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DEVELOPMENT AND VALIDATION OF STABILITY INDICATING RP-HPLC METHOD FOR ESTIMATION OF EMPAGLIFLOZIN IN BULK DRUG AND FORMULATION

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ABSTRACT:

A robust reverse phase high performance liquid chromatographic (RP-HPLC) method was developed and validated for the quantification of Empagliflozin in both bulk and formulated forms, with a comprehensive evaluation of its stability under diverse conditions. Effective separation of Empagliflozin and its degradation products was achieved using an Agilent C18 column and a mobile phase comprising methanol and 0.1% formic acid in an equal fraction elution mode at a flow rate of 0.8 mL/min. Detection was carried out using a diode array detector (DAD) at ambient temperature. The method demonstrated linearity over a concentration range of 5-25 µg/mL, with Empagliflozin eluting at 3.4 minutes. The limits of detection (LOD) and quantification (LOQ) were determined to be 0.0509µg/mL and 0.1544µg/mL, respectively. Method validation parameters including precision, accuracy, linearity, selectivity, specificity, and robustness all met the requisite acceptance criteria. Stability-indicating capability was confirmed through subjecting Empagliflozin to various stress conditions, including acidic, alkaline, neutral, photolytic, oxidative, and thermal environments. This validated method ensures reliable and accurate assessment of Empagliflozin, making it suitable for routine analysis and stability testing.

Keywords: Empagliflozin, RP-HPLC, Method Validation, Stability-Indicating, Degradation Products, etc.

1. INTRODUCTION

Diabetes mellitus, commonly referred to as diabetes, encompasses a range of endocrine disorders characterized by persistent hyperglycaemia. This condition is one of the fastest-growing health issues globally, with adult-onset diabetes tripling in prevalence over the past two decades [1]. According to the World Health Organization, diabetes was the seventh leading cause of death in 2021. The International Diabetes Federation (IDF) reports that one in eight individuals worldwide has diabetes, with 463 million adults aged 20-80 affected. This number is projected to rise to 783 million by 2045. Recent IDF data indicates that India, along with other South Asian countries, is significantly impacted by diabetes mellitus (DM), with projections showing India moving from 77 million affected individuals in 2019 to 134 million by 2045. Notably, 11% of the urban population and 7.2% of the rural population in India are diagnosed with type II diabetes [2,3].

Type II diabetes is increasingly prevalent, with innovative treatments emerging to address this growing health challenge. Sodium Glucose Co-transporter-2 (SGLT2) inhibitors, also known as gliflozins, represent a novel approach to managing type II diabetes [4]. Unlike SGLT1 inhibitors, which act on glucose/sodium channels in the intestinal mucosa, SGLT2 inhibitors alter nephron physiology in the kidneys, reducing glucose reabsorption and consequently lowering blood sugar levels. These inhibitors, including Empagliflozin, not only control blood sugar but also help in reducing body weight and lowering blood pressure in diabetic patients. They are particularly beneficial for non-insulin-dependent type II diabetes mellitus (T2DM) patients, often improving cardiovascular outcomes [5].

Chemically, Empagliflozin has the empirical formula C23H27ClO7 and a molar mass of 450.91 amu. It is structurally similar to dapagliflozin, differing by the replacement of an oxolane terminal with an ethyl group of ethyl phenyl ether. Empagliflozin is available in 10 mg or 25 mg tablet forms and is a crucial medication for type II diabetes management [6].

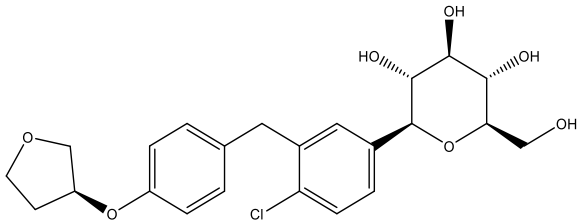

Figure 1. Chemical Structure of Empagliflozin

Various methodologies have been employed to assess the pharmacological activity of Empagliflozin. However, few studies have focused on stability-indicating reverse phase high performance liquid chromatographic (RP-HPLC) methods for its estimation. This study aims to develop a stability-indicating RP-HPLC method that is accurate, cost-effective, and precise for the estimation of Empagliflozin in bulk and formulated forms. Validation of this method will encompass parameters such as precision, accuracy, linearity, selectivity, specificity, and robustness.

This research contributes to the existing analytical techniques by providing a validated and reliable method for the estimation and stability testing of Empagliflozin, ensuring its efficacy and safety in pharmaceutical applications [7].

2. MATERIALS AND METHODS

Instrumentation and Chromatographic Conditions

The performance was conducted using a HPLC comprising a water 2695 solvent delivery module operating in a 4-degree gradient mode. Acquiring data and its analysis were facilitated

by Chemstation software. Such a separation was attained using an Agilent C18 column (150 mm x 4.6 mm, 5 µm) with detection performed via an absorbance detector and autochro-3000 software. The column was maintained at constant surrounding temperature. The mobile phase, consisting of methanol and 0.1% formic acid in a ratio of 58:42, was optimized for detection at 224 nm. The experiment conduction time was 600 sec, with a constant rate of flow 0.8 mL/min utilized throughout the analysis.

Materials and Reagent

Empagliflozin (active pharmaceutical ingredient) was generously provided as a gift sample by Shree Reliable Industrial Training Centre, located in Jalgaon, Maharashtra. Other reagents used in this study, including orthophosphoric acid and formic acid of HPLC grade, as well as methanol and water, were supplied by Merck Specialties Private Limited, situated in Shiv Sagar Estate 'A', Worli, Mumbai, Maharashtra.

Preparation of Solutions

Preparation of Standard Generic Solution

The 5 mg of Empagliflozin performance standard were accurately weighed and transferred into a clean, dry 10 mL volumetric flask. The standard was then completely dissolved in methanol. The flask was filled to the mark with methanol to obtain a standard solution with a concentration of 500 µg/mL. The solution underwent sonication for 15 minutes to ensure complete dissolution and to degas the solution.

Subsequently, equal aliquots of the 500 µg/mL Empagliflozin standard solution were combined with the mobile phase in a 58:42 ratio, reaching a final volume of 10 mL in a volumetric flask. From this mixed solution, 0.1 mL was pipetted into another 10 mL volumetric flask. This aliquot was then diluted to the mark with a solvent mixture consisting of 6mL methanol and 4mL water containing 0.1% formic acid (pH 2.7). Additionally, a separate solvent containing 1% formic acid, adjusted to pH 6.0 with orthophosphoric acid, was prepared for use in further analyses [8, 9].

Preparation of Sample Generic Solution

From the previously prepared standard solution of Empagliflozin (500 µg/mL), an aliquot of 0.1 mL was precisely measured and transferred into a clean 10 mL volumetric flask. The aliquot was then diluted to the mark with the mobile phase, resulting in a final concentration of 10 µg/mL. This dilution step ensures the accurate preparation of the sample generic solution for subsequent analytical procedures [8].

Selection of mobile phase

Each mobile phase was subjected to vacuum degassing and filtration through a 0.45 µm membrane filter before use to remove any particulates and dissolved gases. After preparation, the mobile phase was allowed to reach a stable equilibrium. Various standard solutions of Empagliflozin were analysed using different single solvents and solvent mixtures to optimize separation efficiency and peak stability.

Among the various mobile phases tested, the mixture of methanol and water containing 0.1% formic acid, adjusted to pH 2.7, was selected. This mobile phase provided sharp, well-resolved peaks with symmetrical shapes, meeting acceptable criteria for peak shape and reproducibility. Additionally, this mobile phase consistently demonstrated reliable retention times for Empagliflozin, ensuring reproducible interaction between the stationary and mobile phases [9, 10].

Degradation Studies

To assess the stability of the dosage forms, they were exposed to various adverse conditions, including acidic, basic, oxidative, thermal, and photolytic environments. The stability studies revealed that the dosage forms remained stable under photolytic and hydrolytic conditions. However, significant degradation was observed when the dosage forms were subjected to acidic, basic, and peroxide environments. These findings indicate the susceptibility of the

dosage forms to certain stress conditions and underscore the importance of appropriate storage and handling to maintain the integrity of the product [11].

Acid degradation Studies

To evaluate the acid degradation of Empagliflozin, 0.1 mL of the generic solution (500 µg/mL) was mixed with 10 mL of 0.1N HCl and refluxed for 1 hour at 333 K. After refluxing, the solution was diluted with methanol to a final volume of 50 mL, resulting in a concentration of 200 µg/mL. Subsequently, 0.5 mL of this solution was further diluted to 10 mL with the mobile phase. From this final solution, 10 µL aliquots were injected into the chromatographic system, and the resultant chromatograms were analysed to assess the stability of the samples under acidic conditions [12, 13].

Oxidative degradation studies

0.1 mL of Empagliflozin generic solution was combined with 1 mL of 30% Hydrogen Peroxide (H_2O_2) separately. These solutions were left at room temperature for 1 hour. From the resulting stress solutions, 0.1 mL was moved into a 10 mL volumetric flask and concentration was lower to final volume with the mobile phase, yielding a 10 µg/mL solution. Subsequently, 10 µL aliquots were inserted into the system, and chromatograms evaluated the stability of the sample [13, 14].

Thermal degradation studies

To assess thermal degradation, drug powder was placed in a Petri dish and subjected to 333K in an oven for 1 hour. For RP-HPLC analysis, 10 mg of the resulting drug was accurately weighed and moved into a sterilized, moisture less 10ml volumetric flask. A few mL of methanol was added, and put for sonication for 900 seconds before being made up to 10mL with methanol [14]. From this stress solution, 0.1ml was withdrawn and transferred into a 10mL volumetric flask, which was then filled to the final volume with the mobile phase to create a 10µg/ml solution. Subsequently, 10µL aliquots were inserted into the system, and chromatograms evaluated the stability of sample [15, 16].

Photolytic degradation studies

For the photostability study, drug powder was directly exposed to sunlight in a Petri dish for 24 hours. For HPLC analysis, 10mg of the resulting drug was precisely measured and moved into clean moisture less 10 ml volumetric flask. A few mL of methanol was added, and put for sonication for 900 seconds before being made up to 10 mL with methanol. From this stress solution, 0.1 ml was withdrawn and transferred into a 10 mL volumetric flask, which was then filled to the final volume with the mobile phase to create a 10 µg/ml solution. Subsequently, 10 µL aliquots were inserted into the system, and chromatograms evaluated the stability of the sample [17].

Neutral Degradation Studies

0.1 ml of Empagliflozin generic solution was mixed with 10 ml of water and fading for 1800 second at 60ºC. The concentration of resulting solution lowered to 50 mL with methanol to create a 200 µg/ml solution [18]. Subsequently, 0.5 mL of this solution was withdrawn and diluted to 10 mL with the mobile phase. From this solution, 10 µL aliquots were inserted into the system, and chromatograms evaluated for the stability of sample [19, 20].

3. RESULT AND DISCUSSION

Initial trials

The observed initial trials using methanol and 0.1% formic acid in the ratio of 58:42 at 224nm. As mention in table 1 trial 1 to 8 where unaccepted due to several reasons and the accepted trial with obtained sharp peak was 9.

Table 1. Initial Trials

Sr. No.	Column used	Mobile phase ratio	Detection wavelength	Flow rate	Injection volume	Run time	Result	Conclusion
1	Agilent C-18 (150mm x 4.6mm, 5µm)	Methanol with 0.1% Formic acid (90:10)	224nm	0.7ml/min	20µl	8min	Resolution was not satisfactory	Method unaccepted
2	Agilent C-18 (150mm x 4.6mm, 5µm)	Methanol with 0.1% Formic acid (80:20)	224nm	0.7ml/min	20µl	8min	Response of the first peak was not satisfactory	Method unaccepted
3	Agilent C-18 (150mm x 4.6mm, 5µm)	Methanol with 0.1% Formic acid (70:30)	224nm	0.7ml/min	20µl	8min	Broad peak	Method unaccepted
4	Agilent C-18 (150mm x 4.6mm, 5µm)	Methanol with 0.1% Formic acid (60:40)	224nm	0.7ml/min	20µl	8min	Larger retention time	Method unaccepted
5	Agilent C-18 (150mm x 4.6mm, 5µm)	Methanol with 0.1% Formic acid (65:35)	224nm	0.7ml/min	20µl	10min	Response of the second peak was not satisfactory	Method unaccepted
6	Agilent C-18 (150mm x 4.6mm, 5µm)	Methanol with 0.1% Formic acid (65:35)	224nm	1ml/min	20µl	10min	System suitability condition is not within the limit	Method unaccepted
7	Agilent C-18 (150mm x 4.6mm, 5µm)	Methanol with 0.1% Formic acid (68:32)	224nm	1ml/min	20µl	10 min	Fronting peak	Method unaccepted
8	Agilent C-18 (150m	Methanol with 0.1%	224nm	1ml/min	20µl	10 min	Resolution was not	Method unaccepted

	m x 4.6mm , 5µm)	Formic acid (58:42)					satisfactory	
9	Agilent C-18 (150mm x 4.6mm , 5µm)	Methanol with 0.1% Formic acid (58:42)	224nm	0.8ml/min	20µl	8 min	Sharpe peak was obtained	Method accepted

Method development

The validation of the method adhered to the guidance of the ICH. Various validation parameters were assessed; including linearity, accuracy, precise and its limit of detect and quantify [21].

Optimized chromatographical conditions

Optimized chromatographical conditions and typical chromatogram were presented in Table 2 and Figure 2

Table 2. Chromatographic Conditions

Parameters	Conditions
Instrumentation	Water HPLC with auto sampler and PDA detector
Injected volume	20µl
Mobility phase	Methanol: 0.1% formic acid (58:42)
Column	Agilent C_{18} (150 mm x 4.6 mm, 5 µm)
Flow rate	0.8 ml/min
Run time	8 minutes
Separation mode	Isocratic mode

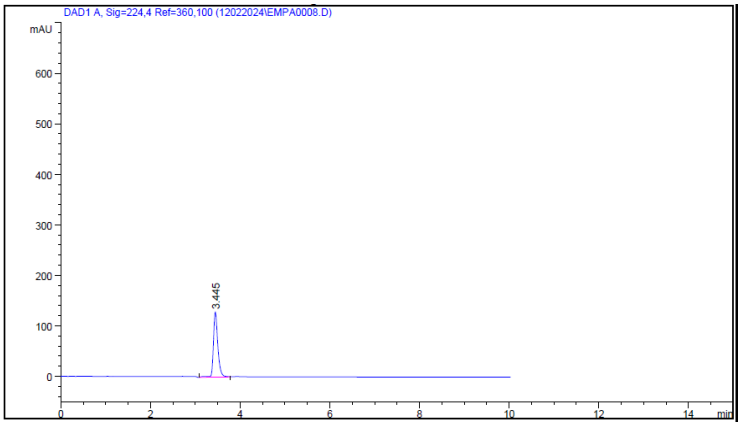

Figure 2: Typical Chromatogram of Empagliflozin

Linearity

The method was set linear up to the concentration range of 5-25 µg/ml. Samples were prepared by aliquoting 5, 10, 15, 20, and 25 µg/ml from a secondary generic solution, labelled as solutions 1 through 5, respectively. Each solution, with a volume of 20 µl, was inserted into the system operating at a flow rate of 0.8 ml/min. The calibration curve was constructed by plotting the peak area ratio against the applied concentration of Empagliflozin. The linearity curve for

Empagliflozin is depicted in Figure 3, and the associated linearity parameters are presented in Table 3. The linear equation for the calibration curves was determined [22].

$$Y=37.69+25.94$$

Where,
Y= Peak area of drug
X= Concentration of drug in µg/mL
R^2= Regression coefficient

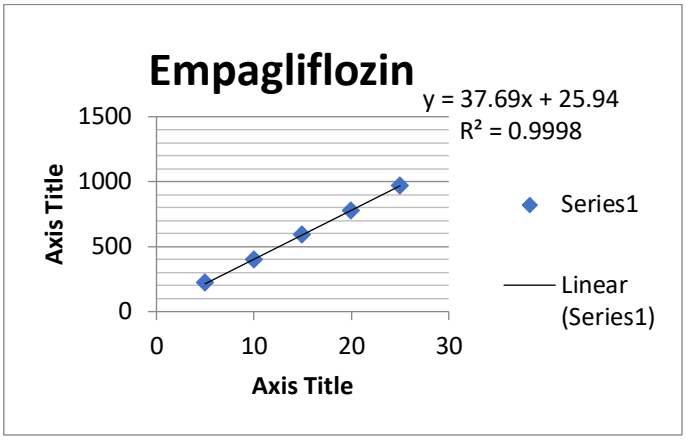

Figure 3: Empagliflozin linearity curve

Table 1: Parameter for Linearity of Empagliflozin

Concentration (µg/mL)	Area
5	219.93
10	397.74
15	588.23
20	778.96
25	971.56

Accuracy

Empagliflozin was precisely weighed and incorporated into a blend of the tablet's excipients at three distinct concentration levels: 80%, 100%, and 120%. For every concentration level, samples were prepared in triple amount, and the recovery percentages were determined respectively. [23, 24]

Precision

In both the real-time and subsequent day precision studies, the method exhibited exceptional precision across the tested range of 5-25µg/ml Empagliflozin. Samples were analysed thrice on the same day, and according to that the percentage relative standard deviation (% RSD) was noted [25].

Robustness

The robustness of the developed RP-HPLC method was obtained by investigating the impact of minor alterations in chromatographic conditions, including variations in rate of flow, its wavelength and composition of mobile phase, on the method performance [26].

Limit of detection (LOD) and limit of quantitation (LOQ)

The LOD (limit of detection) and LOQ (limit of quantitation) LOQ were calculated using a signal/noise ratio of 3:1 and 10:1 respectively. The calculated values for LOD and LOQ were found to be 0.0509 µg/ml and 0.1544 µg/ml, respectively [27].

Table 2: Accuracy Parameter for Empagliflozin

Method	**Level (%)**	**Amount taken (ug/ml)**	**Amount Added (ug/ml)**	**Area Mean S.D.**	**Amount recovered Mean ± S.D.**	**% Recovery Mean± S.D.**
HPLC	80%	10	8	18.19 ±0.03	8.19 ± 0.03	102.38± 0.47
	100%	10	10	20.13 ± 0.06	10.13 ± 0.06	101.25± 0.67
	120%	10	12	22.08 ± 0.09	12.08±0.094	100.67± 0.78

Table 3: Precision Parameter for Empagliflozin

Method	**Concentration (µg/ml)**	**Inter-day Precision**		**Intra-day Precision**	
		Mean ± SD	**% Amount Found**	**Mean ± SD**	**% Amount Found**
HPLC	5	213.88± 1.65	99.73	213.27 ± 3.56	99.41
	15	596.90± 2.07	100.99	598.62 ± 1.65	101.30
	20	994.16 ± 4.83	102.76	993.61 ± 5.59	102.70

Table 4: Robustness Parameter for Empagliflozin

Parameter	**Concentration (µg/ml)**	**Amount of detected (Mean ± SD)**	**% RSD**
Chromatogram rate of flow change 0.7ml	15	524.13 ± 1.08	0.21
Chromatogram rate of flow change 0.9ml	15	595.04± 0.59	0.10
Chromatogram composition change wavelength change 223nm	15	515.7 ± 1.34	0.26
Chromatogram composition change wavelength change 225nm	15	564.27 ± 2.56	0.45
Chromatogram of mobile phase change 57+43ml	15	594.0± 0.82	0.14
Chromatogram of mobile phase change 59+43ml	15	613.15 ± 2.00	0.33

Force degradation studies

The objective of the force degradation studies was to validate the effective separation of Empagliflozin from its degradation products. Various parameters including acidic hydrolysis,

basic hydrolysis, hydrogen peroxide, oxidation, and photolysis were investigated. The outcomes of the forced degradation studies are shown in a representable form in Table 7 [27]

Table 5. Force Degradation Parameters of Empagliflozin

Sr. No.	Degradation Parameter	% Degradation of Empagliflozin
1	Alkali degradation 0. 1 N NaOH	9.87
2	Acid degradation 0. 1 N HCL	4.89
3	3% H_2O_2 degradation	7.46
4	Neutral	2.00
5	Photolytic	0.34

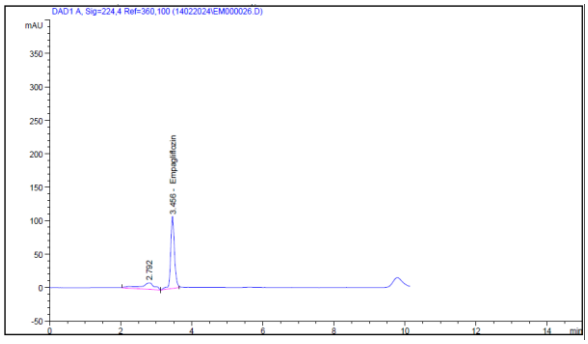

Figure 4: RP-HPLC Alkaline Degradation chromatogram

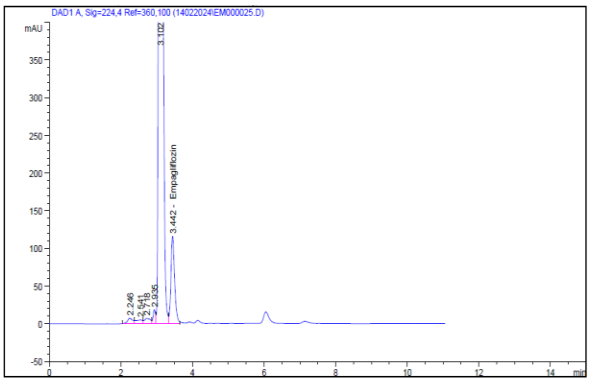

Figure 5: RP-HPLC Acidic Degradation chromatogram

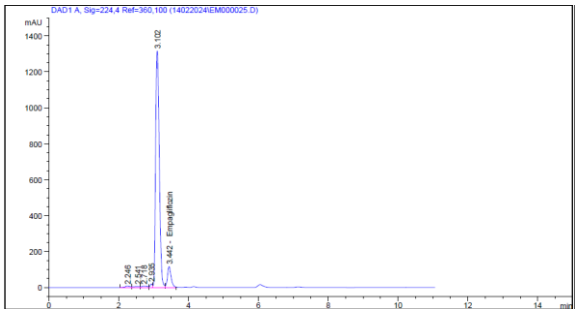

Figure 6: RP-HPLC Oxidative Degradation chromatogram

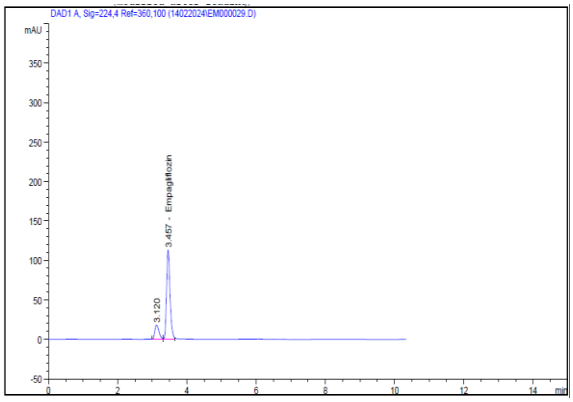

Figure 7: RP-HPLC Neutral Degradation chromatogram

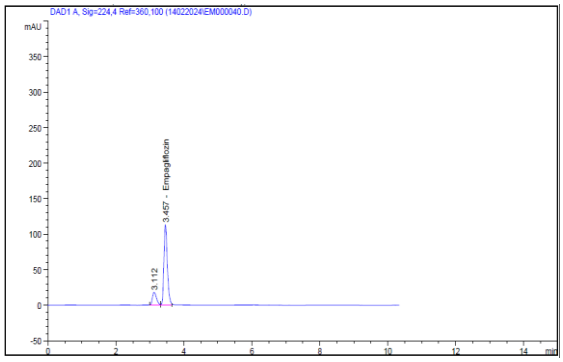

Figure 8: RP-HPLC Photo Degradation chromatogram

4. CONCLUSION

The current work presents the development and validation of stability indicating RP-HPLC method for estimation of Empagliflozin in bulk drug and formulation. Initial trials were conducted using RP-HPLC, leading to the optimization of chromatographic conditions. All validated parameters fell within allowable ranges. The developed and validated method exhibited linearity, precision, accuracy, robustness, high sensitivity, and ruggedness. Based on these findings, it can be inferred that the method is suitable for stability and validation investigations.

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