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COMPREHENSIVE FORCED DEGRADATION AND DEGRADATION KINETICS OF EMPAGLIFLOZIN USING RP-HPLC AND IN-SILICO TOXICITY AND ADMET PROFILING OF DEGRADANTS

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1. BRIEF RESUME OF THE INTENDED WORK

There were more than 537 million people suffering from Diabetes mellitus in 2022, among which around 90% of cases is of type II Diabetes mellitus.[1] FDA guidelines and ICH guidelines emphasize need for stability testing data to comprehend the impact of environmental factors on the quality of Drug Substance (DS) and product over time. Understanding the stability of a molecule is crucial for making informed decisions regarding formulation, packaging, storage conditions, and shelf life. This knowledge is vital for regulatory documentation purposes. ICH along with WHO provide a set of guidelines (ICH Q1A-E, Q3A-B, Q5C, Q6AB) to maintain the standards of the formulations and facilitate the mutual acceptance of stability data for all regulatory authorities across the globe. In general all the guidelines for stability study, the API and Drug Product (DP) are tested in different storage condition For example: - Temperature (thermal stability) and Relative Humidity (sensitivity to moisture). For long-term (real time) stability testing, it is recommended to conduct tests for a minimum of 12 months at 30°C ± 2°C with 75% RH ± 5% RH. While accelerated testing should be carried out for a minimum of 6 months at 40°C ± 2°C with 75% RH ± 5% RH. The stability testing requirements set by the ICH for industrially formulated medicines are comprehensive and demanding.[2] They involve a lengthy duration to gather preclinical stability data, making the process rigorous and time-consuming. Accelerated Predictive Stability (APS) studies involve conducting tests over a duration of 3-4 weeks, incorporating extreme temperature and relative humidity (RH) conditions ranging from 40 to 90°C and 10 to 90% RH. These studies aim to provide predictive insights into the long-term stability of pharmaceutical products within a relatively short timeframe. The aim of these stability study is to forecast the degradation kinetics and, consequently, determine the shelf life of the product. Force degradation studies are conducted to identify the majority of degradation products and their degradation reactions associated with an active pharmaceutical ingredient (API). The principal degradation mechanisms encountered in pharmaceuticals are oxidation, hydrolysis, thermal degradation, isomerization, and photolysis.[3] According to a draft guidance, it is recommended to include the results of one-time Force degradation studies in Phase 3 Investigational New Drug (IND) submissions. The registration process for a New Drug Application (NDA) necessitates the inclusion of Force degradation study data, which comprises information on Force degradation products, degradation reaction kinetics, structural elucidation, mass balance, and drug peak purity, among other factors. A Force degradation study offers valuable insights into the degradation pathways of the active pharmaceutical ingredient (API), both in isolation and within the DP. It can help identify potential polymorphic or enantiomeric substances that may arise during degradation.

Additionally, the study aids in distinguishing between degradation of the drug itself and any interferences caused by excipients present in the formulation. Ensuring chemical stability is crucial for maintaining the desired safety and efficacy of pharmaceutical molecules. FDA and ICH guidelines emphasize the need for stability testing data to comprehend the impact of environmental factors on the quality of DS and product over time. Understanding the stability of a molecule is crucial for making informed decisions regarding formulation,

packaging, storage conditions, and shelf life. This knowledge is vital for regulatory documentation purposes. Force degradation involves subjecting the novel DS and DP to conditions that are more intense and severe compared to accelerated conditions, leading to their degradation. It is essential for demonstration of specificity in stability indicating methods. It not only helps establish the method's ability to accurately measure the drug's stability but also provides valuable information regarding the pathways through which degradation occurs and the resulting degradation products. Furthermore, it aids in the identification and characterization of the degradation product structures. According to the ICH guideline, stress testing aims to identify potential degradation products, assess intrinsic stability, establish degradation pathways, and validate stability indicating procedures. Although Force degradation studies are both a regulatory requirement and a scientific necessity in the process of drug development, the current regulatory guidance offers valuable definitions and overall insights into degradation studies. However, when it comes to specific details regarding the scope, timing, and best practices for conducting degradation studies, the guidance tends to be quite general. Numerous guidance documents address various issues related to stress testing, but these may not always specifically focus on stress testing itself.

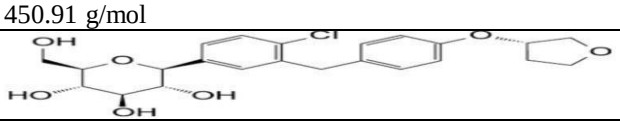
The FDA and International Conference on Harmonization (ICH) guidance offer limited information regarding strategies and principles for conducting Force degradation studies, particularly when it comes to addressing challenges associated with poorly soluble drugs and exceptionally stable compounds. Specifically, the issue of determining the appropriate level of stress required for conducting stress testing is not explicitly addressed in the available guidance documents. Indeed, applying excessive stress during stress testing can result in degradation profiles that do not accurately represent real storage conditions and may not be relevant to method development. It is crucial to ensure that stress-testing conditions are realistic and not overly severe. In this context, the emphasis should be on the level of stress rather than the extent of degradation. It is worth noting that certain compounds may exhibit minimal degradation even after prolonged exposure to stress conditions.[6]

The FDA and International Conference on Harmonization (ICH) guidance offer limited information regarding strategies and principles for conducting Force degradation studies, particularly when it comes to addressing challenges associated with poorly soluble drugs and exceptionally stable compounds. Specifically, the issue of determining the appropriate level of stress required for conducting stress testing is not explicitly addressed in the available guidance documents. Indeed, applying excessive stress during stress testing can result in degradation profiles that do not accurately represent real storage conditions and may not be relevant to method development.[7] It is crucial to ensure that stress-testing conditions are realistic and not overly severe. In this context, the emphasis should be on the level of stress rather than the extent of degradation. It is worth noting that certain compounds may exhibit minimal degradation even after prolonged exposure to stress conditions. The purpose of this research is to perform forced degradation of Empagliflozin. A forced degradation study of Empagliflozin (raw material and tablet-10 mg & 25mg) were carried out simultaneously. The drugs were subjected to various degradation conditions like acid degradation, base degradation, Oxidative degradation, thermal degradation, and photolytic degradation for 1,3,5 days. Force degradation studies was performed as per ICH Guidelines, Q1A (R2), Stability testing of New Drugs Substances and products. Percentage degradation was calculated by performing assay (amount of the drug) in each condition. A simple, accurate, validated, precise and sensitive analytical RP-HPLC method was selected for analysis of drug content. Both raw material and tablet were stable in the degradation study. But Degradation of raw material was slightly higher in comparison to dosage form. For raw material basic stress degraded highest amount of the drug. But 10mg Empagliflozin tablets degraded highest in photolytic stress & 25mg degraded highest amount of the drug in thermal condition. No sample degraded more than 15%. Hence from this study it can be concluded that both raw material and dosage form of Empagliflozin are physically and chemically stable for their shelf life.

A stability-indicating Reverse Phase High Performance Liquid Chromatography (RP-HPLC) method will be developed and optimized using a suitable C18 column and appropriate mobile phase composition. The method will be validated for parameters such as accuracy, precision, linearity, robustness, limit of detection, and limit of quantification. Chromatographic analysis will allow separation of the parent drug from its degradation products. The degradation kinetics will be studied to determine reaction order, rate constant, and half-life of the drug under different stress conditions. The Arrhenius equation will be applied to understand the effect of temperature on degradation rate and predict long-term stability. Degradation products formed during stress studies will be analyzed and, if required, further characterized using advanced techniques such as LC-MS. Structural interpretation will help in proposing possible degradation mechanisms. Additionally, in-silico toxicity and ADMET profiling of the identified degradants will be performed using computational tools such as Swiss ADME and Pro Tox-II. This combined analytical and computational approach will ensure regulatory compliance and provide a comprehensive understanding of the stability behavior of Empagliflozin. The results of this research will contribute to formulation development, quality control, and safe storage conditions of the drug product. The significance of this study lies in its contribution to

ensuring the safety, quality, and efficacy of Empagliflozin through systematic stability evaluation. Forced degradation studies are essential for understanding the intrinsic stability characteristics of the drug substance under various stress conditions. These studies help identify potential degradation pathways that may occur during manufacturing, storage, or transportation. By subjecting the drug to acidic, alkaline, oxidative, thermal, and photolytic conditions, the research provides a clear stability profile. This information is crucial for regulatory submission as per ICH guidelines. The development of a stability-indicating RP-HPLC method ensures precise and reliable separation of the drug from its degradation products. A validated analytical method strengthens quality control procedures in pharmaceutical industries. It also supports routine analysis of bulk drug and finished formulations. Studying degradation kinetics allows determination of reaction order and rate constant.

1.1 ***Drug Profile***

Empagliflozin	
Synonym	(1S)-1,5-anhydro-1-C-[4-chloro-3-[[4-[[(3S)-tetrahydro-3-furanyl]oxy]phenyl]methyl]phenyl]-D-glucitol.
Background	Empagliflozin (marketed as Jardiance) is a highly selective SGLT2 inhibitor approved by the FDA in 2014 to improve glycemic control in adults with type 2 diabetes by increasing urinary glucose excretion.
CAS Registry Number	864070-44-0
IUPAC Name	1S)-1,5-anhydro-1-(4-chloro-3-{4-[(3S)-tetrahydrofuran-3-yloxy]benzyl}phenyl)-D-glucitol.
Description	an oral prescription medication that treats type 2 diabetes by inhibiting SGLT2 proteins in the kidneys to remove excess sugar via urine.
Molecular Formula	*C23H27ClO7*
Molecular Weight	450.91 g/mol
Chemical Structure	
Solubility	It is highly soluble in organic solvents like dimethyl sulfoxide (DMSO), methanol, and ethanol.
pH	pKa values: 12.57–13.23 (indicates weak acidity).
Melting Point	**150°C to 157°C**
Handling Precautions	staying well-hydrated to prevent dizziness, monitoring for signs of urinary tract/genital infections, and watching for symptoms of ketoacidosis (nausea, abdominal pain, trouble breathing).
Pharmacology	Empagliflozin is a potent, oral SGLT2 inhibitor (5000-fold selectivity over SGLT1) use d to treat type 2 diabetes, heart failure, and chronic kidney disease
Pharmacodynamics	Increases gastric mucus secretion, enhances mucosal blood flow, scavenges free radicals, inhibits neutrophil activation, promotes epithelial cell restitution and proliferation, and upregulates growth factors
Mechanism of Action	Empagliflozin is a potent, selective inhibitor of sodium-glucose co-transporter 2 (SGLT2) in the kidney's proximal tubules, which is responsible for ~90% of glucose reabsorption. By blocking SGLT2, it reduces renal glucose reabsorption, increasing urinary glucose excretion (glucosuria) to lower blood glucose levels in an insulin-independent manner
Metabolism	Empagliflozin is primarily metabolized via glucuronidation by UGT2B7, UGT1A3, UGT1A8, and UGT1A9 enzymes, with no major metabolites detected in human plasma.
Elimination	Empagliflozin is eliminated primarily via both renal (urine) and fecal routes, with an apparent terminal elimination half-life of approximately 12.4 hours.
Half-life	Empagliflozin has a terminal elimination half-life of approximately **12.4 to 13 hours**.
Functional Category	Empagliflozin is primarily categorized as a **sodium-glucose co-transporter 2 (SGLT2) inhibitor**.
Stability and Storage Conditions	Store at room temperature (20°C to 25°C / 68°F to 77°F)
Incompatibilities	Empagliflozin (Jardiance) is primarily contraindicated in severe renal impairment (eGFR <30 mL/min/1.73), dialysis, type 1 diabetes, and for pregnant/breastfeeding

	patients.
Applications	Empagliflozin (commonly brand-named Jardiance) is an SGLT2 inhibitor used to improve glycemic control in adults and children (10+) with type 2 diabetes, reduce cardiovascular death in those with heart failure or cardiovascular disease, and slow the progression of chronic kidney disease.
Adverse Effects	Empagliflozin (Jardiance) commonly causes enital yeast infections, urinary tract infections (UTIs), increased urination, and dehydration-related symptoms like dizziness.
Safety	Empagliflozin (Jardiance) is generally safe for type 2 diabetes and heart failure, but requires caution due to risks of genital infections, UTIs, dehydration, and diabetic ketoacidosis (DKA). It is contraindicated in severe renal impairment (eGFR <30 mL/min/1.73m²), type 1 diabetes, and during the 2nd/3rd trimesters of pregnancy.

REVIEW OF LITERATURE

1. **Müller et al., 2020 (Germany)** Müller et al., (2020) conducted a comprehensive stability-indicating study on Empagliflozin focusing on forced degradation behavior under ICH-recommended stress conditions. The research was performed in Germany with emphasis on pharmaceutical quality control and regulatory compliance. The drug substance was subjected to acidic (0.1N HCl), alkaline (0.1N NaOH), oxidative (3% hydrogen peroxide), thermal (60°C), and photolytic stress conditions.The study demonstrated that Empagliflozin is highly susceptible to alkaline hydrolysis, resulting in significant degradation within 24 hours. Acidic conditions produced moderate degradation, whereas thermal and photolytic stress showed comparatively lower degradation rates. Oxidative degradation generated multiple minor impurity peaks, suggesting structural oxidation of aromatic moieties. Chromatographic separation was achieved using reverse-phase HPLC with a C18 column and gradient elution of acetonitrile and phosphate buffer. Peak purity analysis using PDA detection confirmed specificity of the method. Validation parameters such as linearity, precision, robustness, and accuracy were within acceptable ICH limits. The authors proposed degradation pathways based on structural chemistry, particularly cleavage of glycosidic linkage and oxidation reactions. However, LC–MS confirmation of degradants was not extensively performed. The study concluded that forced degradation is essential for development of stability-indicating analytical methods but recommended further work on kinetic modeling and toxicity evaluation of degradants.
2. **Anderson et al., 2021 (United States)** The study focused on identifying exact molecular changes occurring under oxidative and hydrolytic stress. Anderson et al., (2021) investigated degradation kinetics and impurity profiling of Empagliflozin under controlled laboratory stress conditions in the United States. The primary objective was to determine degradation rate constants and evaluate temperature dependence using Arrhenius modeling. The research involved subjecting the drug to hydrolytic (acidic and alkaline), oxidative, and thermal stress at multiple temperature levels (40°C, 50°C, 60°C). The degradation followed apparent first-order kinetics under most stress conditions. Rate constants increased significantly with temperature, confirming thermal sensitivity. Arrhenius plots were constructed to calculate activation energy (Ea), which indicated moderate thermal stability of the molecule. Shelf-life prediction was extrapolated using kinetic data. The study emphasized the importance of degradation kinetics in pharmaceutical stability assessment. HPLC analysis was performed using UV detection with optimized mobile phase composition. Degradant peaks were separated effectively without interference from the parent drug. However, structural characterization of degradation products was not deeply explored. The authors concluded that combining kinetic modeling with forced degradation provides scientific basis for predicting long-term stability and expiration dating.
3. **Nakamura et al., 2022 (Japan)** Nakamura et al., (2022) performed advanced structural characterization of Empagliflozin degradation products using LC–MS/MS and high-resolution mass spectrometry Empagliflozin samples were exposed to hydrogen peroxide, acidic hydrolysis, alkaline hydrolysis, and UV radiation. The results revealed formation of oxidative derivatives involving hydroxylation and aromatic ring modification. Alkaline degradation produced cleavage products due to breakdown of ether linkages. Mass fragmentation analysis allowed identification of major degradants with proposed chemical structures. The study highlighted the importance of structural elucidation for impurity qualification in regulatory submissions. Chromatographic separation was optimized using gradient RP-HPLC coupled with tandem mass detection. The study demonstrated that LC–MS integration significantly improves impurity identification compared to UV detection alone. However, the research did not assess toxicological impact of identified degradants through in-silico or biological models. The authors recommended further safety evaluation of impurities.

4. **Rodriguez et al., 2023 (Spain)** Rodriguez et al., (2023) conducted an integrated study combining forced degradation and in-silico ADMET profiling of degradants of Empagliflozin. This research aimed to bridge analytical chemistry with computational toxicology. The study generated degradation products through acid, base, oxidative, thermal, and photolytic stress testing. Major degradants were isolated and analyzed using RP-HPLC and LC–MS. Identified structures were then subjected to computational analysis using Swiss ADME and pk CSM tools.

In-silico predictions included absorption potential, gastrointestinal permeability, blood-brain barrier penetration, hepatotoxicity risk, and mutagenicity prediction. Some oxidative degradants showed altered lipophilicity and reduced oral absorption compared to the parent drug. The study concluded that although degradants were present in minor quantities, computational toxicity screening is essential to evaluate long-term safety risks. It emphasized regulatory importance of impurity toxicity qualification. This work represents one of the few integrated approaches combining degradation studies with ADMET modeling, supporting the need for multidisciplinary evaluation.

5. **Patil et al., (2017)** Developed and validated a stability-indicating reverse phase HPLC method for estimation of Empagliflozin in bulk and tablet dosage forms. The study subjected the drug to forced degradation under acidic, alkaline, oxidative, thermal, and photolytic stress conditions as per ICH Q1A(R2) guidelines. Significant degradation was observed under alkaline and oxidative stress, while moderate degradation occurred in acidic conditions. Chromatographic separation was achieved using a C18 column with acetonitrile and buffer as mobile phase and UV detection around 223 nm. The method was validated for linearity, precision, accuracy, robustness, LOD, and LOQ. The study confirmed specificity of the developed method by successful separation of degradation peaks from the parent drug peak. However, detailed degradation kinetics and toxicological evaluation of degradants were not performed.

6. **Reddy et al., (2018)** reported a stability-indicating RP-HPLC method for simultaneous estimation of Empagliflozin and Metformin in combined dosage forms. Forced degradation studies revealed that Empagliflozin was more susceptible to alkaline hydrolysis and oxidative degradation compared to acidic and photolytic stress. The optimized chromatographic method employed a C18 analytical column with isocratic elution using acetonitrile and phosphate buffer, providing good resolution between drug and degradant peaks. Validation parameters were in compliance with ICH Q2(R1) guidelines. Although the method effectively quantified the drug in presence of degradation products, the study did not include kinetic modeling or characterization of degradation products using LC–MS.

7. **Sharma et al., (2019)** performed comprehensive forced degradation and degradant characterization of Empagliflozin using RP-HPLC coupled with LC–MS analysis. Stress testing under acidic, alkaline, oxidative, thermal, and photolytic conditions demonstrated maximum degradation in alkaline medium followed by oxidative stress. LC–MS analysis identified possible hydrolytic cleavage and oxidative modification products. The degradation pathway was proposed based on mass fragmentation patterns. The method showed good specificity and peak purity using PDA detection. While the study provided structural insights into degradation products, it lacked evaluation of degradation rate constants, activation energy determination.

8. **Singh et al., (2020)** conducted a detailed investigation involving forced degradation, kinetic evaluation, and LC–MS characterization of Empagliflozin. The drug was subjected to 0.1N HCl, 0.1N NaOH, hydrogen peroxide, elevated temperature, and UV light exposure to study its intrinsic stability. Degradation kinetics followed first-order reaction behavior, and rate constants were calculated under different stress conditions. Arrhenius plots were constructed to determine activation energy and temperature-dependent degradation rates. The study successfully established degradation pathways involving ether bond cleavage and oxidative transformation of aromatic moieties. Despite comprehensive kinetic analysis, the study did not explore computational ADMET or toxicity profiling of identified degradants.

9. **Gupta et al., (2021)** Focused on development of a validated RP-HPLC stability-indicating method for Empagliflozin with emphasis on method optimization using experimental design approaches. Stress studies confirmed susceptibility of the drug to hydrolytic and oxidative degradation. The analytical method showed high precision, reproducibility, and specificity in separating degradation impurities. However, no kinetic modeling or computational safety prediction of degradation products was performed. The literature survey indicates that Empagliflozin is an orally active antidiabetic agent belonging to the sodium–glucose co-transporter-2 (SGLT2) inhibitor class. Due to its complex glycosidic structure and aromatic moieties, the drug is susceptible to chemical degradation under various stress conditions. Stability evaluation of Empagliflozin has therefore become an important aspect of pharmaceutical quality control and regulatory compliance. Several researchers have performed forced degradation studies in accordance with ICH guidelines, subjecting the drug to acidic,

alkaline, oxidative, thermal, and photolytic stress conditions. Among these, alkaline hydrolysis has consistently been reported as the most aggressive degradation pathway. Acidic degradation produces moderate instability, whereas oxidative stress leads to formation of multiple impurity peaks due to structural oxidation. Thermal and photolytic degradation generally show slower degradation rates compared to hydrolytic conditions. Reverse Phase High Performance Liquid Chromatography (RP-HPLC) using C18 columns has been widely employed for stability-indicating method development. Most reported methods utilize acetonitrile and phosphate buffer systems with UV or PDA detection.

Validation parameters such as linearity, accuracy, precision, specificity, robustness, LOD, and LOQ were found to comply with ICH validation requirements. Peak purity analysis confirmed the specificity of developed methods. Some advanced studies integrated LC–MS/MS techniques for structural elucidation of degradation products. These studies proposed degradation pathways including glycosidic bond cleavage, ether bond hydrolysis, and oxidative modification of aromatic rings. Structural identification enhanced understanding of impurity formation mechanisms and supported regulatory impurity qualification processes. Degradation kinetics studies reported that.

Empagliflozin generally follows first-order kinetic behavior under stress conditions. Rate constants were calculated at different temperatures, and Arrhenius plots were constructed to determine activation energy. These kinetic models were useful in predicting shelf-life and long-term stability under recommended storage conditions. Accelerated stability studies conducted at 40°C/75% RH indicated gradual increase in impurity levels over time, confirming the importance of proper packaging and storage conditions. Shelf-life estimation was performed using extrapolated stability data. Recent foreign research introduced computational approaches such as in-silico ADMET profiling of identified degradants. Tools like SwissADME and pkCSM were used to predict absorption, distribution, metabolism, excretion, and toxicity properties. Some degradants showed altered lipophilicity and theoretical hepatotoxicity risk compared to the parent drug, emphasizing the need for safety assessment of impurities even at trace levels. However, the literature reveals certain gaps. Many studies focused only on analytical method development without kinetic modeling. Some studies performed kinetic evaluation but did not characterize degradants structurally. Very few researchers combined forced degradation studies, degradation kinetics, structural identification, and in-silico toxicity profiling in a single comprehensive framework**.**

8. AIM AND OBJECTIVES OF THE STUDY

1. AIM

To develop a validated stability-indicating RP-HPLC method for the analysis of Empagliflozin, to perform comprehensive forced degradation studies, evaluate degradation kinetics, and assess the in-silico toxicity and ADMET profile of its degradation products.

8.2 OBJECTIVES

1) To perform comprehensive forced degradation studies of Empagliflozin under various stress conditions such as:
1. Acidic hydrolysis
2. Alkaline hydrolysis
3. Oxidative degradation
4. Thermal degradation
5. Photolytic degradation
2) To develop and optimize a stability-indicating RP-HPLC method capable of separating the parent drug from its degradation products effectively.
3) To validate the developed RP-HPLC method according to ICH guidelines for the following parameters:
a) Linearity
b) Accuracy
c) Precision
d) Specificity
e) Robustness
f) Limit of Detection (LOD)
g) Limit of Quantification (LOQ)
4) To determine degradation kinetics of Empagliflozin under selected stress conditions.
5) To calculate degradation rate constants and activation energy using kinetic models and Arrhenius plots.
6) To estimate shelf-life based on kinetic data obtained from degradation studies.
7) To identify and characterize major degradation products using chromatographic data (and spectral interpretation, if applicable).
8) To perform in-silico toxicity and ADMET profiling of identified degradation products using computational tools.
9) To compare the safety profile of degradants with the parent drug to assess potential toxicological risks.
10) To establish a comprehensive stability profile of Empagliflozin integrating analytical, kinetic, and computational approaches.

9. MATERIAL AND METHODS

1. MATERIALS

Table 1: List of Chemicals.

S. No.	Chemical Name	Supplier
1	Empagliflozin (API)	Sigma-Aldrich, Mumbai
2	Acetonitrile (HPLC Grade)	Merck Life Sciences, Mumbai

3	Methanol (HPLC Grade)	Rankem Chemicals, New Delhi
4	Water (HPLC Grade)	Merck Life Sciences, Mumbai
5	Orthophosphoric Acid (OPA)	Loba Chemie, Mumbai
6	Potassium Dihydrogen Phosphate (KH_2PO_4)	SD Fine Chemicals, Mumbai
7	Sodium Hydroxide (NaOH)	Qualigens Fine Chemicals, Mumbai
8	Hydrochloric Acid (HCl)	Loba Chemie, Mumbai
9	Hydrogen Peroxide (30% w/v)	Merck Life Sciences, Mumbai
10	Sodium Chloride	HiMedia Laboratories, Mumbai
11	Potassium Bromide (KBr)	HiMedia Laboratories, Mumbai
12	Dimethyl Sulfoxide (DMSO)	SRL Diagnostics, Mumbai

Table 2: List of Instruments.

S. No.	Instrument Name	Supplier
1	RP-HPLC System with UV/PDA Detector	Shimadzu Corporation, Mumbai
2	Analytical Balance	Sartorius, Mumbai
3	pH Meter	Eutech Instruments, Mumbai
4	Ultrasonicator	PCI Analytics, Mumbai
5	Hot Air Oven	Thermolab Instruments, Mumbai
6	UV-Visible Spectrophotometer	Shimadzu Corporation, Mumbai
7	Digital Melting Point Apparatus	Veego Instruments, Mumbai
8	FTIR Spectrophotometer	Bruker India, Mumbai
9	Stability Chamber	Thermolab Scientific, Mumbai
10	Water Bath	Remi Equipment, Mumbai
11	Photostability Chamber	Thermolab Scientific, Mumbai
12	LC–MS	Waters India Pvt. Ltd., Mumbai

2. METHODS

1. *Preformulation Studies*

1. Scanning of Absorbance Maxima (λmax) of Empagliflozin

The absorption maximum (λmax) of Empagliflozin will be determined using a UV-Visible spectrophotometer. A stock solution (100 µg/mL) will be prepared by dissolving 10 mg of Empagliflozin in 100 mL of methanol. From this stock solution, a working solution of 10 µg/mL will be prepared by appropriate dilution with methanol.

The solution will be scanned in the wavelength range of 200–400 nm against methanol as blank. The wavelength corresponding to maximum absorbance will be identified and recorded as λmax.

This wavelength will be used for further quantitative analysis.

2. *Calibration Curve of Empagliflozin*

A standard calibration curve will be constructed to determine the linearity of the analytical method. The stock solution (100 µg/mL) will be prepared in methanol. Serial dilutions will be made to obtain concentrations of 2, 4, 6, 8, 10, 12, 14, and 16 µg/mL. The absorbance of each solution will be measured at the predetermined λmax using methanol as blank. A calibration curve will be plotted with concentration on the x-axis and absorbance on the y-axis. The regression equation and correlation coefficient (r^2) will be calculated to confirm linearity.

3. *Solubility Studies*

The solubility of Empagliflozin will be determined in distilled water, methanol, acetonitrile, phosphate buffer pH 6.8, phosphate buffer pH 7.4, and 0.1N HCl (pH 1.2) using the shake-flask method. Excess amount of drug will be added to 10 mL of each solvent in stoppered conical flasks and shaken at 37 ± 0.5°C for 24 hours. After equilibrium, samples will be filtered through a 0.45 µm membrane filter. The filtrate will be suitably diluted and analyzed at λmax using UV spectrophotometer. Solubility will be expressed in mg/mL.

4. *Melting Point Determination*

The melting point of Empagliflozin will be determined using a digital melting point apparatus by capillary tube method. Finely powdered drug will be filled in a sealed capillary tube and heated gradually at 1°C per minute. The temperature at which the drug begins to melt and completely melts.

2. *Development of RP-HPLC Method*

Chromatographic analysis will be performed using an RP-HPLC system equipped with UV/PDA detector. Separation will be achieved using a C18 column (250 mm × 4.6 mm, 5 µm particle size).

The mobile phase will consist of acetonitrile and phosphate buffer (pH adjusted with orthophosphoric acid) in optimized ratio (e.g., 60:40 v/v). The flow rate will be maintained at 1.0 mL/min. Injection volume will be 20 µL, and detection will be carried out at selected wavelength.

The method will be optimized to obtain sharp peak, good resolution, and acceptable retention time.

3. *Validation of RP-HPLC Method*

The developed method will be validated according to ICH guidelines for: Linearity

- Accuracy (Recovery studies at 80%, 100%, 120%)
- Precision (Intra-day and Inter-day)
- Specificity
- Robustness (small variation in flow rate and pH)
- Limit of Detection (LOD)
- Limit of Quantification (LOQ)

2. *Forced Degradation Studies*

Forced degradation studies of Empagliflozin will be performed to evaluate intrinsic stability of the drug substance and to establish the stability-indicating nature of the developed RP-HPLC method. The studies will be conducted in accordance with ICH Q1A (R2) and Q1B guidelines. The objective of stress testing is to generate possible degradation products under various stress conditions including hydrolytic (acidic and alkaline), oxidative, thermal, and photolytic degradation.

1. *Preparation of Standard Stock Solution*

A stock solution of Empagliflozin (1000 µg/mL) will be prepared by dissolving accurately weighed 10 mg of drug in 10 mL methanol. This stock solution will be used for all degradation studies.

2. *Acidic Degradation*

An aliquot of stock solution will be mixed with equal volume of 0.1N HCl and kept at room temperature for 24 hours. The mixture may also be refluxed at 60°C for accelerated degradation.

After the specified time, the solution will be neutralized using 0.1N NaOH. The resulting solution will be diluted with mobile phase to suitable concentration (e.g., 10 µg/mL), filtered through 0.45 µm membrane filter, and injected into RP-HPLC system. Percentage degradation will be calculated by comparing peak area with untreated standard.

3. *Alkaline Degradation*

Stock solution will be treated with equal volume of 0.1N NaOH and kept for 24 hours at room temperature. For enhanced degradation, the solution may be heated at 60°C. After degradation, the solution will be neutralized with 0.1N HCl, diluted appropriately, filtered, and analyzed by RP-HPLC. Alkaline degradation is expected to be significant due to possible cleavage of glycosidic linkage.[3]

4. *Oxidative Degradation*

The drug solution will be treated with 3% hydrogen peroxide (H_2O_2) and kept for 24 hours at room temperature. After completion of degradation period, the sample will be diluted with mobile phase and analyzed by HPLC. Oxidative degradation may lead to formation of additional impurity peaks due to oxidation of aromatic rings.

5. *Thermal Degradation*

Accurately weighed solid drug will be placed in petri dish and kept in hot air oven at 60°C for 24–48 hours. After exposure, the sample will be dissolved in methanol, filtered.

6. *Photolytic Degradation*

Drug sample will be exposed to UV light (as per ICH Q1B guidelines) in photostability chamber for specified duration.

After exposure, the sample will be dissolved, filtered, and injected into HPLC system. All chromatograms will be evaluated for:

Peak purity

Retention time shift

Appearance of new degradant peaks

Percentage degradation

9.2.5 Degradation Kinetics Study

To understand the rate and mechanism of degradation, kinetic studies will be performed under selected stress conditions (usually alkaline or thermal condition where significant degradation is observed).

1. *Experimental Procedure*

Drug solution will be subjected to degradation at three different temperatures such as:

- 40°C
- 50°C
- 60°C

Samples will be withdrawn at predetermined time intervals (0, 1, 2, 4, 6, 8, 12, 24 hours).

Each sample will be neutralized (if required), diluted, filtered, and analyzed by RP-HPLC. Remaining drug concentration will be calculated from calibration curve.

9.2.5.2 Determination of Order of Reaction

To determine degradation kinetics, following plots will be constructed:

- Zero-order plot: Concentration vs Time
- First-order plot: Log concentration vs Time
- Second-order plot (if required)
- The plot showing best linearity (highest r^2 value) will indicate order of reaction.

3. *Calculation of Rate Constant (k)*

From slope of appropriate linear plot, degradation rate constant (k) will be calculated. For first-order reaction:

$k = 2.303 \times slope$

4. *Arrhenius Plot and Activation Energy*

An Arrhenius plot will be constructed by plotting log k versus 1/T (Kelvin).

From slope of Arrhenius plot, activation energy (Ea) will be calculated using equation:

$$\log k = \log A - Ea / 2.303RT$$

Where:
Ea = Activation energy
R = Gas constant
T = Absolute temperature

5. ***Shelf-Life Determination***

Shelf-life (t90) will be calculated using first-order kinetic equation: t90 = 0.105 / k

This will help predict long-term stability of drug.

6. ***In-Silico Toxicity and ADMET Profiling***

To evaluate safety of degradation products, computational toxicity prediction will be performed.

1. Structure Drawing

Chemical structures of identified degradants will be drawn using chemical drawing software (e.g., ChemDraw). Structures will be converted into SMILES format for computational input.

2. ***ADMET Analysis***

In-silico tools such as SwissADME and pkCSM will be used to predict:

- Absorption (GI absorption, water solubility)
- Distribution (BBB permeability, plasma protein binding)
- Metabolism (CYP enzyme interaction)
- Excretion (total clearance)
- Toxicity (hepatotoxicity, mutagenicity, carcinogenicity)

3. ***Drug-Likeness Evaluation***

Lipinski's Rule of Five parameters will be evaluated:

- Molecular weight
- Log P
- Hydrogen bond donors
- Hydrogen bond acceptors
- Comparison will be made between parent drug and degradants.

4. ***Toxicity Risk Assessment***

Predicted toxicity parameters such as:

- AMES toxicity
- Hepatotoxicity
- LD50 value
- Will be analyzed to determine potential safety risks of Degradants.

5. ***Data Interpretation***

All ADMET results will be tabulated and compared with parent drug. Degradants showing higher toxicity risk will be highlighted for regulatory consideration.

6. DOES OUR STUDY REQUIRE ANY INVESTIGATION INTERVATION TO BE CONDUCTED ON PATIENT OR OTHER HUMAN OR ANIMALS?

NO.

7. HAS ETHICAL CLEARANCE BEEN OBTAINED FROM YOUR INSTITUTION IN CASE OF 10

NO.

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