, which gives symmetry within limits, significant reproducible retention time.

2.1.3 Chromatographic parameters:

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This method was developed by using column Thermo scientific BDS C18 Hypersil (150cm × 4.6 mm x 5 µ). Method includes port A mobile phase was 0.1% OPA in Water (100%), port B mobile phase was ACN: Methanol (40:60). Flow rate was adjusted at 0.8 mL/min at wavelength of 224.0 nm. The sample preparation, mobile phase was prepared, degassed and sonicated. Buffers were filtered through 0.45µm nylon membrane filter at room temperature [6].

2.1.4 Procedure of mobile phase preparation and establishment of chromatogram:

Mobile phase solution A was prepared by mixing 1ml OPA in Water 1000 mL water & Mobile phase solution B was prepared by mixing of 400 mL volume of acetonitrile in 600 mL volume methanol. By using 0.45µm nylon membrane filter each buffer was filtered and combination of solvents sonicated using ultrasonic bath.

Standard solution conc. 40 ppm prepared from standard stock solution into the diluent. Stationary phase saturated with the mobile phase was until the normal baseline achieved. Into 10 mL volumetric flask from Empagliflozin stock solution concentrations of 20-60 µg/ mL solutions were prepared using diluent. At 20µL injection volume, the above solutions injected triplicate and peak areas obtained [7].

2.1.5 Chromatographic parameters:

From the various trials the obtained chromatographic parameters are shown in Table no.2 as below:

Table 2: Chromatographic parameters

Column	Thermoscientific BDS C18 Hypersil (150cm × 4.6 mm x 5 µ)
Mobile phases	Mobile phase Sol A: 0.1% OPA in Water (100%), Mobile phase Sol B: ACN: Methanol (40:60 %v/v).
Flow	1.0 mL/min
Injection volume	20 µL
Sampler temperature	Ambient 25ºC
Run Time	6.0 minutes
Oven Temperature	Ambient 25ºC
Wavelength	224 nm
Detector	UV
Software	Empower

Typical chromatogram for Empagliflozin shown in Figure no.3 as below.

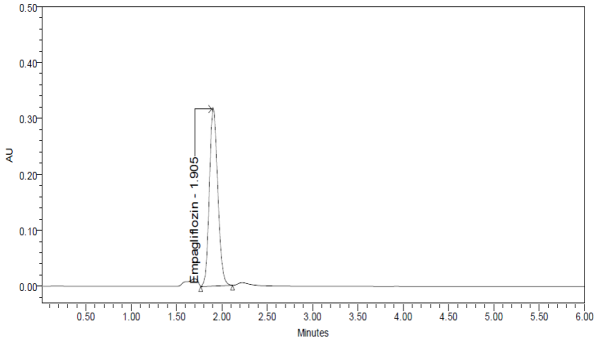

Figure 3: Typical chromatogram of Empagliflozin by HPLC.

The Retention time of Empagliflozin was observed 1.90 minutes.

2.2 Validation of Analytical Method:

The method was validated using various analytical parameters to ensure the reproducibility, reliability of the method [8].

2.2.1 Preparation of Standard Solution:

The standard solutions prepared as follows:

In 25 mL volumetric flask weighed 25.23 mg of Empagliflozin added 10 mL of methanol sonicated to dissolve and make up to the mark with methanol. Further in 50 mL volumetric flask added 2.0 mL of standard stock solution and make up to the mark with methanol (Concentration: 40 ppm). For achievement of system suitability criteria five replicates injected of this standard solution in same system.

2.2.2 LINEARITY:

Linearity of Empagliflozin achieved from developed method. Five levels of concentrations as per ICH Q2(R2) guidelines in a range of 20-60µg/mL injected and calibration curve developed. For this the different levels of EMPA working standard (40 µg/mL) were injected. The observed linearity certifies and complies with the Beer's law [8,9]. From linearity study the equation obtained was y=55411x+5704 with correlation coefficient (r) was 0.999. for Linearity curve for Empagliflozin shown in Figure no.4 as below.

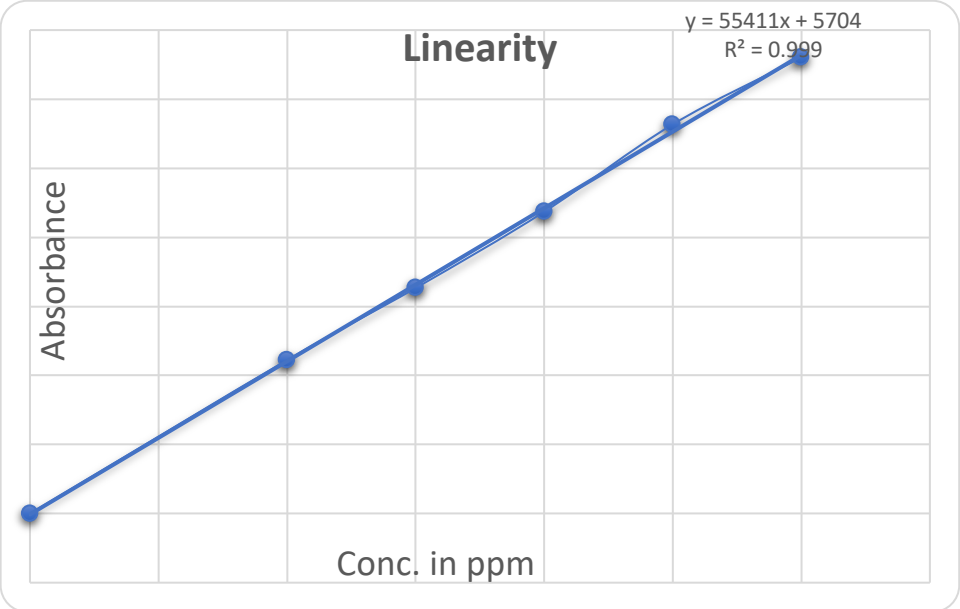

Figure 4 : Linearity curve of Empagliflozin.

Linearity levels obtained for Empagliflozin shown in Table no.3 as below.

Table 3: Linearity levels for Empagliflozin as per ICH Q2(R2) guidelines.

Level	Concentration (ppm)	Area
I	20	1111792
II	30	1632616
III	40	2184843
IV	50	2817451
V	60	3301352

Linearity study results obtained for Empagliflozin shown in Table no.4 as below.

Table 4: Linearity study results

Sr. No.	Parameters	Results
1	Range	20-40µg/mL
2	Regression equation	y=55411x+5704
3	Correlation coefficient (R^2)	0.999

2.2.3 PRECISION

Precision for developed method was checked by different studies like Repeatability, intermediate precision studies (Interday, Intraday)

2.2.3.1 REPEATABILITY:

In this study for one concentration of drug six replicates injected using HPLC[10]. As a result %RSD was observed within acceptance criteria (Less than 2%).

2.2.3.2 INTERMEDIATE PRECISION:

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In this study, on two successive days 3 replicates of standard concentration injected on HPLC. As a result %RSD of EMPA found to be less than 2%.
Results of Intermediate study (Intraday) are shown in Table no.5 as below.

Table 5: Intermediate precision of EMPA (Intraday)

	Method Precision Empagliflozin (Intraday)						SD	AVG	%RSD
Intraday	**STD**	2055339	2062676	2064996	2057595	2061765	3924.947	2060474	0.19
	SPL	2054236	2059435	2059653	-	-	3066.513	2057775	0.14

Results of Intermediate study (Interday) are shown in Table no.6 as below.

Table 6: Intermediate precision of EMPA (Interday)

	Method Precision Empagliflozin (Interday)						SD	AVG	%RSD
Interday	**STD**	2055339	2062676	2064996	2057595	2061765	3924.947	2060474	0.19

2.2.4 Accuracy:
Recovery study was performed to verify the accuracy of method as per ICH guidelines.
2.2.4.1. Preparation of Sample Solutions:
Drug powder equivalent to about 25mg of Empagliflozin weighed and transferred 25 mL volumetric flasks. Weights taken for preparation of sample are shown in Table no.7 as below.

Table 7: Weights taken for sample solution preparation

Flask no.	Weight in mg
1	21.51 mg
2	21.90 mg
3	21.62 mg
4	27.11 mg
5	26.62 mg
6	26.57 mg
7	30.95 mg
8	31.55 mg
9	31.66 mg

In same flasks 5 mL methanol added, mixed properly using sonicator and make up to the mark with methanol. Filtered by using 0.45µm PVDF syringe filter and further 2mL solution was transferred in 50 mL volumetric flask and make up to the mark with methanol.

Recovery of drug was calculated by standard addition method with three concentration levels (80%, 100% and 120%). As a result % recoveries observed at 99.35% to 101.54%. Results of Accuracy are shown in Table no.8 as below.

Table 8: Results of Accuracy

Recovery Level (in %)	Drug added (in mg)	Drug recovered (in mg)	% Recovery	% Recovery Mean	SD	%RSD
80	21.51	21.51	100.05	99.35	0.92	0.93
	21.90	21.52	98.29			
	21.62	21.54	99.75			
100	27.11	27.48	101.39	100.28	1.00	1.00
	26.62	26.47	99.44			
	26.57	26.57	100.01			
120	30.95	31.99	103.37	101.54	1.59	1.57

	31.55	31.81	100.84			
	31.66	31.79	100.41			

2.2.5. LOD & LOQ:
Determined by S/N ratio method in HPLC.

2.2.5.1 LOD (Limit of Detection)
Results of LOD are shown in Table no.9 as below.

Table 9: Results of LOD:

		Area	s/n	T.F.	R.T.
LOD_EMPA	Conc. of std 0.132 ppm	19114	11.75	0.91	1.944

2.2.5.2 LOQ (Limit of Quantitation)
Results of LOD are shown in Table no.10 as below.

Table 10: Results of LOQ

		Area	s/n	T.F.	R.T.
LOQ_EMPA	Conc of std 0.4 ppm	39988	20.81	1.03	1.954
	Conc of std 1 ppm	70052	46.73	1.07	1.952

Results of S/N ratio of LOD & LOQ are shown in Table no.11 as below.

Table 11: Calculation of S/N ratio for LOD & LOQ by HPLC.

Title	EMPA (S/N Ratio)
LOD	11.75
LOQ	20.81

2.2.6 ROBUSTNESS
The robustness of the method was evaluated by change in parameters used in method like flow rate, wavelength etc. [10,11] From this robustness studies it was found that no change in system suitability parameters and %RSD was less than 2%. The Robustness results of Empagliflozin shown in table 12.

Table 12: Changes of Flow rate (± 0.2 ml) & Wavelength (± 2 nm)

Chromatographic Changes			
Factor	**Level**	**RT values**	**Tiling Factor**
Flow Rate		**EMPA**	**EMPA**
0.8 mL/min	-0.2	2.40	1.24
1.0 mL/min	0.0	1.97	1.17
1.2 mL/min	+0.2	1.62	1.17
Wavelength		**EMPA**	
Low Wavelength 222nm	-2	1.94	-
224 nm	0	1.97	-
High Wavelength 226nm	+2	1.94	-

2.3 RESULTS & DISCUSSION:
Results of Method validation are shown in Table no.13 as below.

Table 13: Results of Method validation by HPLC

Sr. No.	Parameters	Results
1	Linearity range	20-60 ng/band
2	R^2 (Correlation coefficient)	0.999
3	Precision	
	Intraday Pression (%RSD)	0.19 & 0.14
	Interday precision (%RSD)	0.19
4	Accuracy (%Recovery)	99.35% to 101.54%
5	Limit of Detection (S/N Ratio)	11.75
6	Limit of Quantitation (S/N Ratio)	20.81
7	Robustness (RT)	
	a) Change in Flow Rate (±2 ml/min)	
	+0.2min	1.62
	-0.2min	2.40
	b) Change in Wavelength	
	Low Wavelength 222nm	1.94
	High Wavelength 226nm	1.94
8.	% label claim of Marketed formulation	98.99%

2.4 Forced Degradation:
As per ICH guidelines the forced degradation studies performed. In this forced degradation study effects of various stress conditions like acid, base, hydrolytic, oxidative, dry heat (thermal) and light exposure on drug were studied.

2.4.1 Study of Forced degradation for drug Empagliflozin:
In six different flasks, drug 25 mg weighed and transferred dissolved in diluent methanol, make up to the mark with the diluent. From this stock solution transferred in another 50.0 ml volumetric flasks 1, 2, 3, 4,5 and 6. After this in flask 1,2and 3, 2 mL of 3 N HCl, 3 N NaOH, H_2O_2 were transferred and refluxed at 80°C for 1 hr. In flask 4, HPLC water is added and kept at dark for 3 hr. And also, flask 5 containing 2 mL of stock solution was make up to the mark with the diluent, kept at 80ºC using water bath for 1 hr. for thermal degradation. For UV exposure flask 6 was

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kept in ultraviolet radiations at 294 nm for 24 hrs. in a UV-chamber. After the time interval all flasks make up to the mark with diluent and analyzed for effect of each stress condition on drug.

Chromatograms for effect of each stress condition on drug are as follows.

2.4.1.2 Acid degradation:

In Acid degradation, on exposure to 3N HCl 2.63% degradation was observed.

Results of Acid Degradation are shown in Table no.14 as below.

Table 14: Results of Acid Degradation

Area of Standard	2691152	% Degradation = 2.63%
Area after Acid Degradation	2620119	

Chromatogram of Acid Degradation are shown in Figure no.5 as below.

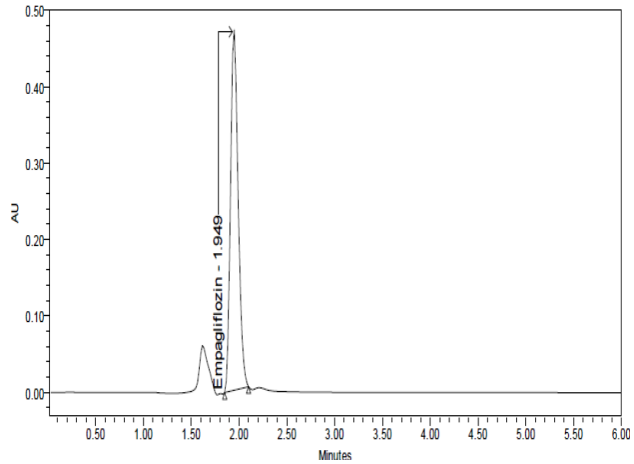

Figure 5: HPLC Chromatogram for acid degradation.

2.4.1.3 Alkaline/ Base degradation:

In alkaline degradation, on exposure of 3N NaOH 1.25% degradation was observed.

Results of Acid Degradation are shown in Table no.15 as below.

Table 15: Results of Base Degradation

Area of Standard	2691152	% Degradation = 1.25%
Area after Base Degradation	2657417	

Chromatogram of Base Degradation are shown in Figure no.6 as below.

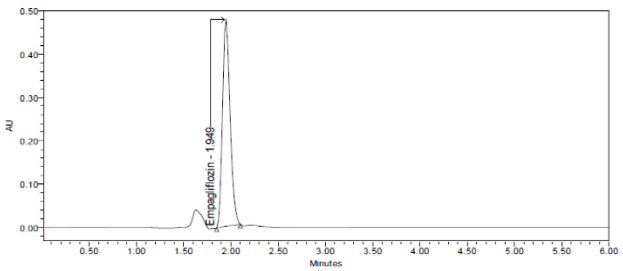

Figure 6: HPLC Chromatogram for alkaline/base degradation.

2.4.1.4 Oxidation degradation:

In oxidation degradation, on exposure of 6% H_2O_2 4.20% degradation observed.

Results of Oxidation Degradation are shown in Table no.16 as below.

Table 16: Results of Oxidation Degradation

Area of Standard	2691152	% Degradation = 4.20%
Area after Oxidation Degradation	2578088	

Chromatogram of Oxidation Degradation are shown in Figure no.7 as below.

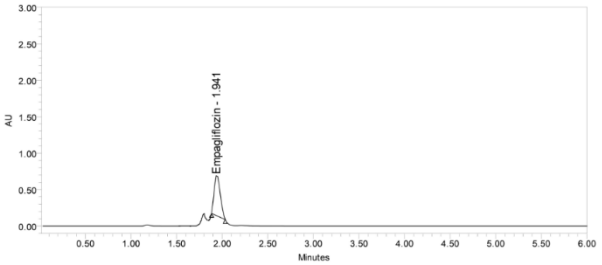

Figure 7: HPLC chromatogram for oxidation degradation.

2.4.1.5 Photolytic degradation:

In Photolytic degradation, on exposure of UV light (294 nm), 0.71% degradation observed.

Results of Photolytic Degradation are shown in Table no.17 as below.

Table 17: Results of Photolytic Degradation

Area of Standard	2693819	% Degradation = 0.71%
Area after Photolytic Degradation	2674600	

Chromatogram of Photolytic Degradation are shown in Figure no.8 as below.

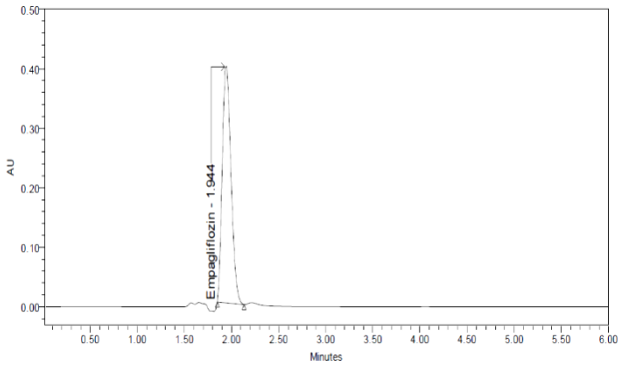

Figure 8: HPLC chromatogram for photolytic degradation.

2.3.1.6 Thermal degradation:

In thermal degradation on exposed to 80°C temp for 1 hr, 1.48% degradation observed.

Results of Thermal Degradation are shown in Table no.18 as below.

Table 18: Results of Thermal Degradation

Area of Standard	2693819	% Degradation = 1.48%
Area after Thermal Degradation	2653799	

Chromatogram of Thermal Degradation are shown in Figure no.9 as below.

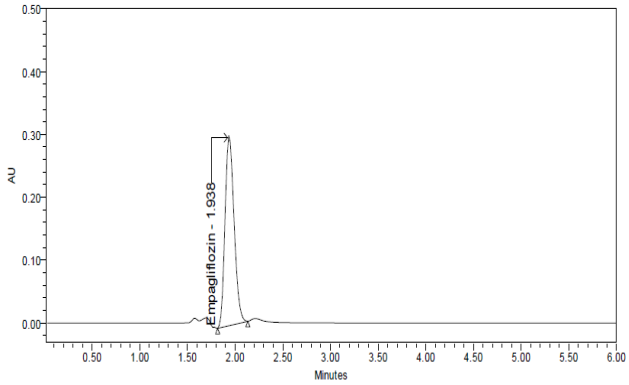

Figure 9: HPLC chromatogram for thermal degradation.

2.4.1.7 Neutral degradation:

In Neutral degradation on exposed at room temperature for 45 min, 0.14% degradation observed.

Results of Thermal Degradation are shown in Table no.19 as below.

Table 19: Results of Neutral Degradation

Area of Standard	2691152	% Degradation = 0.14%
Area after Neutral Degradation	2687190	

Chromatogram of Neutral Degradation are shown in Figure no.10 as below.

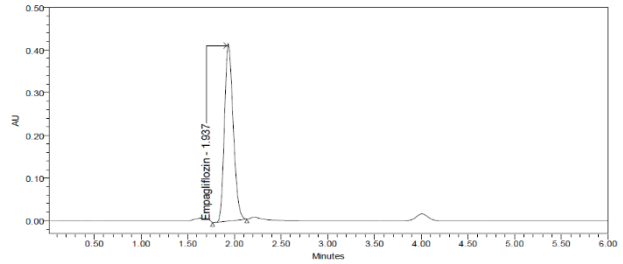

Figure 10: HPLC chromatogram for neutral degradation.

Summary of Results of Degradation studies are shown in Table no.20 as below.

Table 20: Summary of degradation studies.

Sr. No.	Degradation Condition	Duration	Degradation (In %)
1.	Acid (3 N HCl)	80°C temp for 1 hr.	2.63%
2.	Base (3 N NaOH)	80°C temp for 1 hr.	1.25%
3.	Oxide (6 % H_2O_2)	80°C temp for 1 hr.	4.20%
4.	Neutral	RT for 45 min	0.14%
5.	Thermal	80°C temp for 1 hr.	1.48
6.	Photolytic Degradation	24 hrs.	0.71

2.5: CONCLUSION:

This method was developed and validated as per ICH Guidelines Q2(R2). Method was shown that it may be useful in determination of assay of a Empagliflozin drug product and substance. By using regulatory information, ICH guidance and pharmacopeial standards the method validation was performed. This method successfully met the pre-defined acceptance criteria for various validation parameters like Linearity and range, accuracy, precision, limits of detection, limit of quantitation, Robustness etc. Therefore, it was considered suitable for routine analysis as well as stability testing of drug.

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