STUDY ON DEGRADATION BEHAVIOR AND IMPURITY PROFILING OF BULK DRUGS AND THEIR FORMULATION OF SOME SELECTED ANTI-DIABETIC DRUGS

A Thesis submitted to Gujarat Technological University

for the Award of

Doctor of Philosophy

in

Pharmacy

By

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under supervision of

Dr. Suresh Kumar

GUJARAT TECHNOLOGICAL UNIVERSITY
AHMEDABAD

[September–2024]

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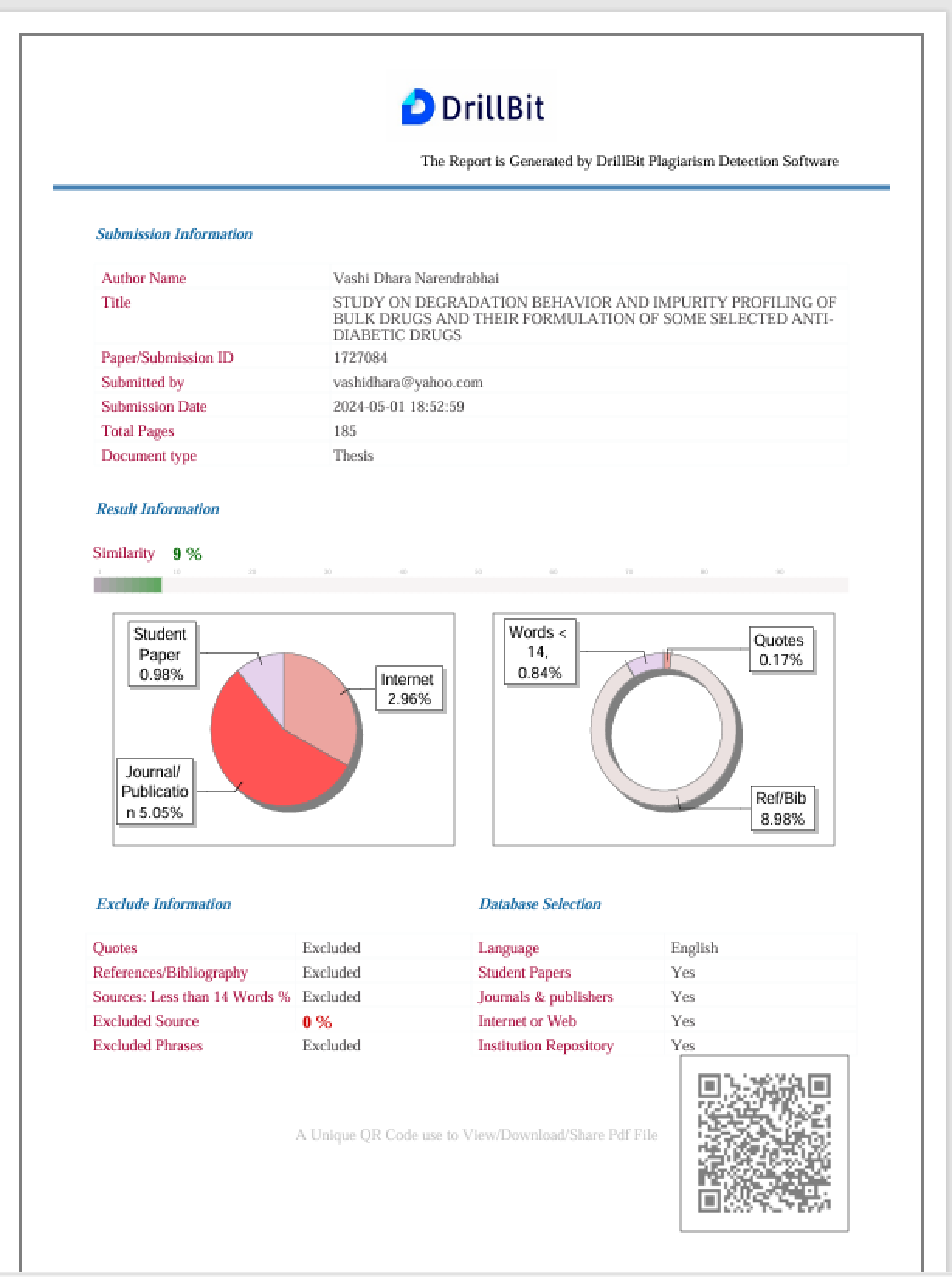

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ABSTRACT

A simple, precise, accurate, specific, linear, rugged and robust method was developed and validated for the estimation of degradation impurities of Empagliflozin and Dapagliflozin in API and tablet formulation. Chromatographic conditions were used as Stationary phase YMC ODS A C-18 (150mm x 4.6mm), Mobile phase Acetonitrile: Water in the ratio of 50:50 and flow rate were maintained at 0.5 ml/min and 0.7 ml/min, detection wave length was 224 nm and 273 nm for Empagliflozin and Dapagliflozin, respectively. Column temperature was set to 35ºC and diluent was mobile phase. Validation was performed according to ICH Guidelines Q2(R1).The degradation study of both the drugs under stressed conditions was examined following ICH guidelines Q1(R2).Empagliflozin and Dapagliflozin were subjected to oxidative, acidic, alkaline, neutral, photolytic, and thermolytic degradation conditions. The drug was stable in Oxidative, thermal, and photolytic conditions, and no degradation products were observed. For Empagliflozin two degradation products were formed in acid (DP-1,RT:2.28 min) and Alkali stress hydrolysis conditions (DP-2, RT:2.25 min) and for Dapagliflozin two degradation products formed in acid (DP-1,RT: 6.90 min) and Alkali stress hydrolysis conditions (DP-2, RT: 2.3 min). All the degradation Impurities were observed and well separated in same developed method. Unknown impurity formed during stability studies was isolated using preparative HPLC and structure was characterized by ^{1}H NMR, FTIR and Mass spectroscopy studies. Empagliflozin and Dapagliflozin with its tablet formulation were more sensitive towards acid and Alkali degradation. Total two impurities were detected in acidic and alkali condition for Empagliflozin, based on data chemical structure of DP 1 was assumed as (1S,2S,3R,4R,5S)-1-(3-((S)-4-((S)-tetrahydrofuran-3-yloxy) benzyl)-4-chlorophenyl)hexane-1,2,3,4,5,6-hexaol and DP 2 was (2S,3S,4R,5R, 6S)-6-(3-(4-(tetrahydrofuran-3-yloxy) benzyl) -4-chlorophenyl) -3,4,5-trihydroxy -tetrahydro -2H-pyran-2-carbaldehyde. For Dapagliflozin acid degradation impurity DP1 was (2R,3S,4R,5R,6S)-6-(3-(4-ethoxybenzyl)-4-chlorophenyl)-3,4,5-trihydroxy-tetrahydro-2H-pyran-2-yl) methyl acetate and DP2 was 2R,3S,4R,5S)-1-(3-(4-ethoxybenzyl)-4-chlorophenyl)-2,3,4,5,6-pentahydroxyhexan-1-one. This method was specific as no interference was observed because of excipients and degradation product.

Dedicated to the god,
Who empowers me for doing the best
My family and Friends,
Whose affection and Love are infinite,
Special Thanks to my Late father, in loving Memory

ACKNOWLEGMENT

I offer flowers of gratitude to the Almighty GOD who has been the source of strength in my life and blessed me with the courage to ladder the success. I pay reverence to the omniscient, omnipresent, omnipotent, The God who has perpetually patronized me with the contentiousness and love. This thesis is the end of my journey in obtaining my Ph.D degree, but it's also starting a new journey in this real competitive world. I have not travelled in a vacuum in this journey. This thesis has been kept on track and been seen through to completion with the support and encouragement of numerous people including my well-wishers, my friends, family, colleagues and various institutions. At the point of successful completion of my thesis I would like to thank all those people who made this thesis possible and an unforgettable experience for me. At the end of my thesis, it is a pleasant task to express my thanks to all those who contributed in many ways to the success of this study and made it an unforgettable experience for me. I wholeheartedly take this opportunity to place on record my profound gratitude to my respected parents who encouraged me to enlarge my knowledge, never give up and do new experiences. This research project would not have been possible without the support of my parents. I want to dedicate this thesis to my parents ***Late Narendrabhai Vashi*** *and* ***Mrs. Geetaben Vashi*** *because they made my ambitions possible, providing me all I needed. They always show their faith in me and always fulfills my requirements. I would like to thank my husband* ***Mr. Mitul Desai*** *and my son* ***Krishiv*** *who motivated me and boosted my morale when I was stressed. Without their unconditional love and support this project would not have been possible. A special thank also to my Brother* ***Mr. Dhruvin Vashi*** *and Sister in- Law* ***Mrs. Divya Vashi*** *and my son* ***Krishiv*** *who made me feel loved and never alone. Thank for the examples and the advices you gave to me become successful person.*

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Vashi Dhara N.

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ABBREVIATIONS

Abbreviation	**Description**
HBr	Hydrobromide hydrate
% RSD	Relative Standard Deviation
µg	Microgram
µl	Microliter
µm	Micrometre
cm	Centimetre
FTIR	Fourier Transform Infrared Spectroscopy
gm	Gram
HCl	Hydrochloric Acid
HPLC	High Performance Liquid Chromatography
HPTLC	High Performance Thin Layer Chromatography
GC	Gas Chromatography
i.d.	Internal Diameter
IP	Indian Pharmacopoeia
IR	Infrared
IUPAC	International Union of Pure and Applied Chemistry
ICH	International Conference for Harmonization
LC	Liquid Chromatography
LC-MS	Liquid Chromatography-Mass Spectroscopy
LOD	Limit of Detection
LOQ	Limit of Quantification
mg	Milligram
ml	Millilitre
mm	Millimetre
NaOH	Sodium Hydroxide
H_2O_2	Hydrogen Peroxide
nm	Nanometre
NP/RP	Normal Phase / Reverse Phase
^0C	Degree Celsius

R^2	Correlation Coefficient
SD	Standard Deviation
TEA	Triethyl Amine
GAA	Glacial Acetic Acid
TLC	Thin Layer Chromatography
USP	United State Pharmacopoeia
UV	Ultra Violet
EP	European Pharmacopoeia
WHO	World Health Organization
ACN	Acetonitrile
Abs	Absorbance
Conc.	Concentration
Df	Degree of Freedom
FDA	Food and Drug Administration

CHAPTER 1

INTRODUCTION

1. INTRODUCTION

1.1 History of Dyslipidaemia associated with type 2-Diabetes Mellitus

Since ancient times, diabetes has had a negative impact on human life. In texts from around 1550 B.C., the Egyptians recorded a disease that is thought to be diabetes. The condition was well known to the ancient Indians (approximately 400–500 A.D.), who had even distinguished between two varieties of it. They looked studied whether ants were drawn to a person's urine as a diagnostic for diabetes, which they termed "honey urine." [1] Diabetes is an "iceberg disease" that affects at least 61.4 million people in India and 347 million people worldwide, with a prevalence of 10% of which 90% is type 2 diabetes. [2] 1.2 million people died in 2008 as a result of excessive blood sugar. The most prevalent kind of Diabetes, Type 2 Diabetes Mellitus, is characterized by either an insulin shortage, insulin resistance, or both. Obesity, which frequently coexists with Type 2 Diabetes Mellitus, is another condition that exhibits insulin resistance. The metabolism of lipids and lipoproteins, which is changed in diabetes and may result in Dyslipidaemia, is significantly influenced by insulin. [3] Twenty years ago, type 2 Diabetes in children was not known to exist. In fact, type 1 diabetes was originally referred to as "Juvenile Diabetes" and it was called "adult-onset Diabetes." However, because of bad eating practices, inactivity, and excess weight, more cases started to occur in kids and teenagers in the last 20 years. As a result, "type 2 Diabetes" was coined for adult-onset diabetes.

1.2 Physiology behind Heart Disease and Cholesterol

To understand cholesterol, heart disease, and Diabetes and potential linkages, take a look at some of the physiology behind all this. Your heart's principal function is to circulate oxygen-rich blood throughout your body. Blood is returned to your heart through veins after being pumped to the body through arteries. Plaque can accumulate when an artery has been damaged. [4] One of the many substances that make up this plaque and circulate in the blood is cholesterol, a waxy substance made by the liver. It becomes more difficult for blood to flow through an artery as it should when plaques get thicker and clog more and more of the vessel. Extra blood sugar complicates this process even more by making the blood more viscous and challenging to pump Additionally, one of the major contributors to that first artery damage can be too much sugar in the circulation (because excess sugar causes inflammation). Significant heart surgery is required. [5]

1.2.1 Cholesterol

The aforementioned justification can lead one to believe that cholesterol is "bad." That is inaccurate. Every cell contains cholesterol, which is essential for maintaining the structure of the cell and for proper brain function. Additionally, it is necessary for the production of several vitamins and hormones, including as vitamin D, testosterone, estrogen, and cortisol. Very low cholesterol has been associated with a higher risk of dying, as well as neurological diseases like poor memory and Parkinson's and emotional issues including depression, frustration, and suicide (ironically often from heart disease).

The "Lipoproteins" that carry cholesterol through the blood are proteins. Two different kinds of lipoproteins transport cholesterol all over the body:

- LDL (low-density lipoprotein), sometimes called “bad” cholesterol, makes up most of your body’s cholesterol. High levels of LDL cholesterol raise your risk for heart disease and stroke. “Small dense” – which are harmful

- HDL (high-density lipoprotein), or “good” cholesterol, absorbs cholesterol and carries it back to the liver. The liver then flushes it from the body. High levels of HDL cholesterol can lower your risk for heart disease and stroke. “large fluffy” – which are not problematic and may be protective

Diabetic Dyslipidaemia: is a term that refers to alteration of blood lipids, or in other words, changes to your cholesterol levels. In Dyslipidaemia type 2-Diabetes Mellitus Excess insulin and high blood sugar levels increase fatty acid synthesis in the liver, which means your body produces more cholesterol, triglycerides and fatty acids. This does not occur from foods that contain cholesterol, it occurs due to high blood sugar, high insulin and hormonal changes.

1.3 Introduction of Diabetes mellitus

Diabetes mellitus, also called Diabetes, is a term for several conditions involving how your body turns food into energy. When you eat a carbohydrate, your body converts it into sugar glucose and releases it into your bloodstream. Insulin, a hormone released by the pancreas, helps in the transfer of blood glucose into your cells, which use it as an energy source.[6] An absolute or relative shortfall in insulin synthesis or action results in Hyperglycaemia, which is the characteristic of the heterogeneous group of illnesses referred to as Diabetes Mellitus, the retina, kidney, neurological system, heart, and blood vessels are just a few of the end organs that are harmed, dysfunction, or fail as a result of the chronic Hyperglycaemia of Diabetes Mellitus.[7]

1.3.1 Types of Diabetes Mellitus

- **Type 1 Diabetes Mellitus:** A person with this condition must inject insulin since their body is unable to create insulin on its own. In the past, this condition was known as juvenile Diabetes or Insulin-Dependent Diabetes Mellitus (IDDM).
- **Type 2 Diabetes Mellitus**: It is caused by insulin resistance, a disorder in which cells improperly utilize insulin, which is occasionally accompanied by a complete lack of insulin. Previously, this type was referred to as "adult-onset Diabetes" or "non-insulin-dependent Diabetes Mellitus."

Before biochemical hyperglycaemia develops in type 2 Diabetes, there is frequently a prolonged period of increased cardiovascular risk. During this time, obesity and insulin resistance are frequently present, together with hypertension and dyslipidaemia, and this condition is known as metabolic syndrome. In type 2 diabetes, is a disorder in body's ability to control and utilize sugar (glucose) as fuel[8]. This chronic (long-term) disorder causes the bloodstream to circulate with an excessive amount of sugar. Over time, cardiovascular, neurological, and immune system issues might result from excessive blood sugar levels.

- **Gestational Diabetes:** Third major type, develops in pregnant women who haven't previously been diagnosed with diabetes and has high blood sugar levels. It might result in type 2 DM.
- **Other types of Diabetes include those caused by:**
  Mature-Onset Diabetes of the Young (MODY) and neonatal Diabetes Mellitus are two examples of genetic disorders of the beta cells, the portion of the pancreas that produces insulin (NDM) Pancreatic disorders or illnesses that harm the pancreas, such as cystic fibrosis and pancreatitis excess hormone production brought on by some medical diseases, such as Cushing's syndrome's cortisol, which interferes with insulin's ability to do its job drugs like glucocorticoids that inhibit the function of insulin or substances that damage beta cells[9].

1.3.2 Sign and Symptoms of Type 2 Diabetes Mellitus

People can frequently have diabetes without being aware of it. The main reason for this is that the symptoms at first seem normal. However, the earlier diabetes is identified, the more likely it is that serious problems, which can be caused by having diabetes, can be avoided Increasing thirst.[10]

- Often urinating
- Increased appetite
- Unwanted loss of weight
- Fatigue
- Blurred vision
- Slowly heaving wounds
- Many infections
- Tingling or numbness in the hands or feet
- Areas of skin that have browned, typically in the neck and armpits

Figure No: 1.1 Symptoms of diabetes

1.3.3 Causes of Type 2 Diabetes Mellitus

Insulin resistance develops in the liver, muscle, and fat cells. These cells don't absorb enough sugar because insulin doesn't interact with them normally. A sufficient amount of insulin cannot be produced by the pancreas to control blood sugar levels. Although the exact cause of this is uncertain, being overweight and being sedentary are major risk factors.

1.3.4 Risk factors

Type 2 Diabetes risk factors include the following: [11]

- **Excess Weight:** Being obese or overweight significantly increases the risk of developing type 2 diabetes.
- **Fat Distribution:** A higher risk is associated with abdominal fat accumulation compared to fat stored in the hips and thighs. Men with a waist measurement over 40 inches (101.6 centimetres) and women with a measurement over 35 inches (88.9 centimetres) have an increased risk.
- **Lack of Physical Activity: Risk** increases with decreased physical activity levels. Exercise helps manage weight, utilizes glucose as fuel, and enhances insulin sensitivity.
- **Family History:** Having a parent or sibling with type 2 diabetes increases the risk.
- **Ethnicity and Race:** Some races and ethnicities, such as Black, Hispanic, Native Americans, Asians, and Pacific Islanders, are more prone to developing type 2 diabetes compared to white individuals.
- **Blood Lipid Levels:** Low levels of high-density lipoprotein (HDL) cholesterol (the "good" cholesterol) and high levels of triglycerides are linked to an increased risk.
- **Age:** Risk increases with age, especially after 35 years old.
- Prediabetes: Having a blood sugar level above normal but not high enough for a diabetes diagnosis indicates prediabetes.
- **Pregnancy-related Risks:** Women who had gestational diabetes or gave birth to a baby weighing more than 9 pounds (4 kilograms) are at higher risk.
- **Polycystic Ovary Syndrome (PCOS):** Those with PCOS, a common disorder characterized by irregular menstrual cycles, excessive hair growth, and obesity, have a higher risk[12].
- **Skin Darkening:** Darkened skin, especially around the neck and armpits, may indicate insulin resistance, which is linked to an increased risk of diabetes.

1.3.5 Pathophysiology of Type 2 Diabetes Mellitus

Diabetes Mellitus includes two basic subgroups, and each has a specific pathology[13]

T1DM: Genes play a significant role in the immune system's mistaken attack on pancreatic cells in Type I Diabetes Mellitus (T1DM).

T2DM: Genetic and lifestyle factors strongly interact with Type II Diabetes Mellitus. (T2DM) Obesity or being overweight increases the risks.

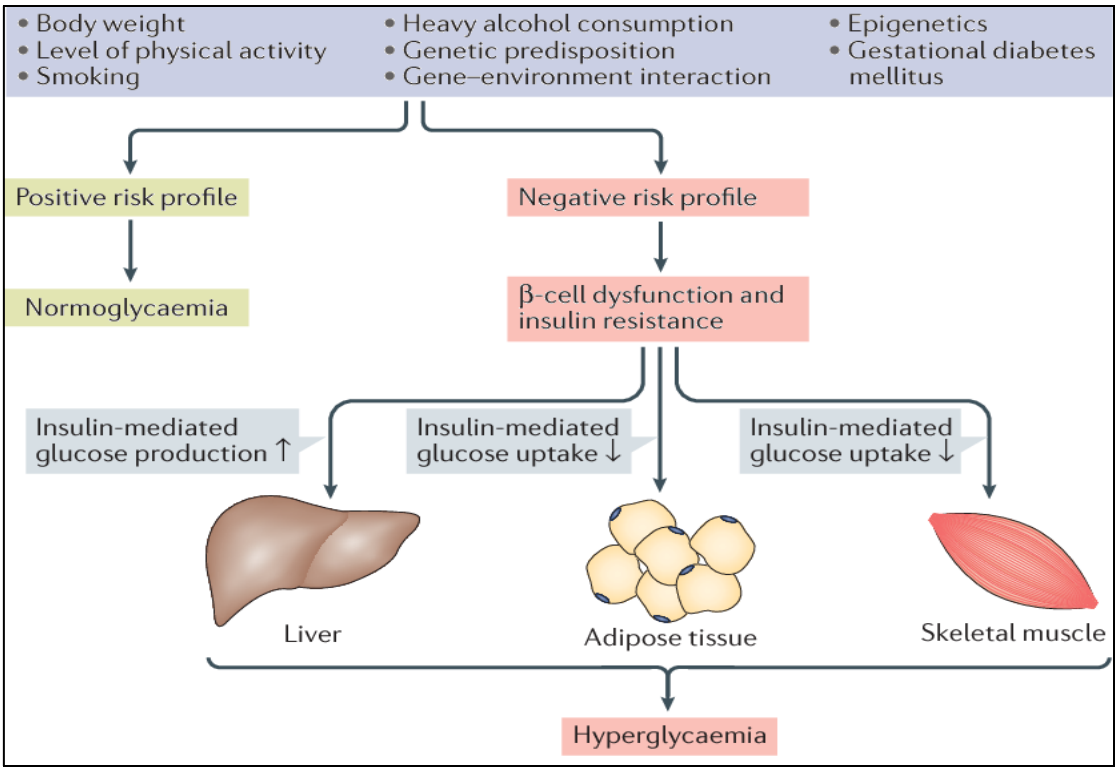


Figure No: 1.2 Pathophysiology of T2DM

The most significant hormone, insulin, is typically the cause of this type of health issue. It is clear that the body stops producing the required amount of insulin. The issue of beta cell insufficiency is also linked to insulin resistance. Peripheral insulin resistance predicts that hypoglycaemia or low blood sugar issues won't develop even if the average amount of blood containing insulin increases. Numerous adjustments to insulin receptors may alter the actions of insulin, which could account for this effect[14]. In the Pathogenesis of Type 2 Diabetes, obesity is one of the main issues connected to insulin resistance. Patients are only ever advised to take insulin in a very small number of situations, and only when other treatments have failed to provide the intended effects due to oral medications' failure to create the required levels of insulin.[11]

1.3.6 Diagnosis Test of T2DM

- **Glycated Haemoglobin (A1C) test**: The average blood sugar level over the previous two to three months is shown by this blood test. This is how the results are interpreted: Normal is 5.7% or less
    - Prediabetes has a diagnosis rate of 5.7% to 6.4%.
    - Diabetes is diagnosed when two tests show a 6.5% or higher level.
- **Random blood sugar test:** Millimoles of sugar per litre (mmol/L) of blood or milligram of sugar per deciliter (mg/dL) are used to express blood sugar levels. No matter when you last ate, a level of 200 mg/dL (11.1 mmol/L) or above indicates diabetes, particularly if you also exhibit symptoms of diabetes like severe thirst and frequent urination[15].
- **Fasting blood sugar test:** After fasting for the previous night, blood is drawn. This is how the results are interpreted:
    - Normal ranges are below 100 mg/dL (5.6 mmol/L).
    - Prediabetes is defined as a blood sugar level of 100 to 125 mg/dL (5.6 to 6.9 mmol/L).
    - Diabetes is identified when two different tests show 126 mg/dL (7 mmol/L) or above.
- **Oral glucose tolerance test:** Except for when a woman is pregnant, this test is less frequently utilised than the others. You must abstain from food for the entire night before drinking a sweet beverage in the doctor's office. For the following two hours, blood sugar levels are checked at regular intervals. This is how the results are interpreted:
    - A normal level is less than 140 mg/dL (7.8 mmol/L).
    - Prediabetes is defined as blood sugar levels between 140 and 199 mg/dL (7.8 and 11.0 mmol/L).
    - After two hours, 200 mg/dL (11.1 mmol/L) or more is indicative of diabetes.
- **Screening:** All person’s age 35 and older, as well as the following groups, should undergo routine testing for type 2 diabetes with diagnostic tests:
    - Individuals under 35 who are overweight or obese and have one or more diabetes risk factors
    - Pregnant women with gestational diabetes
    - Those with prediabetes who have received a diagnosis

- Children who are overweight or obese, have a history of type 2 diabetes in their family, or who otherwise have a risk.[16]

1.4 Classification

Table No: 1.1 Classification of Drugs used in Diabetes Mellitus[17]

Sulfonylureas	Glyburide Glipizide Glimepiride
Rapid-acting prandial insulin releasers	Prandin Nateglinide
Biguanides	Metformin
Thiazolidinediones	Pioglitazone Rosiglitazone
Alpha-glucosidase inhibitors	Acarbose Miglitol
Dipeptidyl peptidase-4 inhibitors (DPP-4)	Sitagliptin Saxagliptin Linagliptin Alogliptin Vildagliptin
Glucagon-like peptide1 agonists (GLP-1)	Exenatide Liraglutide Exenatide ER Albiglutide Dulaglutide
Sodium-glucose co-transporter 2 inhibitors (SGLT2)	Canagliflozin Dapagliflozin Empagliflozin Ertugliflozin Remogliflozin

1.5 Mechanism of action

1.5.1 Mechanism of action of Dapagliflozin:

Dapagliflozin is a recently developed medicine used to treat early and late type 2 diabetes. Dapagliflozin is a selective sodium glucose cotransporter or sodium-glucose linked transporter (SGLT) agent. It increases urine glucose excretion and inhibits glucose reabsorption in the kidney[18]. All glycaemic indicators develop as a result of glucose excretion and falling plasma levels.

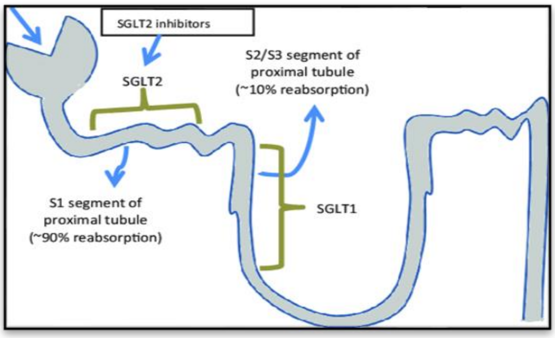


Figure No: 1.3 Mechanism of Dapagliflozin

This method of action, which is unrelated to the effects of insulin, depends on blood glucose levels as well as other thiazolidinedione actions (mediated by GLUTs). As a result, there is little chance of hypoglycaemia and little danger of beta cells becoming overstimulated or worn out. Because its mechanism of action is dependent on normal renal glomerular-tubular function, the effectiveness of SGLT-2 is reduced in those with renal impairment.[19]

1.5.2 Mechanism of action of Empagliflozin:

Empagliflozin has a unique mechanism of action by inhibiting glucose and sodium reabsorption in the proximal tubule of the kidney, they promote urinary glucose excretion and natriuresis. In diabetes, these effects cause glucose lowering, BP reduction, and weight loss. [20] The mechanism of action of other glucose lowering agents usually involves an increase in insulin secretion (i.e., sulfonylureas, and glucagon-like peptide-1 receptor agonists) or decrease in insulin resistance (i.e., metformin, and thiazolidinediones), which develops in either suppression of hepatic glucose production or increased tissue glucose uptake. [21] In contrast, empagliflozin promote urinary glucose and sodium disposal, which represents a well-defined and new metabolic mechanism of action. The mechanism of action is unique for empagliflozin and potentially used as an adjunct therapy to lower glucose levels when used with other glucose lowering therapies. [22]

1.6 Marketed Formulations

1.6.1 Empagliflozin Marketed Formulation

Manufactured and marketed by: Boehringer Ingelheim india Pvt Ltd, Maharashtra

Brand Name: Jardiance

Label Claim: Each Tablet Contains:

Empagliflozin……25 mg

Excipients………...Q.S

Figure No: 1.4 Marketed Formulation of Empagliflozin

1.6.2 Dapagliflozin Marketed Formulation

Manufactured and marketed by: Intas Pharmaceuticals Ltd.

Brand Name: DAPARYL 10

Label Claim: Each Tablet Contains:

Dapagliflozin ……10 mg

Excipients………...Q.S

Figure No: 1.5 Marketed Formulation of Dapagliflozin

1.7 Introduction to Analytical methods

1.7.1 Definition of Analytical Method:

- Analytical methods are used to determine the amounts of chemical elements or chemical compounds. There are many different analysis techniques, from straightforward weight analysis (gravimetric analysis) to extremely complex techniques using highly specialised gear from titrations (Titrimetric). The study of pharmaceutical analysis has advanced quickly with the passage of time and the application of sophisticated tools. [23] It is essential to provide simple analytical processes for challenging compositions**.**
- However, because of the following factors, it is highly challenging to establish an accurate analytical procedure for each component of a complicated dose formulation.
- Substances that are therapeutically and chemically compatible and have extremely identical chemical natures are included in combination dose forms.
- They are present in the formulation at widely different quantities, depending on their relative efficacy and the patient's therapeutic demands;
- They contain additives, excipients, and breakdown products, which makes it considerably more complex to develop the analytical technique[24].

1.7.1.1 Type of Analytical methods

Instrumental methods of chemical analysis are currently the foundation of experimental chemistry. Figure classifies the several methods for performing a pharmaceutical analysis.

The advantages of employing instrumental techniques over chemical ones include the following:

- It is more dependable,
- Very quick,
- Accurate, and precise,
- It can analyse complex mixtures
- Analyse with multiple components
- A small sample size.
- It is widely used in research and quality control,

- It can be used to study drugs and their metabolites in vivo and in vitro.
- It can also be used to create new drug molecules.

Table No: 1.2 Types of Analytical Methods

Instrumental Method	Property used	Application
UV-Visible Spectrophotometer	Absorption of radiation	Identification of Functional group and Quantitation of unsaturated Compound
FTIR Spectroscopy	Absorption of radiation	Quantitative Analysis if organic Compound at high Concentration Level
Atomic Absorption	Absorption of radiation	Quantitation of Metals or Metalloids
Flame Photometry	Emission of radiation	Quantitation of Alkaline metal or Alkaline earth Metal
X-ray Diffraction	Diffraction of radiation	Identification of Crystal lattice structure, Determination of percent of crystallinity in Polymers
Nuclear Magnetic Resonance (NMR)	Nuclear spin energy level of a Molecule in an applied magnetic field	Identifies type of hydrogen and carbon in organic molecule. Analysis of trace Impurity and Degradants
Thermal Analysis (DTA/DSC)	Difference in Temperature/Heat Energy	Determination of Melting point and polymorphism, Drug-Excipients Compatibility
Mass Spectroscopy LC-MS/GC-MS	Mass to Charge Ratio	Molecular weight determination, quantification of Analyte in Liquid or Gas sample. Analysis of Trace impurity and degradants

1.7.1.2. Selection of Analytical methods

A critical first step in every quantitative inquiry is the technique selection. For this, it will be crucial to carefully consider the following factors: [24]

1. The type of analysis required.

2. Problems resulting from the nature of the subject of the research.
3. The potential for interruption from uninteresting sections of the subject matter.
4. The concentration range needs to be considered.
5. The need of precision.
6. The amenities provided.
7. The time required to complete the analysis.
8. The number of such analyses that must be performed.

1.7.2 Analytical Method Development

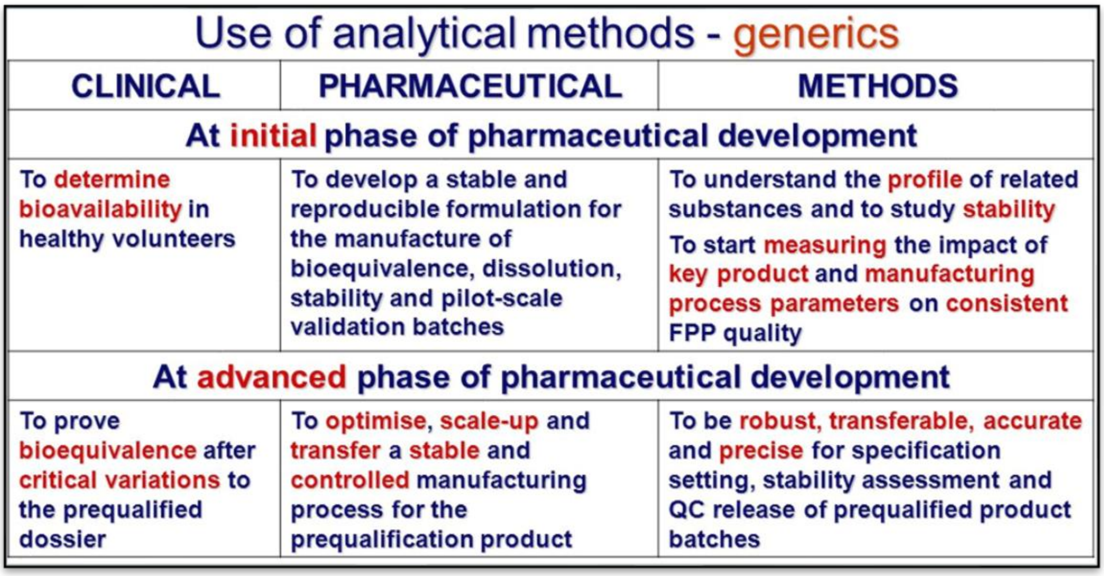
Each year, a greater number of drugs are introduced to the market. These drugs may be brand-new creations or structurally altered variations of already available drugs. The dates of a drug's release onto the market and when it is added to pharmacopoeias commonly coincide. This is a result of possible hazards related to the prolonged and widespread use of these drugs, reports of novel toxicities that forced their recall from the market, the rise of patient resistance, and the introduction of superior drugs by competitors. In certain cases, the pharmacopoeias may not contain standards and analytical methods for specific drugs[25]. It becomes necessary to develop improved analytical methods for such drugs as a result.

The method validation and assessment procedures imply the procedures for documenting or ensuring that an analytical technique provides analytical results for the intended usage.

For the purpose of validation analytical methods, the following are required.

- Obtaining recognition from international organisations.
- ensuring quality
- Conditions that must be fulfilled before receiving ISO 17025 accreditation.
- Registration is required for all pesticide and medicinal compositions.
- Only when conducting proficiency testing should validation procedures be applied.
- The validated/evaluated method goes through quality control processes before being examined further.

Concurrent with the advancement of the medicinal product, method development is a continuous activity. [26]



Use of analytical methods - generics

CLINICAL	PHARMACEUTICAL	METHODS
At initial phase of pharmaceutical development		
To determine bioavailability in healthy volunteers	To develop a stable and reproducible formulation for the manufacture of bioequivalence, dissolution, stability and pilot-scale validation batches	To understand the profile of related substances and to study stability To start measuring the impact of key product and manufacturing process parameters on consistent FPP quality
At advanced phase of pharmaceutical development		
To prove bioequivalence after critical variations to the prequalified dossier	To optimise, scale-up and transfer a stable and controlled manufacturing process for the prequalification product	To be robust, transferable, accurate and precise for specification setting, stability assessment and QC release of prequalified product batches

Figure No: 1.6 Analytical Methods Development process

1.7.3 Introduction to Drug Analysis

Drug analysis involves testing on raw materials (purity criteria), pharmaceutical or veterinary formulations, and a number of additional, more complex matrices such foods, drinks, and foodstuffs for clinical, forensic, or veterinary applications.[27]A number of matrices, such as blood, urine, and tissues, may be used in these tests. Pharmaceutical evaluations of pharmaceutical formulations and raw ingredients are the sole focus of pharmaceutical analysis; occasionally, clinical evaluations are also included. The detection of impurities, excipients, the stability of active substances (and their intermediates or end products of degradation), as well as other factors including content homogeneity, solubility, and dissolving rate, are all included in pharmaceutical analysis**.**

Some spectrophotometric analyses focus on the properties of the target substance (its natural spectrum), a chemically derived substance, or the results of a sample preparation or separation procedure. Recent advances in chemometrics and derivative spectrophotometry have also made studies of mixtures of components conceivable. Only when the wavelength being used is unaffected by background absorption or interference from other species are direct examinations of target chemicals possible.

1.7.4 Introduction to Chromatography

Chromatography is a method used to separate and identify the different components present in a mixture. While there are various types of chromatography, they all follow the same basic principles. The components of the mixture are separated as the mobile phase moves through the stationary phase, based on their differing affinities for the two phases. Components that are more strongly attracted to the mobile phase will move faster, while those that are more strongly attracted to the stationary phase will be retained.

Proper selection of the stationary and mobile phases is essential for effective separation of the components. The choice of phases determines the speed at which they move; for example, using a polar solvent as the mobile phase will cause polar molecules to move more quickly.

The two primary chromatography types: [28]

- A non-volatile liquid stationary phase supported on an inert solid surface is used in partition chromatography, which is a type of chromatography. The components are split between the two phases according to how soluble they are in relation to one another. Paper chromatography and gas liquid chromatography are two examples. The more soluble or volatile the component, the faster it will move.
- A stationary solid phase and a mobile liquid or gas phase are used in adsorption chromatography, a form of chromatography. While other, lesser elements travel through the mobile phase more quickly, some mixture constituents are drawn to the solid surface. Thin-layer chromatography is one instance. Because the stationary phase is frequently a polar solid, the more polar solutes are more readily adsorbed than the less polar solutes.

1.7.4.1 Thin Layer Chromatography

A thin layer of an adsorbent material, often silica gel, aluminium oxide (alumina), or cellulose, is covered with an inert substrate, such as a sheet of glass, plastic, or aluminium foil, in thin-layer chromatography. This adsorbent layer is referred to as the stationary phase. The sample is deposited on the plate, and a solvent or solvent mixture (referred to as the mobile phase) is transported up the plate by capillary action.[29] Because different analytes ascend the TLC plate at different rates, order is established.

1.7.4.2 Paper Chromatography

Paper chromatography is an analytical method for separating coloured chemicals or molecules. Alternative chromatography methods, such thin-layer chromatography, have taken its place in the laboratory, therefore it is now mostly used as a teaching tool (TLC). In two-dimensional paper chromatography, which rotates the paper 90 degrees between solvents, two solvents are employed. This is useful for sorting out complex chemical combinations having the same polarity, such as amino acids. The configuration consists of three components. The mobile phase is a term used to describe a solution that, as a result of capillary action, enters the stationary phase.

While the mobile phase normally consists of a combination of non-polar organic solvents, the stationary phase typically consists of the polar inorganic solvent water. In this case, paper supports the stationary phase, which is water.[30] Polar water molecules are trapped in the host paper's empty cellulose network. TLC employs a layer of adsorbent (typically silica gel or aluminium oxide) as its stationary phase as opposed to paper chromatography, which uses less absorbent paper as its stationary phase.

1.7.4.3. High Performance Liquid Chromatography

High performance liquid chromatography has significantly improved the idea of column liquid chromatography (HPLC). Instead of being permitted to flow through a column naturally under gravity, a solvent is forced through one at high pressures of up to 400 atmospheres. This makes it considerably quicker. [31] The split of a sample into its constituent parts as a result of differences in the relative affinities of various molecules for the mobile phase and the stationary phase used in the separation is the fundamental principle that underlies all chromatographic separations, including HPLC.

Different mode of Separation

- **Normal Phase HPLC:** This method separates analytes based on polarity. It uses a polar stationary phase and a non-polar mobile phase. Common mobile phases include hexane, methylene chloride, chloroform, diethyl ether, or their mixtures, while silica is typically used as the stationary phase. More polar samples are retained on the polar surface of the column packing.
- **Reverse Phase HPLC:** Here, the stationary phase is nonpolar (hydrophobic), while the mobile phase is polar, such as water-acetonitrile or water-methanol

mixtures. The system relies on hydrophobic interactions, so the more nonpolar the material, the longer it will be retained.

- **Size-Exclusion HPLC:** This technique uses a substance with controlled pore sizes in the column packing. Molecules are separated based on their molecular sizes, with smaller molecules entering the pores and eluting later, while larger molecules pass through the column more quickly.
- **Ion-Exchange HPLC**: In this method, the stationary phase has an ion charge that differs from the ions in the sample. It is used for ionic or ionizable materials. The sample's charge determines how strongly it is attracted to the ionic surface and how long it takes to elute. The pH and ionic strength of the aqueous buffer used as the mobile phase influence the elution process.

Instrumentation:

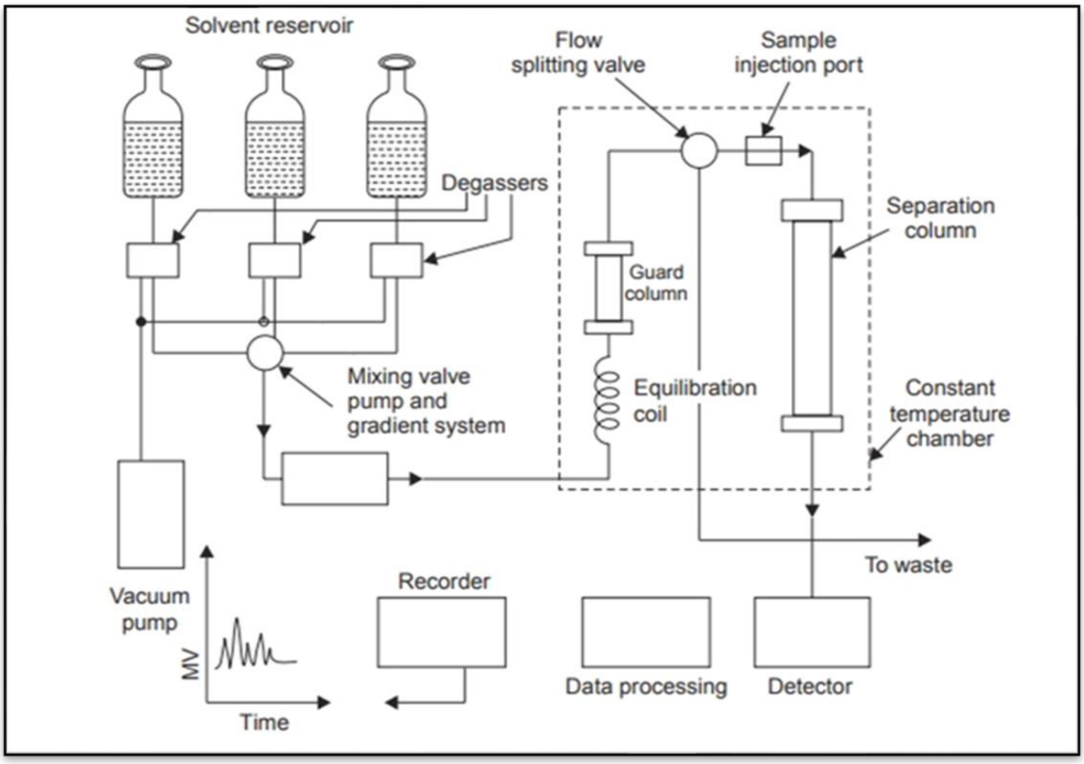


Figure No: 1.7 Instrumentation of HPLC

According to the schematic diagram shown above, an HPLC system consists of several key components, including a pump, injector, column, detector, and integrator or acquisition and display system. The column is the central component where separation takes place.

- **Solvent Reservoir:** This reservoir holds the mobile phase components.
- Mobile Phase: The mobile phase, or solvent, is typically a mixture of polar and nonpolar liquids, with the proportions adjusted based on the sample being analysed.
- **Pump:** The pump draws solvent from the reservoir and pushes it through the column and detector. It can generate high pressures, up to 42000 kPa (about 6000 psi), depending on various factors.
- **Sample Injector:** The injector can be manual or automated and should be capable of injecting liquid samples accurately and reproducibly, typically in volumes between 0.1 and 100 ml.
- **Columns:** Columns are usually made of stainless steel, with internal diameters of 2 to 5 mm and lengths of 50 to 300 mm. They are filled with a stationary phase consisting of 3 to 10 µm particles. Columns with internal diameters less than 2 mm are known as microbore columns. The mobile phase and column temperatures should remain stable during analysis.
- **Detector**: The detector, located at the end of the column, detects analytes as they elute. Various types of detectors, such as UV, fluorescence, mass spectrometric, and electrochemical detectors, are used.
- **Data Collection:** Detector signals are recorded using electronic integrators or chart recorders, with varying levels of complexity and data handling capabilities. Computers can also be used for data collection, providing a chromatogram that is easy to interpret.

Quantitation of HPLC

For measurements of peak height or peak area, just return an answer in terms of detector signal. This response must be related to the target component's concentration or mass. To accomplish, some sort of calibrating work must be done.

The following are the top three quantitation methods:

a) External standard method
b) Internal standard method
c) Method of standard addition

a) **External standard method**:

In order to accomplish accurate quantification using external standards, the chromatographic conditions must be maintained throughout the separation of all standards and samples. A component of the external standard technique is the use of one standard or as many as three solutions. The peak area or height of the sample is directly compared to the applied standard. It is also possible to use the slope of the calibration curve built using standards with known concentrations of the target chemical.

b) **Internal standard method:**

In order to accomplish accurate quantification using external standards, the chromatographic conditions must be maintained throughout the separation of all standards and samples. A component of the external standard technique is the use of one standard or as many as three solutions. The peak area or height of the sample is directly compared to the applied standard. It is also possible to use the slope of the calibration curve built using standards with known concentrations of the target chemical.

- A full standard must be established internally, free from outside influence.
- It must elute somewhat near the intended chemical.
- It must not have been present in the initial sample.
- It ought to be dependable, stable, and unaffected by the mobile phase, column packing, and sample constituents.
- It must behave similarly to the target medication for analytical reasons, including in pretreatments, derivative formulations, etc.
- It must provide a peak area or peak height ratio that is about equivalent to that of the chemical at the concentration at which it is added.
- Selling this chemical in high purity would be ideal.

c) **Method of standard addition**

The standard addition method involves adding a known quantity of a standard chemical to a measured volume of the sample solution to estimate its concentration. This approach is particularly useful when a sufficient amount of sample is available, as it allows for calibration in the presence of excipients or other components. It is essential to maintain the composition and flow rate of the mobile phase constant during analysis. The sample should dissolve completely in the mobile phase. Both the solvent used in the sample solution and the mobile phase must be the same to ensure the accuracy of the analysis.

1.7.5 Introduction of Stability indicating method

Identification of Degradation Pathways: The first step is to carefully examine the drug's structure to identify likely degradation pathways. This includes understanding how the drug may degrade under various conditions, such as oxidation, hydrolysis, or photolysis. [32]

- **Physicochemical Properties:** It is crucial to gather information on the drug's physicochemical properties, including its pKa, log P, solubility, absorptivity, and maximum wavelength. This information helps in selecting appropriate conditions for analysis.
- **Stress Studies:** Stress studies are conducted according to ICH guidelines, involving exposure of the drug to conditions such as high temperature, humidity, and light to induce degradation. These studies help identify potential degradation products and pathways.
- **Initial Separation of Stressed Samples:** Chromatographic methods, particularly HPLC, are commonly used to separate and detect degradation products. The mobile phase composition and column parameters are optimized to achieve good separation and resolution of peaks.
- **Method Development and Optimization:** The method is further developed and optimized by adjusting parameters such as mobile phase ratio, pH, gradient, flow rate, temperature, and column type to improve separation of closely eluting peaks.
- **Identification and Characterization of Degradation Products:** Degradation products are identified and characterized using techniques such as mass

spectrometry, NMR spectroscopy, and IR spectroscopy. Standards for these degradation products are also developed for validation purposes.

- **Validation of SIAM:** The final method is validated according to FDA, USP, and ICH guidelines. Validation parameters include specificity, selectivity, accuracy, precision, linearity, range, and robustness. Peak purity is also assessed to ensure homogeneity of the analyte peak.
- **Peak Purity Assessment:** Peak purity is assessed using techniques such as PDA detection, absorbance ratio method, second order derivative spectroscopy, and spectral overlay to ensure the homogeneity of the analyte peak.
- **Accuracy Assessment:** Accuracy is evaluated by spiking the drug in degraded solutions and measuring the % recovery of the drug. This helps ensure that the method can accurately quantify the drug in the presence of degradation products.
- **Robustness Evaluation:** Robustness of the method is evaluated by testing its performance under different conditions, such as changes in mobile phase composition or flow rate, to ensure its reliability and reproducibility.
- Overall, the development of a SIAM involves a systematic approach to ensure that the method is capable of accurately and reliably detecting and quantifying drug degradation products, thereby ensuring the stability and quality of the pharmaceutical product.

1.7.6. Methodology of Analytical method development

Steps to establishing a method include: [33]

I. Sample details

II. Define the purpose of the separation.

III. Indicate the prerequisites and pre-treatment for the unique process.

IV. Choose and set up the detector

V. Improve the separation circumstance

VI. Examine any potential problems or the need for a specific operation.

VII. purified material recovery.

VIII. Qualitative/quantitative calibration

IX. Validation of the method before dissemination to laboratories

1. **Choosing an appropriate method:** The methods are adopted and adjusted using the literature that is now available and previous methodology. To use the newest approaches instrumentation, sample preparation and instrument conditions are adopted.
2. **Optimization**: Optimization in method development aims to lessen errors that happen throughout the development process while also speeding up and saving money. The steps in the optimization process are:
    - a) Method Selection
    - b) Mobile phase Selection
    - c) Column Selection
    - d) Detector Selection

a) **Method Selection:** The most prominent chromatographic procedures are ion exchange, reverse phase ion pair, and normal phase approaches. When selecting a method for organic chemicals, evaluating the reverse phase approach should come first. The ion pair reverse phase method and ion exchanger chromatographic processes are the final resorts if the normal phase approach fails.

b) **Column selection:** The HPLC column is at the centre of the best separation process. The column section depends on the column length, column diameter, column particle size, and column particle shape.

c) **Mobile phase selection:** It is crucial for the analysis of drugs. The concentration of the organic phase required for the mobile phase can be estimated using the gradient elution method.

d) **Selection of detectors:** The eyes of the chromatography system are the detectors, which quantify the chemicals after the separation of the column. The must have a few characteristics, including high sensitivity, higher linear range, non-destructive nature, quick response time, and application to the majority of solutes without band broadening.

1.7.7. Methodology of Analytical method validation

- **Definition**
  - A process known as validation that provides a high level of assurance that a specific action will consistently produce the anticipated result or product that satisfies its set standards and quality attributes is the establishment of documented proof.
  - It is the process of proving that any procedure, tool, activity, work product, system, or action operates as intended under a given set of conditions and offers the required level of precision, sensitivity, resilience, accuracy, etc.
- **Benefits of Analytical Method Validation[34]**
  - Method validation's key advantage is that it fosters a high level of trust in both users and developers.
  - The validation process may appear to be time- and money-consuming, but it actually ends up being economical, gets rid of repetitive repetition, and enhances time management.
  - Minor modifications to the circumstances, such changing the reagent supplier, reagent grade, or analytical setup, are unavoidable for obvious reasons, but method validation mitigates the shock of such changes and yields a return on the process investment that is greater than the initial investment.
  - The United States Pharmacopoeia (USP) provides a framework for validation that is more effective and efficient for the analytical technique validation of pharmaceutical recommendations from the United States Food and Drug Administration (USFDA), American Association of Official Analytical Chemists (AOAC), and International Conference on Harmonization (ICH).

1.7.8. Analytical Method Validation: The Regulatory Perspective

USFDA

- According to this, validation is the act of producing written proof that provides a high level of assurance that a certain process will consistently produce a product meeting its stated standards and quality criteria.

WHO

- Validation is the process of proving that a process, operation, machine, drug, activity, or system actually has the intended consequences.

European Committee[35]

- The act of proving that any procedure, process, material, activity, or system actually generates the expected results in accordance with the GMP requirements is known as validation.
- The WHO published recommendations titled "Validation of Analytical Procedures Used in the Examination of Pharmaceutical Materials." This information was included in the 32nd report of the WHO expert committee, "Specifications for Pharmaceutical Preparations," which was published in 1992.
- The "Text on validation of analytical procedures (Q2A)" and "Validation of Analytical Procedure Methodology" are two guidelines that have been released by the International Conference on Harmonization (ICH), which has taken the initiative in developing the harmonised tripartite guidelines for adoption in the US, Japan, and EC (Q2B).

1.7.9. Analytical Method Validation Parameters

Before beginning the validation of an analytical procedure, it is necessary to understand the validation parameters. The numerous performance indicators that the Edina validation exercise addressed are grouped here. [36]

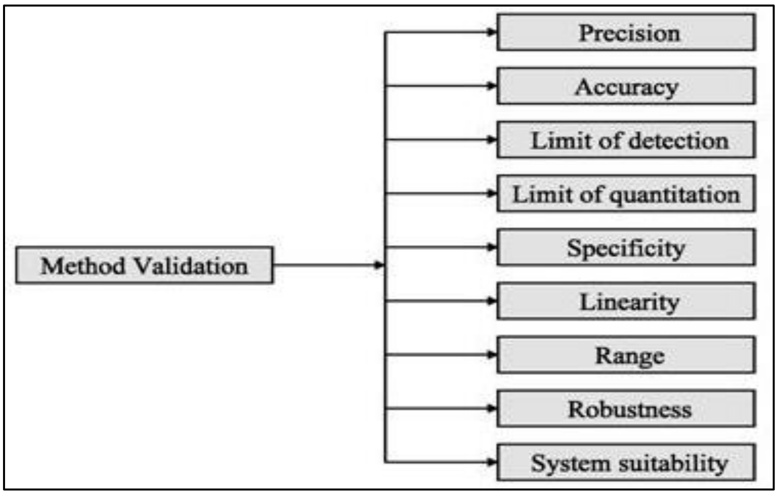


Figure No: 1.8 Method validation parameters as per ICH guidelines

1 **Selectivity and Specificity:** An analytical method must be selective to accurately measure the analyte in the presence of other components. Selectivity refers to the qualitative identification of components, while specificity is the quantitative measurement of analytes.

2 **Linearity and Range:** Beer's law states the linear range of detectability depends on the detector and compound. The method must show linearity for both the working sample and accuracy test samples. Linear least squares regression with a zero intercept is used to analyze data.

3 **Accuracy:** It measures how close test results are to the true value. Percentage recovery after adding a known amount of analyte is a common measure of accuracy.

4 **Precision:** It measures the agreement between repeated test results. Repeatability is when the same analyst uses the same equipment and method, while reproducibility is when different conditions are used.

5 **Limit of Detection:** It's the smallest amount of analyte detectable, but not necessarily measurable. It's determined using the signal-to-noise ratio.

6 **Limit of Quantitation:** It's the lowest analyte concentration that can be accurately measured. It's estimated by multiplying the standard deviation by a factor, often 10.

7 **Robustness:** It's the method's ability to withstand small, deliberate changes in parameters. It shows the method's reliability under normal conditions.

8 **Ruggedness:** It's the reproducibility of test results under various conditions like different labs, analysts, and equipment.

9 **System Suitability:** It's the verification of a method's performance before or during the analysis of unknowns. Parameters such as plate count, tailing factors, resolution, and repeatability are measured and compared to set standards.

These aspects are essential for ensuring the reliability and accuracy of analytical methods in pharmaceutical analysis.

Table No: 1.3: Recommendations and Parameters for System Suitability

Parameters	**Recommendations**[37]
Capacity Factor (k)	The peak must be distinct from other peaks and the vacuum volume, which is often K>2.
Repeatability	RSD ≤ 2 % N ≥ 5 is desirable
Relative retention	As mentioned in the resolution, not essential
Resolution (Rs)	There should be a ratio of greater than two between the peak of interest and the closest eluting potential interfere (impurity, excipients, degradation products, internal standard, etc.).
Tailing factor (T)	T ≤ 2
Theoretical plates (N)	> 2000

a) Tailing factor (T_f)

The tailing factor, a gauge of peak symmetry, is one for totally symmetrical peaks, and as tailing becomes more visible, its value rises.

As peak asymmetry increases, precision and integration become less trustworthy. The following equation was used to calculate it.

$$AS(T_f) = \frac{0.05W}{2d}$$

Where T_f = tailing factor

0.05 W = peak width at 5 % peak height

D = half of peak width at 5 % peak height

b) Retention factor:

The following definition of retention factor (k), a unitless indicator of a particular compound's retention in a particular chromatographic system under a particular set of circumstances:

$$k = \frac{V_R - V_O}{V_O} = \frac{V'_R}{V_O} = \frac{t_R - t_O}{t_O}$$

Where t_0 is occasionally defined as the retention time of the analyte that was not retained, V_R is the analyte retention volume, V_0 is the volume of the liquid phases in the chromatographic system.

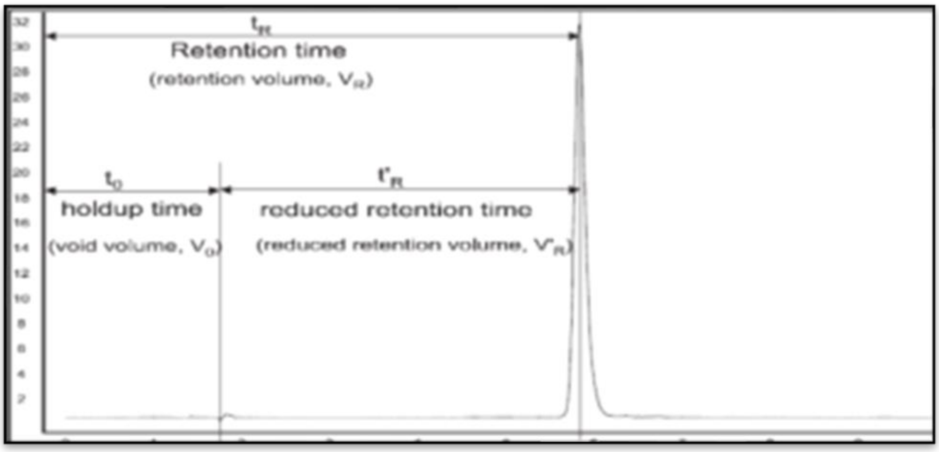


Figure No: 1.9 Analyte retention descriptors

c) Efficiency

Efficiency quantifies the peak dispersion in a specific column, making it essentially a column attribute. The efficiency (E) of the imagined number of plates (N)

$$N = 16\left(\frac{t_R}{w}\right)^2$$

where w is the baseline peak width and t_R is the analyte retention time

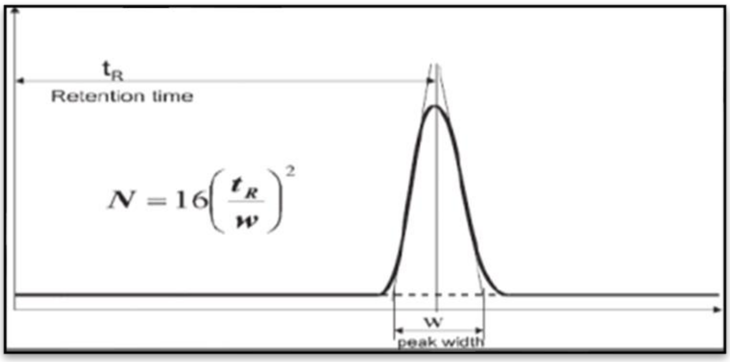


Figure No: 1.10 Diagram of the efficiency measures (Number of theoretical plates in column)

d) Selectivity (α)

Selectivity is the capacity of a chromatographic system to differentiate between two different analytes (α). It is defined as the ratio of equivalent capacity factors.

$$\alpha = \frac{k_2}{k_1}$$

e) Resolution (R)

Resolution (R), a combined measure of the separation of two compounds, includes peak dispersion and a type of selectivity. [38]

Defining resolution as:

$$R = 2\frac{t_{R.2} - t_{R.1}}{w_2 - w_1}$$

1.8 Impurity Profiling:

1.8.1 Impurities:in pharmaceuticals are unwanted chemicals that remain with the Active Pharmaceutical Ingredients (APIs) or develop during formulation or develop upon ageing of both APIs and formulated APIs to medicines.[39]

Classification according to ICH guidelines: [40]

1. Organic Impurities (Process and drug-related)
2. Inorganic Impurities (Reagent, ligands, catalysts)
3. Residual Solvents (Volatile solvents)

1 Organic Impurities:

Organic impurities can arise during the manufacturing process or storage of the drug substance. They are classified as follows:

- **Starting Materials or Intermediate Impurities:** These impurities are almost always present in active pharmaceutical ingredients (APIs) unless meticulous care is taken at every step of the multistep synthesis process.
- **By-products:** It is rare to achieve a single end product with complete yield during the predefined, controlled manufacturing process; there is often a chance of by-products being formed along with the desired end product.

- **Degradation Products:** Improper storage of bulk drugs can lead to drug degradation and the formation of degraded products, which act as impurities in pharmaceutical formulations.
- **Synthesis Related Impurities:** Any additional chemical entity generated during the manufacturing process from raw materials, solvents, intermediates, or byproducts.
- **Formulation Related Impurities**: During formulation, drugs are exposed to various stages and conditions. Alterations in predefined conditions can lead to the degradation of components. Solutions and suspensions are prone to degradation due to hydrolysis.

2 **Inorganic Impurities:** [41]

- **Reagents, Ligands, and Catalysts:** The occurrence of these impurities is rare. If proper manufacturing procedures are not followed, there is a rare chance of their occurrence.
- **Heavy Metals:** Water is widely used in various manufacturing processes, providing the main source of heavy metals such as arsenic, cadmium, chromium, sodium, magnesium, and manganese. Demineralized water and glass-lined reactors can be used to avoid heavy metal contamination.
- **Other Materials (Filter Aids, Charcoal):** Centrifuge bags are routinely used during the manufacturing of bulk drugs in many industries. In some chemical reactions, activated carbon is used, which can introduce impurities during synthesis. Regular monitoring of fibers and black particles in bulk drugs is essential to avoid contamination.

3 **Residual Solvents:**[42]Residual solvents are organic or inorganic liquids used during the manufacturing process that are difficult to remove completely through the work-up process.

1.8.2 Impurities Present in Formulation

Numbers of impurities arise in product formulation due to varieties of conditions that can lead degradation or other deleterious reaction. In the formulation use of water can not only contribute its own impurities but it is also provide a ripe situation for the hydrolysis and catalysis. [43]

Impurity forms during formulation are:

1. Method related:

This is the impurity which is related from method. For e.g. Impurity is formed during the sterilization of the parental dosage form of Diclofenac sodium, Indolin-2-one I by Autoclave. Impurity is produced during formation of indolinone derivative and sodium hydroxide takes place due to the intramolecular cyclic reaction of the Diclofenac sodium.

2. Environmental related:

These factors are responsible to reduce the stability of the drug substance such as: [44]

- **Exposure to adverse temperature:** Heat sensitive drug substance produced adverse effect in presence of high temperature. For e.g. vitamins which undergo degradation in liquid formulation that cause the decrease in potency of vitamin.
- **Light especially UV light:** Some Drug substance become unstable in presence of light. For e.g. Ergometrine and methyl ergometrine injection is unstable or shows the complete degradation when kept in the direct sunlight.
- **Humidity:** It affect hygroscopic products, which are sensitive in the humid environment. It also affect the bulk powder and solid dosage form for e.g. Aspirin and Ranitidine.

3. Dosage form related impurities:

Sometimes the dosage form factors that influence the stability of the drug. In general, liquid dosage forms are much susceptible to both degradation and microbial contamination. In which, water content, pH of the solution, compatibility of the anions and cations, mutual interaction of the ingredients, and the primary containers are the critical factors for the impurities.

A) Mutual interaction amongst ingredients.

Mutual interaction among the ingredient is also the major problem which cause the instability in the drug product. Most of the vitamins are very liable and become unstable on storage specially in liquid dosage form. For e.g. during the formulation, one formulation is in simple distilled water and other is in typical formulated vehicle that include disodium edetate and benzyl alcohol both have similar mutual interaction causing the degradation of the product.

B) Functional group- related typical degradation.

- **Ester hydrolysis:** It is the reaction when the ester reacts with the water to produce ethanoic acid and ethanol.
- **Hydrolysis:** It is a reaction which occurs due to water. Mainly this is a reaction of breaking bond in a molecule by using water. For e.g. sodium acetate is a salt which get hydrolyze by adding water and then separate in to the sodium ions and acetate ions. Oxidative degradation: It is the degradation in which the cleavage of c=c with the introduction of new carbon and oxygen bond. For e.g. hydrocortisone, methotrexate, conjugated dienes, nitrite derivatives and aldehydes all are susceptible to the oxidative degradation.
- **Photolytic cleavage:** it occurs due to the direct exposure of the sunlight. For e.g. in the preparation of ciprofloxacin eye drop, sunlight induce the photocleavage reaction producing ethylenediamine analogue of ciprofloxacin.
- **Decarboxylation:** In this case when we heat the p-aminosalicylic acid then it lose the carbon dioxide from the carboxyl group.

1.8.3 Identification and Characterization of Impurities

The impurities can be identified by the following methods:[45]

1. **Reference standard method:** This technique use for identification and detection of active ingredient in dosage form but also for the impurities, degradation product, starting materials, and excipients. In this method reference standard is prepared for use as the standard in an assay, identification, or purity test which can use to evaluate both the process and product performance.
2. **Spectroscopic method:** Following methods are used for Characterization of Impurities:
   - **Ultraviolet spectrometry:** It is used UV region 200-400 in Electromagnetic spectrum. Individual impurity shows Characteristic absorption of light at specific wavelength, Which is differ then original drug substance.
   - **Infrared spectroscopy:** It is use for solid, liquid or gaseous samples to identify Drug substance and detection of impurities by matching with

reference spectra. In this technique samples are not destroy during identification process.

- **Mass spectrometers:** in this technique, m/z ratio is measure and according to mass abundant impurity can be identified and quantified. Hyphenated technique like GC-MS and LC-MS can use for identification with better resolution of impurity with structurally similar substance.
- **GC-MS:** Gas chromatography-mass spectrometry (GC-MS) is a technique that combines the separation capabilities of gas chromatography with the detection capabilities of mass spectrometry. It is used to identify different substances within a test sample. The sample is vaporized and injected into a heated system, where it is carried through a column by an inert gaseous mobile phase. The compounds are separated based on their volatility and interact with the column's stationary phase. The separated compounds are then detected by a mass spectrometer, which provides detailed structural information about each compound. GC-MS is particularly useful for analyzing volatile and semi-volatile compounds.
- **LC/MS:** Liquid chromatography-mass spectrometry (LC/MS) is another hyphenated technique that combines the separation power of high-performance liquid chromatography (HPLC) with the detection power of mass spectrometry. In LC/MS, the liquid eluent from the HPLC column is passed into the mass spectrometer after removing the liquid carrier. This technique is used for handling normal eluent flow rates of 0.5-2.0 ml/min, which are not compatible with the pumping systems of mass spectrometers. LC/MS has become popular due to its ability to analyze a wide range of compounds with high sensitivity and specificity.
- **NMR Spectroscopy:** by this technique, sample can be determine at its atomic and nuclear level. It is very sensitive in nature.
- **Raman spectroscopy:** It is used to study vibrational, rotational and other low frequency modes in a system. It have good sensitivity and detect the process related impurities.

1.8.4 Methodology

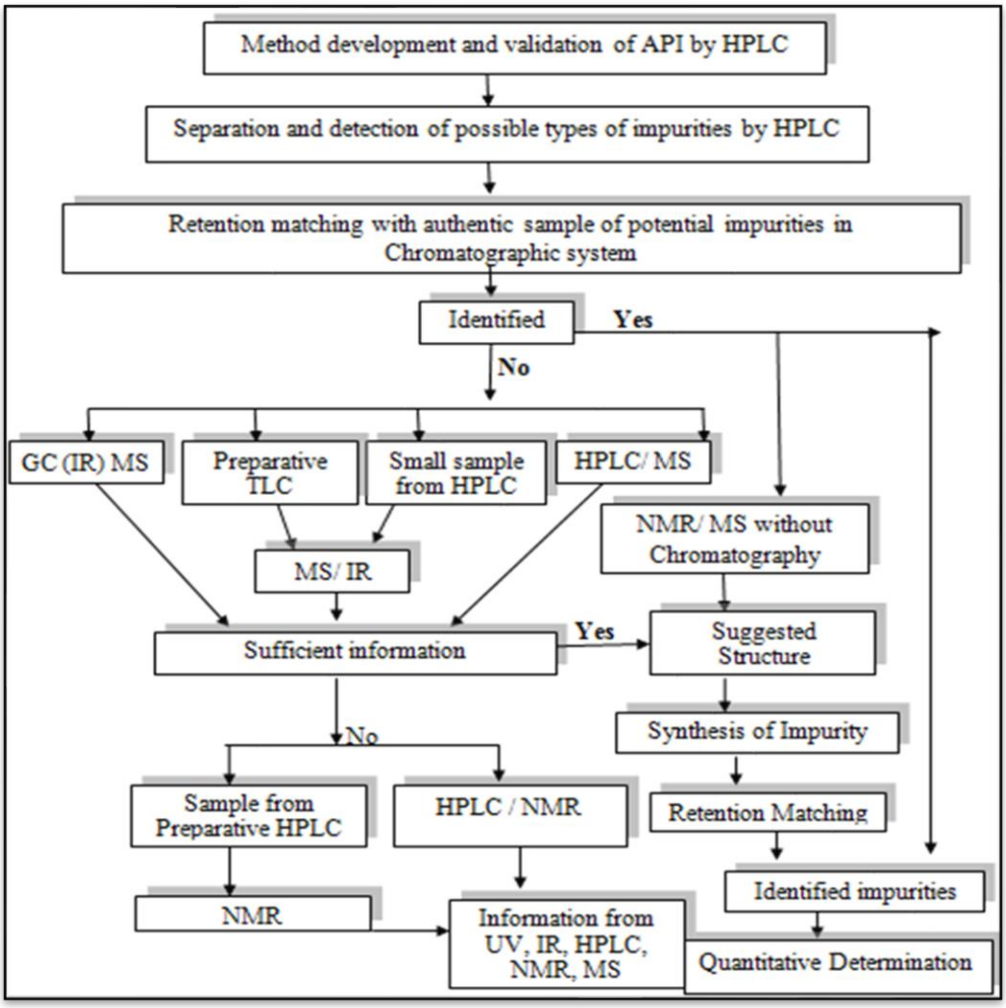


Figure No: 1.11: Steps for identification and characterization of impurities

1.8.4.1 Separation Methods of Impurity: [46]

The following separation methods are as follows.

- **Thin-layer chromatography:** In this chromatography the glass, plastic, and aluminium foil which are coated with adsorbent material such as silica gel, aluminium oxide and cellulose is used as stationary phase. Solvent system used as mobile phase which is travel up by capillary action to separate the mixtures and which after the sample has been applied on the plate then a solvent mixture is drawn up by the capillary action. Different analyte have different affinity towards stationary phase and mobile phase according to affinity components are get separated.
- **Gas chromatography**: In this chromatography sample are used in gaseous form for separation and quantification. This technique is used in combination with

other chromatography technique for better result i.e Gas-Liquid chromatography and Gas –Mass chromatography. Most of the time gas chromatography is used for the testing of purity or separating the different components of the mixtures.

- **High-pressure liquid chromatography:** It is a column chromatography used to separate, identify, and quantify the compounds. HPLC have different type's stationary phases and a pump that moves the mobile phase and also there is a detector to provide the characteristic information about the compound such as API and its impurities. It is helpful to check the quality of the API starting material and also signify the unknown impurities.
- **Capillary electrophoresis:** It is also known as capillary zone electrophoresis. It is used to separate the ionic species by their charge and size in the small capillary filled inside with an electrolyte. It is based on the different separation principles and also used for the quality control of the pharmaceutical products.
- **Supercritical fluid chromatography (SFC):** It is the chromatography in which we separate one component from other component by using the super critical fluid. Carbon dioxide is used as a supercritical fluid where ethanol or methanol used as a co-solvent. In this we provide the critical temperature of 31^oc with critical pressure of 72 bars.

1.8.4.2 Isolation of Impurities:

Generally chromatographic and non-chromatographic techniques are used for the isolation of the impurities such as.

- **Solid-phase extraction method:** It is the method which is used to trace the organic compound as well as remove the interfering compound to obtain a clear extract. Mainly this technique is used for the extraction and purification of the compounds. Main use of this method is to clean up the sample before use for the chromatographic technique to quantify the analyte in the sample. This technique is widely applied for the isolation of analytes from a liquid matrix.
- **Liquid-liquid extraction method:** It is also known as solvent extraction and partitioning method. In this method compounds gets separated according to their relative solubility in two different immiscible liquids usually water and organic solvents. The method is performed in the separating funnel. Commonly solvents

used for liquid-liquid extraction are ethyl acetate, methylene chloride and hexanes.

- **Accelerated solvent extraction method:** It is the better technique used for the extraction of solid and semi-solid samples. All the process done at the elevated temperature and pressure to get the fast and efficient removal of analysts from the samples. It performs the experiment in less time with using smaller quantity of solvent.

1.9. International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use.

Table No: 1.4 ICH Quality Guidelines[47]

Q1A-Q1F	Stability Testing
Q2	Validation of Analytical Procedures
Q3A-Q3D	Impurities
Q4-Q4B	Pharmacopoeia
Q5A-Q5E	Quality of Biotechnological Products:
Q6A-Q6B	Specifications
Q7	Good Manufacturing Practice
Q8	Pharmaceutical Development
Q9	Quality Risk Management
Q10	Pharmaceutical Quality System
Q11	Development and Manufacture of Drug Substances
Q12	Lifecycle Management
Q13	Continuous Manufacturing of Drug Substances and Drug Products
Q14	Analytical Procedure Development

1.9.1 ICH Q3 Guidelines for Impurities

<table>
<tr><th>Q3A-Q3D</th><th>Impurities</th></tr>
<tr><td colspan="2">Q3A (R2) - Impurities in New Drug Substances</td></tr>
<tr><td colspan="2">Q3B (R2) – Impurities in New Drug Products</td></tr>
<tr><td colspan="2">Q3C (R5) – Impurities : Guideline for Residual Solvents</td></tr>
<tr><td colspan="2">Q3D – Impurities : Guideline for Elemental Impurities</td></tr>
</table>

Figure No: 1.12 ICH Q3 Guidelines for Impurities

1.9.2 Impurities in New Drug Substances Q3A(R2)

Impurities in new drug substances are addressed from two perspectives: [48]

The Chemistry Aspects of impurity evaluation encompass the classification and identification of impurities, the generation of reports, the inclusion of impurity listings in specifications, and a concise overview of analytical procedures.

On the other hand, **Safety Aspects** involve providing specific guidance for qualifying impurities that were either absent or present at significantly lower levels in batches of a new drug substance used in safety and clinical studies.

Table No: 1.5 ICH Guidelines for Impurities in New Drug Substance Q3A(R2)

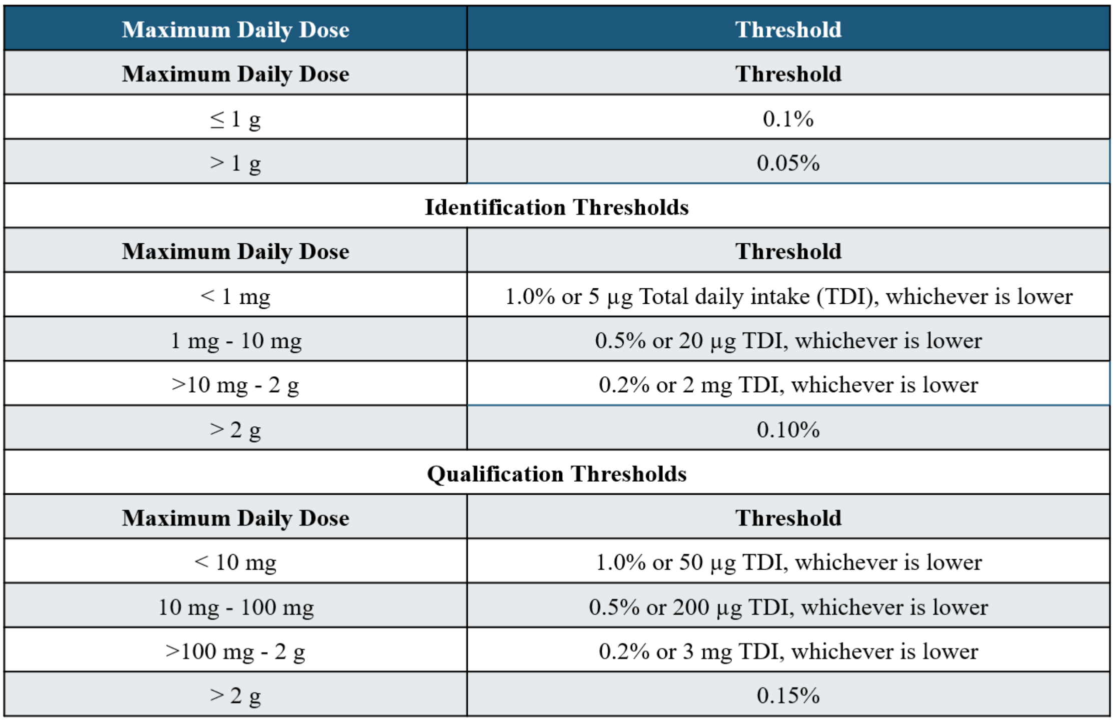
Maximum Daily Dose	Reporting Threshold	Identification Threshold	Qualification Threshold
≤ 2g/day	0.05%	0.10% or 1.0 mg per day intake (whichever is lower)	0.15% or 1.0 mg per day intake (whichever is lower)
> 2g/day	0.03%	0.05%	0.05%

1.9.3 Impurities in New Drug Products Q3B(R2) [49]

This guideline specifically focuses on impurities in new drug products that fall into two categories: degradation products of the drug substance and reaction products of the drug substance with an excipient and/or immediate container closure system. These impurities are collectively referred to as "degradation products" in the guideline.

Impurities found in the new drug substance do not typically need to be monitored or specified in the new drug product unless they are also degradation products. Impurities that arise from excipients in the new drug product or are extracted or leached from the container closure system are not addressed in this guideline.The guideline does not apply to new drug products used during the clinical research stages of development. It also excludes certain types of products, including biological/biotechnological products, peptides, oligonucleotides, radiopharmaceuticals, fermentation products and semi-synthetic products derived therefrom, herbal products, and crude products of animal or plant origin. Additionally, the guideline excludes extraneous contaminants that should not be present in new drug products and are more appropriately addressed through good manufacturing practice (GMP) regulations. Polymorphic forms and enantiomeric impurities are also not covered by this document.

Table No: 1.6 ICH guidelines for Impurities in New drug product Q3B(R2)



Maximum Daily Dose	Threshold
Maximum Daily Dose	**Threshold**
≤ 1 g	0.1%
> 1 g	0.05%
Identification Thresholds	
Maximum Daily Dose	**Threshold**
< 1 mg	1.0% or 5 µg Total daily intake (TDI), whichever is lower
1 mg - 10 mg	0.5% or 20 µg TDI, whichever is lower
>10 mg - 2 g	0.2% or 2 mg TDI, whichever is lower
> 2 g	0.10%
Qualification Thresholds	
Maximum Daily Dose	**Threshold**
< 10 mg	1.0% or 50 µg TDI, whichever is lower
10 mg - 100 mg	0.5% or 200 µg TDI, whichever is lower
>100 mg - 2 g	0.2% or 3 mg TDI, whichever is lower
> 2 g	0.15%

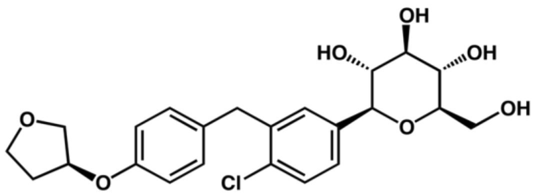
CHAPTER 2

DRUG PROFILE

2. DRUG PROFILE

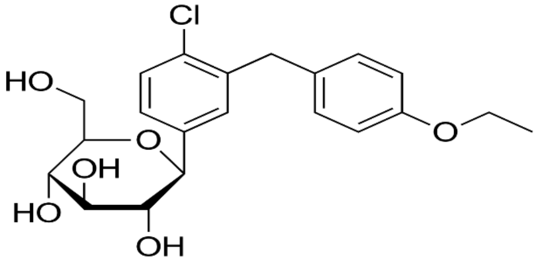
2.1. Empagliflozin [50]

Table No: 2.1 Drug Profile of Empagliflozin

Name of Drug	**Empagliflozin**
Pharmacopeial Status	-
CAS No.	864070-44-0
Chemical Properties	
Chemical Structure	
Molecular Formula	$C_{23}H_{27}ClO_7$
IUPAC Name	(2*S*,3*R*,4*R*,5*S*,6*R*)-2-[4-chloro-3-[[4-[(3*S*)-oxolan-3-yl]oxyphenyl]methyl]phenyl]-6-(hydroxymethyl)oxane-3,4,5-triol
Physicochemical Properties	
Molecular Weight	450.9 g/mol
Melting point (°C)	151 °C – 153 °C
Log P	1.79
pKa	12.57
Solubility	Slightly soluble in Water, soluble in Methanol, Freely soluble in DMSO and Acetonitrile

2.2 Dapagliflozin[51]

Table No: 2.2 Drug Profile of Empagliflozin

Name of Drug	**Dapagliflozin**
Category	Anti-diabetic
Pharmacopeial Status	N/A
Chemical Properties	
Table No.	461432-26-8
Table No.	
Table No.	$C_{21}H_{25}ClO_6$
Table No.	(2S,3R,4R,5S,6R)-2-[4-Chloro-3-(4-ethoxybenzyl)phenyl]-6 (hydroxymethyl) tetrahydro-2H- pyran-3,4,5-triol
Physicochemical Properties	
Molecular Weight	408.9 g/mol
Melting Point	74-78 ℃
Characteristics	White to off-white solid
pKa	12.6
log P	2.7
Solubility	Soluble in Methanol, Acetonitrile and Sparingly soluble in Water

CHAPTER 3

LITERATURE REVIEW

3. LITERATURE REVIEW

3.1 Empagliflozin

3.1.1 Reported methods for Empagliflozin and its combination

Table No: 3.1 Reported methods for Empagliflozin and its combination

Sr. No	Title	Method and Description
1.	Development and Validation of Simple UV Spectrophotometric Method for the Determination of Empagliflozin[52]	**UV** **Solvent:** water: methanol (9:1 v/v) **λmax :** 224 nm **Retention Time:** NA
2.	A rapid and sensitive RP-HPLC method for the quantitative analysis of Empagliflozin in bulk and pharmaceutical dosage form[53]	**RP-HPLC** **Column:** C18 **Mobile Phase:**Acetonitrile: Water (60:40 v/v) **Detection Wavelength**: 223nm **Flow Rate**: 1.0 ml/min **Retention Time:** Empagliflozin: 5.417 min
3.	Development and validation of a novel stability-indicating RP-HPLC method for the determination of Empagliflozin in bulk and pharmaceutical dosage form[55]	**RP-HPLC** **Column:** C18 **Mobile Phase:** Methanol: Acetonitrile (50: 50 v/v) **Detection Wavelength**: 265nm **Flow Rate**: 20µl/min **Retention Time:** Empagliflozin: 2.184 min
4.	A New validated RP-HPLC method for the determination of Metformin HCl and Empagliflozin in its Bulk and	**RP-HPLC** **Column:** C18 **Mobile Phase:** Methanol: phosphate buffer (pH 3)

	Pharmaceutical Dosage Forms[56]	(70:30 v/v) **Detection Wavelength**: 240nm **Flow Rate**: 1.0 ml/min **Retention Time:** Empagliflozin: 3.907 min Metformin: 2.403 min
5.	Stability indicating RP-HPLC method development and validation for estimation of Empagliflozin and Metformin HCl[57]	**RP-HPLC** **Column:** C18 **Mobile Phase:** 0.05M Potassium Dihydrogen Phosphate buffer (pH- 3.5, adjusted with 1% Orthophosphoric acid) and Acetonitrile (50:50% v/v) **Detection Wavelength**: 227nm **Flow Rate**: 1.0 ml/min **Retention Time:** Empagliflozin: 4.388 min Metformin: 2.635 min
6.	Development and validation of stability indicating RP-HPLC method for the simultaneous estimation of Metformin hydrochloride and Empagliflozin in bulk and in a synthetic mixture[58]	**RP-HPLC** **Column:** C18 **Mobile Phase:** 0.1% OPA (Ortho phosphoric acid): Acetonitrile (45:55v/v) **Detection Wavelength**: 233nm **Flow Rate**: 1.0 ml/min **Retention Time:** Empagliflozin: 3.413 min Metformin: 2.270 min

3.2 Dapagliflozin

3.2.1 Reported methods for Dapagliflozin and its combination

Table No: 3.2 Reported methods for Dapagliflozin and its combination

Sr. No	Title	Method and Description
1.	A New RP-HPLC Method Development and Validation of Dapagliflozin in Bulk and Tablet Dosage Form [59]	**RP-HPLC** **Column:** C18 **Mobile Phase:** Phosphate Buffer: Acetonitrile (60:40 v/v) **Detection Wavelength**: 237 nm **Flow Rate**: 1.0 ml/min **Retention Time:** Dapagliflozin: 3.461 min
2.	RP-HPLC Method for Estimation of Dapagliflozin from its Tablet [60]	**RP-HPLC** **Column:** C18 **Mobile Phase:** Acetonitrile: 0.1% Triethylamine (pH - 5.0) (50:50 v/v) **Detection Wavelength**: 224 nm **Flow Rate**: 1.0 ml/min **Retention Time:** Dapagliflozin: 5.136 min **Degradation: (%)** Acid Hydrolysis (0.1 N HCl, 60ºC, 3 hr):**18.06** Base Hydrolysis (0.1 N NaOH, 60ºC, 3 hr):**8.67** Oxidation (3 %, 60ºC, 3 hr):**9.67**
3.	Validation of a Newly Developed Stability Indicating RP-Liquid Chromatographic Method for the Quantitative	**RP-HPLC** **Column:** C18 **Mobile Phase:** Methanol: Sodium 1-octanesulphonate (70:30 v/v) **Detection Wavelength**: 203 nm

	Determination of Dapagliflozin [61]	**Flow Rate**: 1.0 ml/min **Retention Time:** Dapagliflozin: 5.500 min **Degradation: (%)** Acid Hydrolysis (0.1 N HCl, 35ºC, 48hr):**1.7** Base Hydrolysis (0.1 N NaOH, 35ºC, 48hr):**1.2** Oxidation (30 %, 35ºC, 48hr): **18.1** Photo Degradation (UV light 48 hr):**17.9** Thermal Degradation (70ºC, 48 hr):**2.7**
4.	Development and Validation of Stability Indicating HPLC Method for Estimation of Dapagliflozin in Marketed Formulation [62]	**RP-HPLC** **Column:** C18 **Mobile Phase:** Acetonitrile: Water (80:20 v/v) **Detection Wavelength**: 225 nm **Flow Rate**: 1.0 ml/min **Retention Time:** Dapagliflozin: 6.744 min **Degradation: (%)** Acid Hydrolysis (0.1N HCl, 60ºC, 30 min):**32.15** Base Hydrolysis (0.1N NaOH, 60ºC, 30 min): **27.23** Oxidation (5 %, 60ºC, 12 hr):**37.31** Photo Degradation (Sun light 30 min):**39.41** Thermal Degradation (60ºC, 30 min):**25.47**
5.	Development and Stability Indicating HPLC Method for Dapagliflozin in API and Pharmaceutical Dosage Form [63]	**RP-HPLC** **Column:** C18 **Mobile Phase:** Acetonitrile**:** Di-potassium hydrogen phosphate (pH-6.5 adjusted with OPA) (40:60 v/v) **Detection Wavelength**: 222 nm **Flow Rate**: 1.0 ml/min **Retention Time:** Dapagliflozin: 3.160 min **Degradation: (%)**

		Acid Hydrolysis (0.1 N HCl, 60ºC, 2 hr): **7.93** Base Hydrolysis (0.1 N NaOH, 60ºC, 2 hr): **9.19** Oxidation (3 %, 35ºC, 3 hr): **10.15** Photo Degradation (Sun light 1 hr): **11.12** Thermal Degradation (60ºC, 30 hr): **8.73**
6.	Development and Validation of stability-Indicating RP-HPLC Method for Determination of Dapagliflozin [64]	**RP-HPLC** **Column:** C18 **Mobile Phase:** Acetonitrile: Ortho phosphoric acid (55:45 v/v) **Detection Wavelength**: 245 nm **Flow Rate**: 1.0 ml/min **Retention Time:** Dapagliflozin: 5.41 min **Degradation: (%)** Acid Hydrolysis (2 N HCl, 60ºC, 30 min): **7.4** Base Hydrolysis (2 N NaOH, 60ºC, 30 min): **6.4** Oxidation (20 %, 60ºC, 30 min): **5.1** Photo Degradation (UV light, 7 days): **1.4** Thermal Degradation (105ºC, 6 hr): **4.4**
7.	Stability Indicating RP-HPLC Method for Determination of Saxagliptin and Dapagliflozin in Bulk and Tablet Dosage Forms [65]	**RP-HPLC** **Column:** C18 **Mobile Phase:** Methanol: 0.1 % O- phosphoric acid (60:40 v/v) **Detection Wavelength**: 220 nm **Flow Rate**: 1.0 ml/min **Retention Time:** Dapagliflozin: 5.41 min Saxagliptin:7.30 min **Degradation: (%)** Acid Hydrolysis (0.1 N HCl, 80ºC, 3 hr): **24 -27** Base Hydrolysis (0.1 N NaOH, 80ºC, 3 hr):**17-28** Oxidation (3 %, 80ºC, 3 hr): **5 – 10**

3.3 Impurity profiling of some anti-Diabetic drugs

3.3.1 Reported methods for Impurity profiling of some anti-Diabetic drugs

Table No: 3.3 Reported methods for Impurity profiling of some anti-Diabetic drugs

Sr. No	Title	Method and Description
1.	Identification, isolation, and synthesis of seven novel impurities of anti-diabetic drug Repaglinide[66]	**UPLC** **Column:** C8 **Mobile Phase:** Gradient program: Solvent A: 0.05% of Formic acid in water Solvent B: 0.05% Formic acid in Acetonitrile **Detection Wavelength**: 210 nm **Flow Rate**: 1 ml/min **Method of Characterization:** IR, ^{1}H NMR, ^{13}C NMR, MS, Crystal X-ray diffraction **Retention Time:** Impurity 8: 2.20 min Impurity 9: 4.06 min Impurity 10: 5.21 min Impurity 11: 5.36 min Impurity 13: 6.67 min Impurity 14: 7.00 min Impurity 16: 7.55 min
2.	Development and Validation of Miglitol and its impurities by RP-HPLC and Characterization using Mass Spectrometry Techniques [67]	**RP- HPLC** **Column:** C18 **Mobile Phase:** Gradient program: Solvent A: 10 mM Di-potassium hydrogen orthophosphate Solvent B: Acetonitrile

		(pH – 8.0) **Detection Wavelength**: 210 nm **Flow Rate**: 1 ml/min **Method of Characterization**: LC/MS **Retention Time:** Miglitol: 24.070 min Impurity A: 17.037 min Impurity B: 18.776 min Impurity C: 19.480 min Impurity D: 20.604 min Impurity E: 21.170 min Impurity F: 26.621 min
3.	Reversed-phase liquid chromatography with mass detection and characterization of saxagliptin degradation related impurities[68]	**HPLC** **Column:** C18 **Mobile Phase:**Gradient program: Solvent A: Water Solvent B: Acetonitrile **Detection Wavelength**: 213 nm **Flow Rate**: 1 ml/min **Method of Characterization**: MS **Degradation related impurities (DRI):** **DRI 1** (Acid Hydrolysis, HCl 1N, 60°C for 6 hours) **DRI 2** (Acid Hydrolysis, HCl 1N, 60°C for 6 hours) **DRI 3** (Base Hydrolysis, NaOH 1N, 60°C)
4.	Identification, isolation and characterization of potential process-related impurity and its degradation product in Vildagliptin[69]	**UHPLC** **Column:** C18 **Mobile Phase:** Solvent A :10 mM Sodium dihydrogen phosphate monohydrate (pH :7.0) Solvent B: Solvent A + Acetonitrile (7:3 v/v).

		Detection Wavelength: 210 nm **Flow Rate**: 1 ml/min **Method of Characterization**: IR, ^{1}H NMR, ^{13}C NMR, MS **Retention Time:** Vildagliptin: 18.55 min Impurity E: 21.92 min Impurity F: 6.01 - 6.69 min
5.	A developed HPLC method for the determination of Alogliptin Benzoate and its potential impurities in bulk drug and Tablets [70]	**RP-HPLC** **Column:** C18 **Mobile Phase:** Solvent A: Water/Acetonitrile/Trifluoro-acetic acid (1900:100:1 v/v/v) Solvent B: Acetonitrile/Water/Trifluoro-acetic acid (1900:100:1 v/v/v) **Detection Wavelength**: 278 nm **Flow Rate**: 1 ml/min **Method of Characterization**: ^{1}H-NMR, ESI-MS Impurity A, Impurity B, Impurity C, Impurity D, Impurity E, Impurity F, Impurity G
6.	Stability-Indicating Liquid Chromatographic Method for Determination of Saxagliptin and Structure Elucidation of the Major Degradation Products Using LC -MS[71]	**RP-HPLC** **Column:** C18 **Mobile Phase:** Potassium dihydrogen phosphate buffer (pH 4.6): Acetonitrile: Methanol (40: 30: 30, v/v/v) **Detection Wavelength**: 208 nm **Flow Rate**: 1 ml/min **Method of Characterization:** LC-MS DR1: Alkaline impurity DR2: Acidic Impurity

7.	Impurity profile study of Repaglinide[72]	**HPLC** **Column:** C18 **Mobile Phase:** 0.01 M Potassium dihydrogen phosphate: Acetonitrile (50:50 v/v) (pH 3.5) **Detection Wavelength**: 200 nm **Flow Rate**: 1 ml/min **Method of Characterization**: IR, ^{1}H NMR ^{13}C NMR, MS **Retention Time:** Repaglinide: 24 - 26 min Impurity 1: 10 – 12 min Impurity 2: 13 – 15 min Impurity 3: 17 – 19 min Impurity 4: 30-32 min
8.	LC determination of Glimepiride and its related impurities [73]	**RP- HPLC** **Column:** C8 **Mobile Phase:** Phosphate buffer (pH 7.0): Acetonitrile: Tetrahydrofuran (73:18:09, v/v/v) **Detection Wavelength**: 228 nm **Flow Rate**: 1 ml/min **Method of Characterization**: FTIR, UV, NMR, NMR, MS **Relative retention time:** Impurity A:0.35 min Impurity B: 0.95 min Impurity C:1.1 min Impurity D:1.3 min Impurity E:0.12 min
9.	Impurity Profiling and Regulatory Aspects of Sitagliptin Active	**HPLC** **Column:** C8

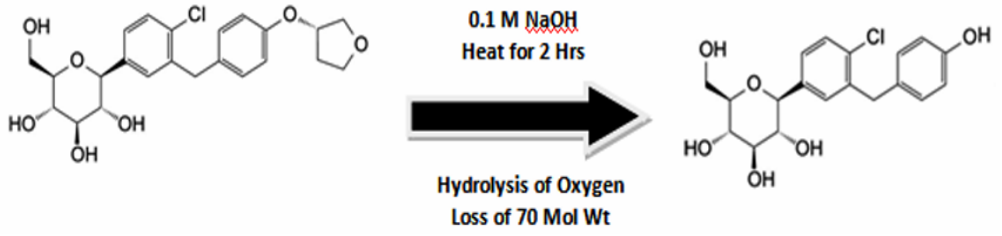
	Pharmaceutical Ingredient[74]	**Mobile Phase**: Gradient program: Solvent A: buffer: Acetonitrile (9: 1) v/v Solvent B: Acetonitrile: buffer (7:3, v/v) **Detection Wavelength**: 210 nm **Flow Rate**: 1 ml/min **Method of Characterization:** NA **Retention Time:** Sitagliptin phosphate: 10.80 min Ketoamide impurity: 26.54 min Enamine impurity: 32.63 min
10.	Validated stability indicating HPLC method for determination of Process Related Impurities in Empagliflozin drug substances[75]	**RP-HPLC** **Column:** C8 **Mobile Phase:** Gradient program: Solvent A: 0.1% Orthophosphoric acid Solvent B: Acetonitrile **Detection Wavelength**: 230 nm **Flow Rate**: 1.2 ml/min **Method of Characterization:** NA **Retention Time:** Empagliflozin: 11.52 min Impurity A: 1.31 min Impurity B: 4.64 min Impurity C: 5.02 min Impurity D: 5.85 min
11.	Development, Validation and resolving mass balance issue by using alternative oxidizing reagents for the determination of Metformin Hydrochloride Impurities in	**RP- HPLC** **Column:** C8 **Mobile Phase:** Mixture of 0.025 M Potassium dihydrogen ortho phosphate and 0.005 M 1-octane sulphonic acid sodium salt monohydrate (pH:5.0) buffer: Methanol (80:20, v/v)

	API and Pharmaceutical Dosage Form[76]	**Detection Wavelength**: 218 nm **Flow Rate**: 1 ml/min **Method of Characterization:** NA **Retention Time:** Metformin: 11.259 min Impurity A: 3.145 min Impurity B: 20.652 min Impurity C: 25.402 min Impurity D: 5.690 min Impurity E: 9.139 min
12.	A Validated Stability Indicating HPLC method for the Determination of Impurities in Pioglitazone Hydrochloride[77]	**RP-HPLC** **Column:** C8 **Mobile Phase**: Gradient Program: Solvent A: Phosphate buffer (pH 3.1) Solvent B: Acetonitrile **Detection Wavelength**: 225 nm **Flow Rate**: 1.5 ml/min **Method of Characterization:** NA **Retention Time:** Pioglitazone: 7.415 min Impurity A: 2.280 min Impurity B: 4.300 min Impurity C: 12.006 min Impurity D: 13.730 min Impurity E: 19.360 min Impurity F: 38.610 min
13.	Simultaneous determination of Glimepiride and Metformin hydrochloride impurities in sustained release pharmaceutical drug product by HPLC[78]	**RP-HPLC** **Column:** C8 **Mobile Phase:** Pentane sulfonic acid sodium salt buffer: Acetonitrile (90: 10 v/v) (pH-3.5) **Detection Wavelength**: 230 nm

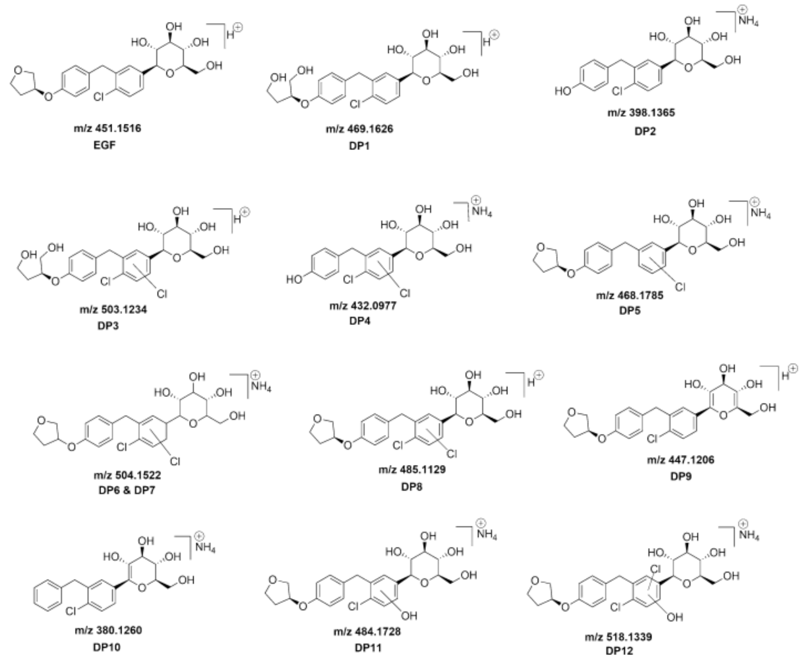
		Flow Rate: 1.5 ml/min **Method of Characterization:** NA **Retention Time:** Glimepiride: 23.50 min Metformin: 7.43 min Glimepiride sulfonamide (GS): 18.61 min Glimepiride urethane (GU): 19.91 min Glimepiride -3- Isomer (GI): 23.73 min Metformin related Compound : 1.98 min
14.	Impurity profile study of Pioglitazone and Glimepiride combination drug product by liquid chromatography [79]	**RP-HPLC** **Column:** C8 **Mobile Phase:** 0.1 N Sodium dihydrogen phosphate dihydrate buffer: Methanol: Acetonitrile (50: 5: 45 v/v/v) (pH-3.0) **Detection Wavelength**: 230 nm **Flow Rate**: 1.2 ml/min **Method of Characterization:** NA **Retention Time:** Glimepiride: 21.49 min Pioglitazone: 5.03 min GS: 2.89 min GU: 3.83 min GI: 22.07 min Pioglitazone impurity (PI): 9.35 min

3.3.2 Reported methods for Impurity profiling of SGLT-2 Inhibitor Drugs

Table No: 3.4 Reported methods for Impurity profiling of SGLT-2 Inhibitor Drugs

Sr. No	Title	Method and Description
1.	Development and Validation of Stability Indicating HPTLC-MS Method for Estimation of Empagliflozin in Pharmaceutical Dosage Form [80]	**HPTLC and LC-MS/MS** **Stationary Phase:** Silica gel 60 F254 plates **Mobile phase:** Toluene: Methanol (7:3 v/v) **Detection Wavelength:** 228 nm. **Degradation Condition:** Acidic : 0.1 M HCl at 80 °C reflux for 2hr Alkali : 0.1 M NaOH at 80 °C reflux for 2 hr Oxidative Condition: 3% H2O2 Thermal: as per guidelines Photolytic: UV light **Rf value:** Dapagliflozin: 0.16,0.32,0.66 Acidic degradation product: 0.13,0,25,0.53,0.67 Alkali degradation product:0.24,0.32,0.65 Oxidative degradation product:0.27,0.65 Thermal degradation product:0.27,0.47,0.67 UV degradation product:0.32,0.55,0.65
Findings: Degradation product in Alkali condition:  During hydrolysis of Empagliflozin bond was broken down between the benzene ring and the tetrahydrofuran ring which is there in the Empagliflozin, this bond was		

<table>
<tr><td colspan="3">broken due to the hydrolysis which is taken place at the oxygen group and hydrogen was added to oxygen and there is loss of tetrahydrofuran hetero cyclic ring.</td></tr>
<tr><td>2.</td><td>Structural characterization of forced degradation products of Empagliflozin by high resolution mass spectrometry[81]</td><td>UHPLC-MS
Colum: Eclipse plus C18 (2.1 X 50 mm, 1.8 µm)
Mobile phase:
water with 0.1% formic acid: Methanol (50:50 %v/v)
Flow rate :1 ml/min
Detection Wavelength: 226 nm.
Degradation Condition:
Acidic : 5 N HCl at 80 °C reflux for 72 hr
Alkali : 5 N NaOH at 80 °C refllux for 72 hr
Oxidative: 30% H_2O_2 at Room temperature for 7 days
Thermal: 80 °C in hot air oven for 5 days (No degradation observed)
Retention time:
Empagliflozin: 3.389 min
Acid degradation product:
DP1:1.282 min
DP2:1.459 min
DP3:1.933 min
DP4:2.152 min
DP5:2.481 min
DP6:4.415 min
DP7:5.053 min
DP8:5.588 min
Alkali degradation product:
DP9:4.743min
DP10:5.4 min
Oxidative degradation product:
DP11:1.783 min
DP:12:2.221 min</td></tr>
</table>

<table>
<tr><td colspan="3">Findings:
Empagliflozin Acid, Alkali and Oxidative degradation product

 </td></tr>
<tr><td>3.</td><td>Characterization of forced degradation products of Canagliflozin by LC/QTOF/MS/MS and in silico toxicity predictions [82]</td><td>UHPLC, LC/QTOF/MS/MS
Colum: CSH C18 (100 mm × 2.1 mm, 1.7µm)
Mobile phase: 0.1% formic acid as aqueous phase and ACN: MeOH (70: 30, % v/v) as the organic phase in a linear gradient program
(T min/%B; 0/20, 1/20, 5/55, 6.5/55, 8.5/90, 9.5/90, 11/20 and 12/20)
Co-Solvent: (ACN: water, 50:50, % v/v)
Flow rate :0.3 ml/min
Detection Wavelength: 291 nm
Degradation Condition:
Oxidative: 30% H2O2 at room temprature
Acidic: 5 N HCl at 75°C

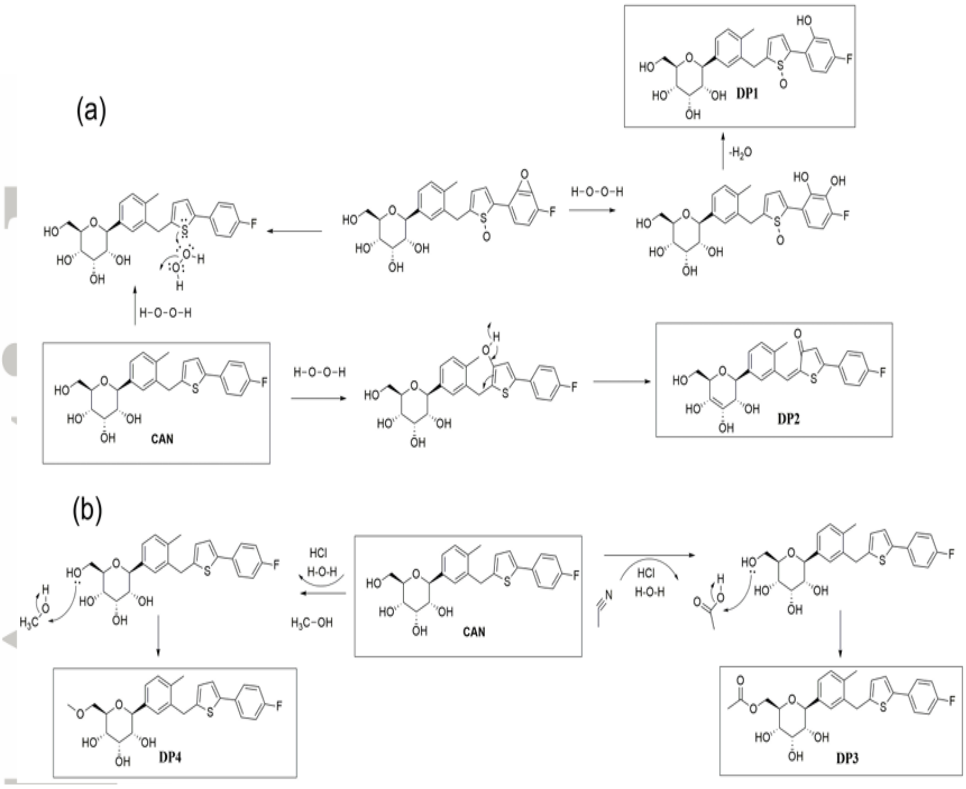
Retention time:
Empagliflozin: 9.10 min
Oxidative Degradation product 8.20 min
Acidic Degradation product 10.90 min</td></tr>
</table>

<table>
<tr><td></td><td></td><td>Degradation product:
Oxidative Degradation product:DP1 & DP2
Acidic Degradation product:DP3 & DP4</td></tr>
<tr><td colspan="3">

Findings:

- This is a typical case of degradation, where co-solvents Acetonitrile: water (50: 50, % v/v) and Methanol: water (50: 50 %v/v) reacts with canagliflozin in acid hydrolytic condition leading to the formation of pseudo-DPs, DP3 and DP4 respectively.
- The formation of DP3 (acetylation of CAN) indicates the probable involvement of diluent (ACN: water, 50:50, % v/v) under acid hydrolysis stress condition.
- Then the diluent effect (co-solvent effect) was further confirmed by subjecting CAN to acid (5N HCl) hydrolysis in the presence of different co-solvent system (MeOH: water, 50: 50, % v/v), in which condition DP4 was formed (methylation of CAN).

Degradation Product of Canagliflozin



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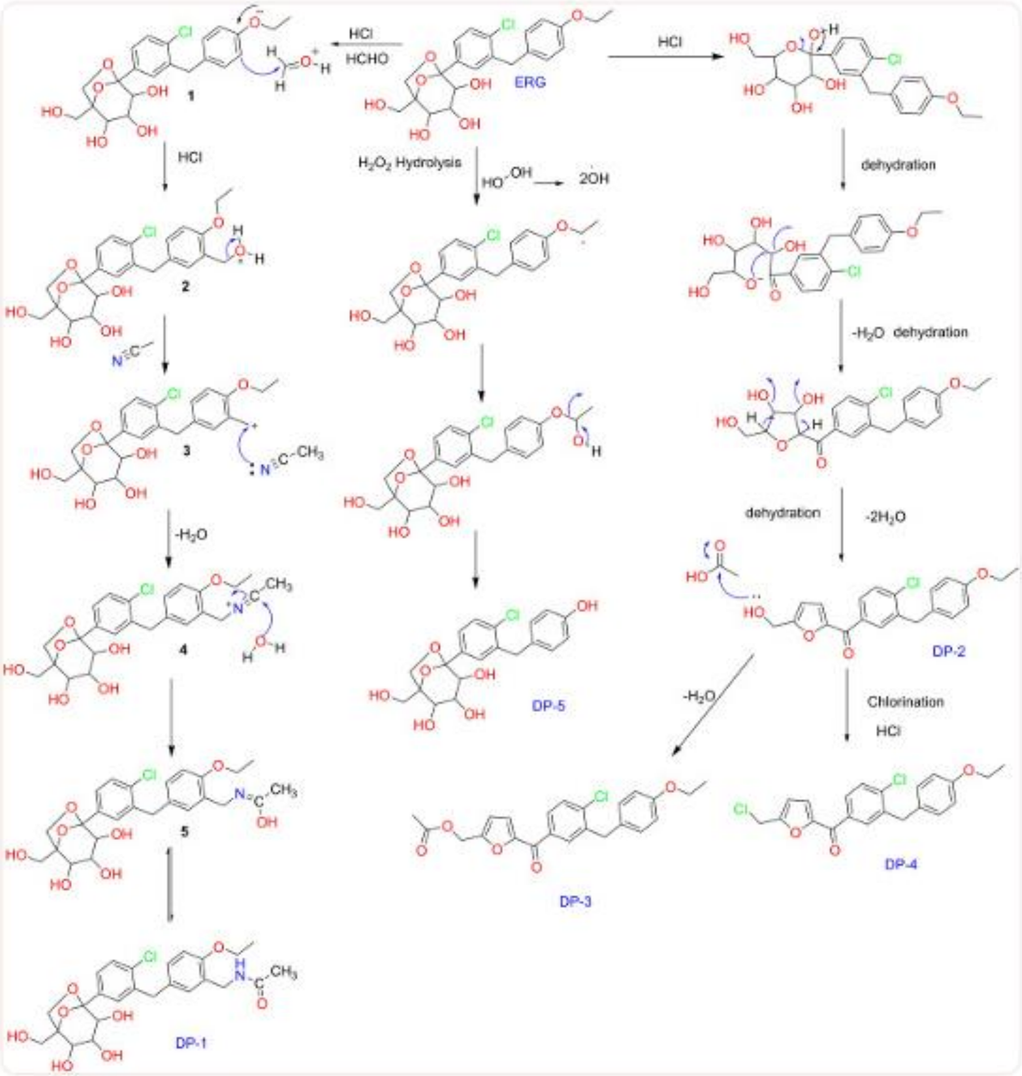
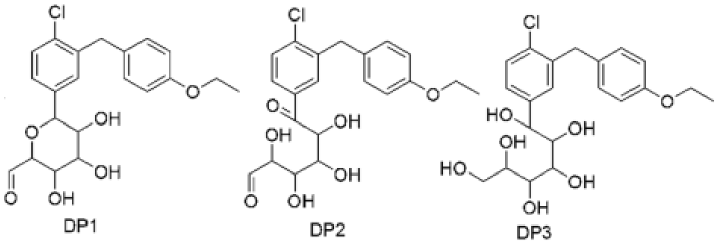
4.	Identification, isolation, and structural characterization of novel forced degradation products of Ertugliflozin using advanced analytical techniques[83]	**UHPLC-MS** **Colum: Luna C18 column (150 mm × 25 mm, 5 µm)** **Mobile phase:** Mobile phase-A :0.1% formic acid in water Mobile phase-B:0.1% formic acid in acetonitrile. gradient program Time (min)/B conc (%): 0/3, 0.5/3, 2.5/98, 3.5/98, 3.6/03, 4/03 **Co-Solvent:** (ACN: water, 50:50, % v/v) **Flow rate :**0.4 ml/min **Detection Wavelength:** 279 nm. **Degradation Condition:** **Oxidative:**30% H_2O_2 at room temprature **Acidic:** 1 N HCl at 60°C **Retention time:** **Empagliflozin:** 10.80 min **Oxidative Degradation product** 9.20 min **Acidic Degradation product** 11.40 min **Degradation product:** **Acidic Degradation product:**DP1,DP2, DP3 & DP4 **Oxidative Degradation product:**DP5
Findings: • DP1, Initial acid mediated electrophilic substitution of protonated formaldehyde to the ortho position as in compound 1 followed by Ritter chemistry with acetonitrile afforded DP1.sugar moiety in the target molecule undergoes gradual decomposition to form formaldehyde under hot acidic condition. This upon react with the target molecule under acidic medium (as given in the manuscript) pro motes the formation of 3. This in turn react with acetonitrile (used as solvent for the solubility purpose) to form DP-1 through Ritter chemistry.		

- Formation of DP3/DP4, sugar moiety in the target molecule undergoes gradual decomposition to form formaldehyde /acetaldehyde and acetic acid in different proportions
- The formed acetic acid involved in the esterification of the DP2 under the existing hot acidic condition.
- In a similar way, the free hydroxymethyl group undergoes chlorination via SN2 mechanism. Accordingly, the acid protonates the hydroxyl group and is successively displaced by the chloride to form the corresponding DP4.

Degradation Product of Ertugliflozin



5.	Degradation Pathway Proposal, Structure Elucidation, and In Silico Toxicity Prediction of Dapagliflozin Propane Diol Hydrolytic Degradation Products [84]	**HPLC and LC-MS/MS** **Colum:** Agilent Zobrax Eclipse C8 analytical column (dimension 150 mm × 4.6 mm, 5 µm particle size). **Mobile phase:** Phosphate buffer: Acetonitrile (pH 3.5) (50:50, v/v) **Flow rate :** 1 ml/min **Detection Wavelength:** 237 nm. **Degradation Condition:** **Acidic Condition:**2 N HCl at 60 °C reflux for 1 day **Alkali Condition:** 2 N NaOH at 60 °C reflux for 1 day **Retention time of Dapagliflozin:** 8.2 min **Acidic Degradation product Retention Time:** 9.3 min (DP3) **Alkali Degradation product Retention Time:** 6.9 min(DP1),7.5 min (DP2)
Findings: Acid and Alkali degradation product  • DP1 was formed by attack of -OH with loss of H_2 and from Dapagliflozin with intact oxane ring. • DP2 form by attack of OH- which lead to oxane ring opening. • DP 3 was formed from Dapagliflozin by attack of H+ on oxygen of oxane ring which lead to oxane ring opening in acid degradation condition.		

CHAPTER 4

RATIONALE,
AIM AND OBJECTIVE

4. RATIONALE, AIM AND OBJECTIVE

4.1 RATIONALE

- Isolation and characterization of impurities is required for acquiring and evaluating data that establishes **biological safety** which reveals the need and scope of impurity profiling of drugs in pharmaceutical research.
- Anti diabetic drugs may also have Impurities like process related impurities, degradation impurities in bulk drugs as well as in formulations.
- Work on Impurity profiling of Sodium glucose co-transporter 2 (SGLT2) inhibitors category of Anti-diabetics like Empagliflozin, Dapagliflozin etc, is one of the such aspect and need to be done.
- So, In present work, we aimed to develop an analytical method which is capable to identify impurities present in above listed drugs and enable us to isolate and characterize those impurities as well.

4.2. AIM OF PRESENT WORK

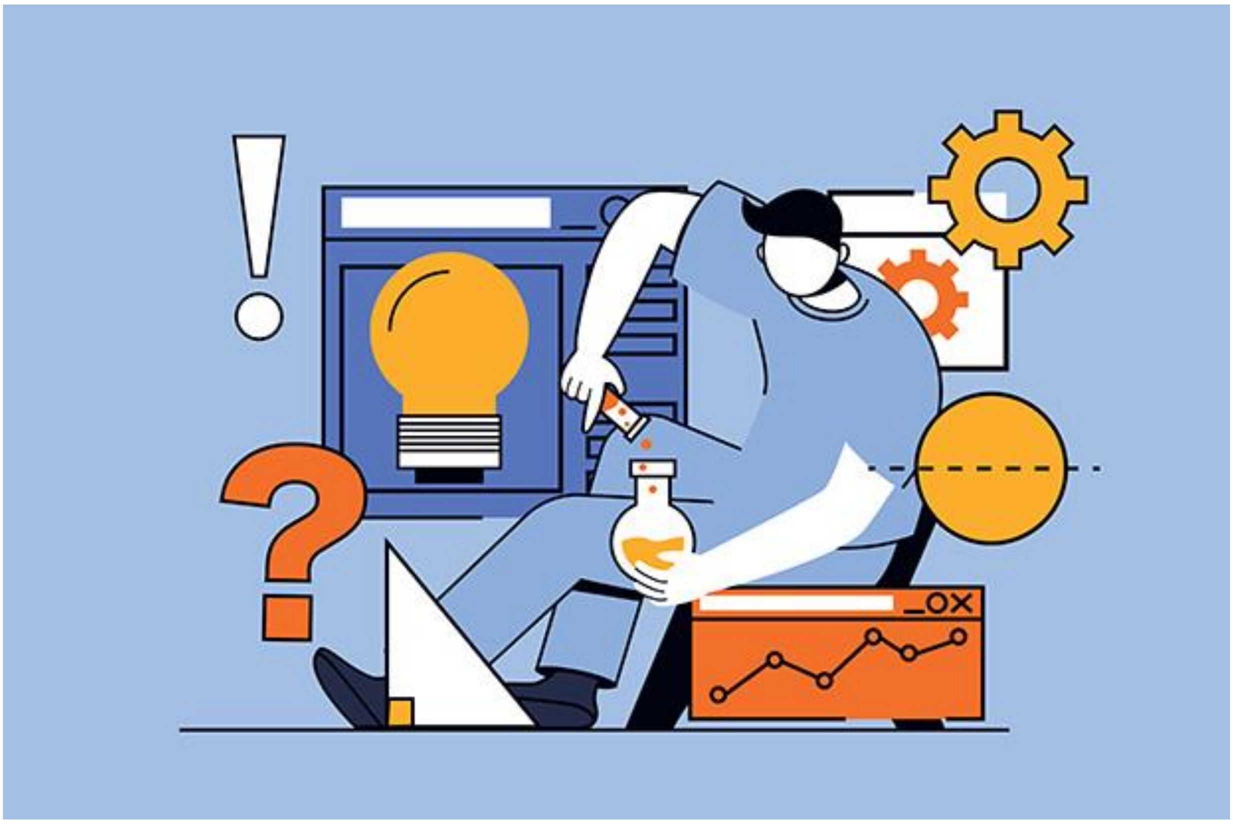
The aim of the work undertaken is to identify process impurities and degradation impurities present in selected drugs and formulations used in the treatment of Diabetes.

4.3. OBJECTIVE OF WORK

- To develop and validate method for selected anti-diabetic drugs as per ICH Guidelines.
- To carry out stress degradation study of selected drugs as per ICH guidelines.
- To isolate major degradation related impurities by Preparative Techniques.
- To characterize major impurities by IR, ^{1}H NMR and Mass Spectroscopy.

CHAPTER 5

MATERIAL AND METHODS



5. MATERIAL AND METHOD

5.1 INSTRUMENT AND APPARATUS

Table No: 5.1 shows a list of instruments and apparatus utilized in method development.

Table No: 5.1 List of instruments and apparatus

Sr. No.	Instrument	Model No.	Manufacturer
1	UV Spectrophotometer	UV-1800	Shimadzu
2	HPLC	LC-2010A HT	Shimadzu (Japan)
3	FT-IR	FTIR-8400S	Shimadzu (Japan)
4	Ultra Sonicator	D 120/1H	Trans-O-Sonic
5	Melting Point	VMP-PM	VEEGO
6	Analytical Weighing Balance	CP2250D	Sartorius, Germany
7	Hot Air Oven	-	Ele Instrument PVT LTD
8	Detector	UV	Shimadzu
9	Column	C-18 (150mm x 4.6 mm)	YMC ODS A

5.2 MATERIALS AND REAGENTS

Table No: 5.2 shows a list of materials and reagents utilized in method development.

Table No: 5.2 List of reagents

Sr. No	Reagents	Grade	Manufacturer
1	Acetonitrile	HPLC Grade	Finar
2	Methanol	HPLC Grade	Finar
3	Water	HPLC Grade	Milli-Q
4	Sodium Hydroxide	AR Grade	Ranken
5	Hydrochloric Acid	AR Grade	Ranken
6	Hydrogen Peroxide	AR Grade	Ranken

5.3. PROCUREMENT OF DRUGS

- Active Pharmaceutical Ingredient (API) Empagliflozin API was Purchased from Simson Pharma Limited, Mumbai.
- Active Pharmaceutical Ingredient (API) Dapagliflozin API was Purchased from Benzchem Enterprize, Vadodara.

5.4. IDENTIFICATION AND CHARACTERIZATION OF API

1. Melting Point
2. IR Spectra.
3. Solubility of Drugs

5.4.1. MELTING POINT DETERMINATION

The melting point of Empagliflozin and Dapagliflozin was performed by using melting point apparatus. First of all, the Capillary was sealed from the bottom using heat applied to the bottom, after that powder was filled into the capillary,and then the capillary was located into the light paraffin bath into the apparatus. Now atwhich temperature Empagliflozin and Dapagliflozin starts to melt and at the temperature at which Empagliflozin and Dapagliflozin completely melted that range recorded

5.4.2 INFRWERED SPECTRAL ANALYSWAS

Identification of Empagliflozin and Dapagliflozin carried out by performing IR Spectroscopy of given API of Empagliflozin and Dapagliflozin for that FTIR-8400S instrument was used. Sample preparation was KBr Dispersion by taking KBr as a Blank. Following was the FTIR Spectrum which shows stretching and bending peak which confirms the presence of functional groups in Empagliflozin and Dapagliflozin.

5.4.3 SOLUBILITY OF DRUGS

The solubility of a drug parameter while developing an analytical method. Solubility study of a drug was done to determine a common solvent. The sample of Vildagliptin, Remogliflozin Etabonate & Metformin Hydrochloride were taken in different test tubes and observed for solubility in various solvents described in the Table.

Table No: 5.3 Solubility as per IP'2010 Specification [85]

Sr. No.	Description terms	Relative Quantities of solvent for 1 part of solute	mg/ml
1	Very soluble	Less than 1 part	10 mg/10 ml
2	Freely soluble	From 1 to 10 parts	10 mg/100 ml
3	Soluble	From 10 to 30 parts	10 mg/100-300 ml
4	Sparingly soluble	From 30 to 100 parts	10 mg/300-1000 ml
5	Slightly soluble	From 100 to 1000 parts	10 mg/1000-10000 ml
6	Very slightly soluble	From 1000 to 10000 parts	10 mg/10000-100000 ml
7	Practically insoluble	More than 10000 parts	10 g/>100000 ml

5.5 UV SPECTRA OF DRUGS

- **Sample Preparation:** Take 10 mg Empagliflozin and 10 mg Dapagliflozin API separately in a 100 ml volumetric flask & add 50ml Methanol and sonicate for 10 to 15 min to dissolve. Methanol was used to bring the final volume up to the required level. Further, take 1 ml Empagliflozin, 1 ml Dapagliflozin in 10 ml Volumetric flask separately and dilute with Methanol. Spectrum of Solution taken in the range of 200- 400 nm by taking Methanolas a blank at 1 cm path length.
- **Selection of Detection Wavelength**

  As per the solubility, Drugs were soluble in Methanol, DMSO and Acetonitrile but economically Methanol was more preferable than DMSO and Acetonitrile. In Methanol, Drugs were given good absorbance. Therefore, Methanol was selected as solvent for further process.

- MODEL: Shimadzu - UV-1800 double beam spectrophotometer connected to a computer loaded with UV Probe 2.34 software.

5.6 SELECTION OF ELUTION MODE

Reverse phase chromatography was not only simple, convenient but also better in terms of efficiency, stability and reproducibility. C18 column was selected because less retentive and it was less polar compare to C4 and C8 columns. C18 column allows eluting non-polar compounds more quickly compere to polar compounds. In additions to this, UV detector was used, which allows easy detection of the compounds in UV transparent organic solvents. A 150 mm x 4.6 mm column of 5 µm particles packing was preferred as a starting point for method development. isocratic mode was chosen due to simplicityin application and robustness with respect to longer column stability. This configuration provides a large number of theoretical plates values for most separation.

5.7 MOBILE PHASE OPTIMIZATION

The mobile phase selection was based on the observation like best peak separation, theoretical plate, resolution, peak symmetry etc. So, number of trials was taken for the selection of mobile phase. Various solvent ratios of Acetonitrile and HPLC grade water were tried as mobile phase for separation in HPLC (Make: Shimadzu, Model: LC-2010A HT)

5.8 PREPARATION OF STANDARD SOLUTIONS OF API

5.8.1 Preparation of Standard Stock Solution of Empagliflozin (1000 µg/ml)

100 mg of Empagliflozin was weighed and transferred to 100 ml volumetric flask. It was dissolved in Methanol and volume was made up to the mark with diluent to give a solution containing 1000 µg/ml.

5.8.2 Preparation of Working Standard Solution of Empagliflozin (100 µg/ml)

Take 10 ml from Stock solution of Empagliflozin and transferred to 100 ml volumetric flask. It was dissolved in Methanol and volume was made up to the mark with diluent to give a solution containing 100 µg/ml.

5.8.3 Preparation of Standard Stock Solution of Dapagliflozin (1000 µg/ml)

100 mg of Dapagliflozin was weighed and transferred to 100 ml volumetric flask. It was dissolved in Methanol and volume was made up to the mark with diluent to give a solution containing 1000 µg/ml.

5.8.4 Preparation of Working Standard Solution of Dapagliflozin (100 µg/ml)

Take 10 ml from Stock solution of Dapagliflozin and transferred to 100 ml volumetric flask. It was dissolved in Methanol and volume was made up to the mark with diluent to give a solution containing 100 µg/ml.

5.9 PREPARATION OF STANDARD SOLUTIONS FROM MARKETED FORMULATION

5.9.1 Preparation of Standard Stock Solution of Empagliflozin Marketed formulation (JARDIANCE) (1000 µg/ml)

20 Tablets of Empagliflozin (JARDIANCE) were weighed and powdered. From the Tablet powder 100 mg equivalent amount of Empagliflozin was transferred into a 100 ml volumetric flask. 50 ml of Methanol was added and sonicated for 20 min to dissolved the entire drug, Filter it and finally made up to the final volume with Methanol to get final concentration 1000 µg/ml.

5.9.2 Preparation of Working Standard Solution of Empagliflozin Marketed formulation (JARDIANCE) (100 µg/ml)

Take 10 ml from Stock solution of Empagliflozin in 100 ml Volumetric Flask. Dilute up to the mark with Methanol to get final concentration 100 µg/ml.

5.9.3 Preparation of Standard Stock Solution of Dapagliflozin Marketed formulation (DAPARYL 10) (1000 µg/ml)

20 Tablets of Dapagliflozin (DAPARYL 10) were weighed and powdered. From the Tablet powder 100 mg equivalent amount of Dapagliflozin was transferred into a 100 ml volumetric flask. 50 ml of Methanol was added and sonicated for 20 min to dissolved the entire drug, Filter it and finally made up to the final volume with Methanol to get final concentration 1000 µg/ml.

5.9.4 Preparation of Working Standard Solution of Dapagliflozin Marketed formulation (DAPARYL 10) (100 µg/ml)

Take 10 ml from Stock solution of Dapagliflozin in 100 ml Volumetric Flask. Dilute up to the mark with Methanol to get final concentration 100 µg/ml.

5.10 FORCED DEGRADATION

5.10.1 Preparation of solutions

5.10.1.1 Preparation of 0.1 N HCl

Precisely take 0.85 ml of HCl and transfer it to 100 ml volumetric flask and add 70% of diluent (water) and sonicate for 5 minutes and then add diluent up-to mark, and then mix well.

5.10.1.2 Standardization of 1 N HCl

Precisely take 8.5 ml of HCl and transfer it to 100 ml volumetric flask and add 70% of diluent (water) and sonicate for 5 minutes and then add diluent up-to mark, and then mix well.

5.10.1.3 Standardization of 2 N HCl

Precisely take 17 ml of HCl and transfer it to 100 ml volumetric flask and add 70% of diluent (water) and sonicate for 5 minutes and then add diluent up-to mark, and then mix well.

5.10.1.4 Preparation of 0.1 N NaOH

Precisely take 0.4 gm of NaOH and transfer it to 100 ml volumetric flask and add 70% of diluent (water) and sonicate for 5 minutes and then add diluent up-to mark, and then mix well.

5.10.1.5 Preparation of 1 N NaOH

Precisely take 4 gm of NaOH and transfer it to 100 ml volumetric flask and add 70% of diluent (water) and sonicate for 5 minutes and then add diluent up-to mark, and then mix well

5.10.1.5 Preparation of 2 N NaOH

Precisely take 8 gm of NaOH and transfer it to 100 ml volumetric flask and add 70% of diluent (water) and sonicate for 5 minutes and then add diluent up-to mark, and then mix well.

5.10.1.6 Preparation of 3 % - 20% H_2O_2

Accurately take 10 ml and 2 ml of 30 % H_2O_2 in different 100 mL volumetric flask and dissolve in water, sonicate, and made up the mark with distilled water to prepare 3% and 20 % H_2O_2 solution, respectively.

5.10.2 Preparation of Standard solutions for API and Marketed formulation Degradation study

5.10.2.1 Acid Degradation Study

Take 1 mL of stock solution of Empagliflozin and Dapagliflozin in 10 mL volumetric flask Separately, add 1 ml of 0.1 N HCl in each volumetric flask and keep it at 60℃ and 80℃ for 6,12,18,24 hr, after that it was neutralized by adding 1 ml 0.1 N NaOH in each Volumetric Flask. Make up the volume up to the mark with Mobile Phase to achieve a final concentration of 100 µg/ml of Empagliflozin and Dapagliflozin individually. Same procedure was repeated for 1 N HCL and 2 N HCl for both the drugs in their API form and its Marketed formulation.

5.10.2.2 Alkaline Degradation Study

Take 1 mL of stock solution of Empagliflozin and Dapagliflozin in 10 mL volumetric flask Separately, add 1 ml of 0.1 N NaOH in each volumetric flask and keep it at 60℃ and 80℃ for 6,12,18,24 hr, after that it was neutralized by adding 1 ml 0.1 N HCl in each Volumetric Flask. Make up the volume up to the mark with Mobile Phase to achieve a final concentration of 100 µg/ml of Empagliflozin and Dapagliflozin individually. Same procedure was repeated for 1 N NaOH and 2 N NaOH for both the drugs in their API form and its Marketed Formulation.

5.10.2.3 Oxidative Degradation Study

Take 1 mL of stock solution of Empagliflozin and Dapagliflozin in 10 mL volumetric flask separately, add 1 ml of 20 % H_2O_2 in pre-labelled flask respectively as per the study protocol, after completion of fix time duration for 24 hr for 60℃ and 80℃. add 0.1 N KMnO4, in a flask for neutralization. Make up the volume up to the mark with Mobile phase to achieve a final concentration of 100 µg/m of Empagliflozin and Dapagliflozin individually. Same procedure was repeated for 30 % H_2O_2 for both the drugs in their API form and its Marketed Formulation.

5.10.2.4 Thermal Degradation Study

Take 10 mg of Empagliflozin and Dapagliflozin in dry state in separate Petri plate and expose in Hot air Oven at 60ºC for 24 hr, after that dilution made up to 10 ml with Methanol to get concentration 100 µg/ml of Empagliflozin and Dapagliflozin individually. Same Procedure repeated for 80ºC for both the drugs in API form. For Marketed formulation take 10 mg equivalent weight for both drugs from their Tablet power in different Petri plate and repeat above procedure.

5.10.2.5 Photo Degradation Study

Take 10 mg of Empagliflozin and Dapagliflozin in dry state in separate Petri plate and expose in UV Chamber for 7 days, after that dilution up to 10 ml with Methanol to get concentration 100 µg/ml of Empagliflozin and Dapagliflozin individually in API form. For Marketed formulation take 10 mg equivalent weight for both drugs from their Tablet power in different Petri plate and repeat above procedure.

5.11. VALIDATION OF PROPOSED METHOD

Parameters to be considered for the validation of method were:

5.11.1 System Suitability Studies (n=6)

The system suitability was evaluated by six replicate analyses at concentration of 100 µg/ml for Empagliflozin and Dapagliflozin individually. The column efficiency, resolution, and peak asymmetry were calculated for the standard solutions.

5.11.2 Linearity and Range (n=6)

The linearity response was determined by analysing 5 independent levels of concentrations in the range of 50-150 µg/ml for Empagliflozin and 5-150 µg/ml for Dapagliflozin.

5.11.2.1 Preparation of Solutions for Calibration curves for Empagliflozin

The calibration curve was constructed with six concentrations ranging from 50-150 µg/ml for Empagliflozin. For that take 5, 7.5,10,12.5,15 ml working standard solution and transfer it in to 5 different 10 ml Volumetric Flask. Dilute all Solutions up to the mark with Mobile phase to get Concentration of 50,75,100,125,150 µg/ml. All solutions were injected in HPLC and the data of peak area versus concentration were treated by linear least square regression analysis.

5.11.2.2 Preparation of Solutions for Calibration curves for Dapagliflozin

The calibration curve was constructed with six concentrations ranging from 5-150 µg/ml for Empagliflozin. For that take 0.5, 2.5, 5, 7.5,10,12.5,15 ml working standard solution and transfer it in to 7 different 10 ml Volumetric Flask. Dilute all Solutions up to the mark with Mobile phase to get Concentration of 5,25, 50,75,100,125,150 µg/ml. All solutions were injected in HPLC and the data of peak area versus concentration were treated by linear least square regression analysis.

5.11.3 Accuracy (n=3)

The accuracy of the approach was assessed using recovery experiments from commercial formulations at three levels (50%, 100%, and 150%) of standard addition as per ICH Guidelines.

Weigh 20 Tablet. Finely crush each Tablet in a mortar & Pastel. Put the powder and the equivalent of 10 mg Empagliflozin in to 100 ml volumetric flask, add 50 ml methanol as diluent. Use moderate shaking to dissolve the material and Filter it. Further dilution made with Methanol up to the mark to get final concentration100 g/ml of Empagliflozin. 'Sample Stock - A' (Test solution)

Take 10 mg Empagliflozin in to 100 ml volumetric flask, add 50 ml methanol as diluent. Sonicate to dissolve the material. further dilution made with Methanol up to the mark to get final concentration 100 g/ml of Empagliflozin. (Standard Stock – A)

From the above 'Sample Stock - A' (Test solution) solution 5 ml of the aliquot was pipette out and transferred to three different 10 ml volumetric flask and spikes with 2.5, 5, 7.5 ml of aliquot from the ' Standard Stock - A'. The volume was adjusted using Methanol.

The solution's concentration for Empagliflozin was 75, 100, and 150 mg/ml was injected in to HPLC by using parameters as per developed method. The Peak area was taken into consideration when calculating the percent recoveries from these data. Data from three concentration levels covering the specified range was determined and % recovery was calculated.

Same procedure was followed for accuracy study of Dapagliflozin using API and its Marketed formulation at three levels (50%, 100%, and 150%) of standard addition.

5.11.4 Precision

5.11.4.1 Repeatability: (n=6)

Repeatability of the developed method was assessed by analysing samples from the same batch 6 times (n=6) with standard solutions containing concentrations 100 µg/ml for Empagliflozin and Dapagliflozin separately and % R.S.D. was calculated for both drugs.

5.11.4.2 Intraday: (n=3)

It was assessed by analyzing samples from the same batch with three standard solutionscontaining concentrations 50,100 and 150 µg/ml Empagliflozin and 75,100 and 125 µg/ml Dapagliflozin. Solutions were analyzed thrice (n=3) on the same day within short interval of time and % R.S.D. was determined when samples were analyzed at several time periods throughout the day: 0 hr, 3 hr and 6 hr.

5.11.4.2 Interday: (n=3)

It was assessed by analyzing samples from the same batch with three standard solutionscontaining concentrations 50,100 and 150 µg/ml Empagliflozin and and 75,100 and 125 µg/ml Dapagliflozin. Solutions were analyzed thrice (n=3) on the three different day and % R.S.D. was determined.

5.11.5 LOD & LOQ

LOD was estimated from the set of 5 calibration curves. LOD was calculated by using formula…

LOD = 3.3 x SD/Slope

LOQ was estimated from the set of 5 calibration curves. LOQ was calculated byusing formula…

LOQ = 10 x SD/Slope

Where, SD = Standard deviation of absorbance

Slope = Slope of calibration curve

5.11.6. Robustness

According to ICH, the robustness of analytical procedure was defined as the capability to remain unchanged by small and deliberate changes in method parameters. In this method there was three different changes were done and % R.S.D. was determined, which were as follows:

A. Changes in Wavelength (± 2nm)

B. Changes in Mobile Phase Composition. (± 2 ml)

C. Changes in Flow rate. (± 0.1 ml/min.)

5.11.7 Specificity

In the case of assay, demonstration of specificity was required to show that the procedure was unaffected by the presence of impurities or excipients. Specificity of an analytical method indicates that the analytical method was able to measure accurately and specifically the analyte of interest without any interference from blank. So, here, the specificity was determined by the comparison of the chromatograms of…

- Blank (Mobile phase).
- Standard solutions of Empagliflozin and Dapagliflozin
- Sample solution of Empagliflozin and Dapagliflozin Marketed Formulation

5.12 ASSAY OF MARKETED FORMULATION (n=3)

5.12.1 Assay of Marketed formulation of Empagliflozin(JARDIANCE TABLETS)

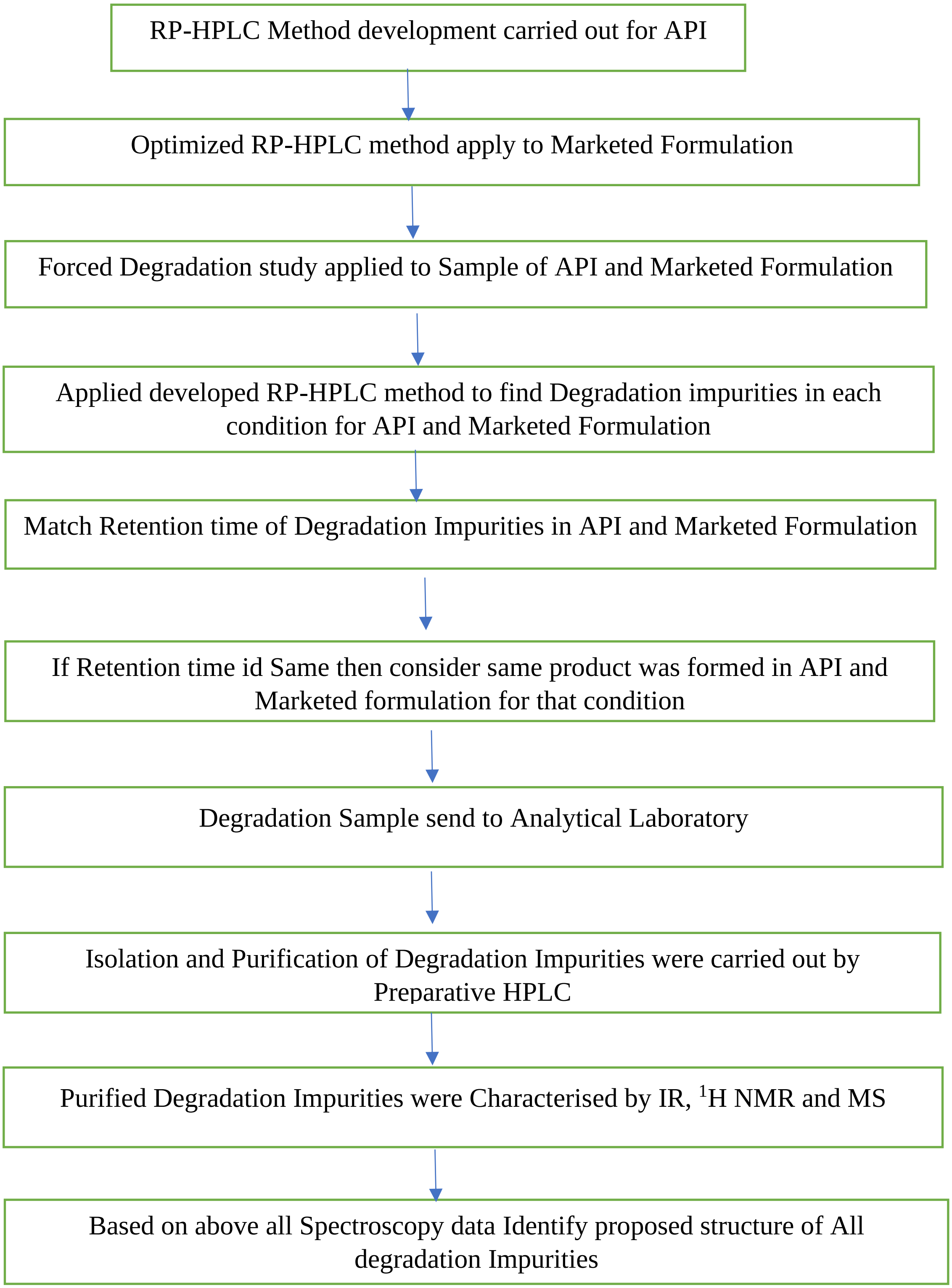
For the estimation of drugs in the commercial formulation (JARDIANCE TABLETS), 20 Tablets were weighted accurately. The average weight was calculated and then crushed to obtain fine powder.A quantity of Tablet powder equivalent to about 25 mg Empagliflozin was transferred to 100 ml volumetricflask; 50 ml Methanol was added and sonicate for 10-15 min, volume was made up tothe mark with diluent to get 250 µg/ml for Empagliflozin and the solution filtered through 0.45µm nylon membrane filter. This solution was used at stock solution 1 ml of aliquot solution was pipetted out and transferred to a 10 ml volumetric flask. Then the volume made up to the mark with diluent to get sample solution containing 25 µg/ml of Empagliflozin. Solutions were analyzed for three times and % R.S.D. was determined.

5.12.2 Assay of Marketed formulation of Dapagliflozin(DAPARYL 10 TABLETS)

For the estimation of drugs in the commercial formulation (DAPARYL 10 TABLETS), 20 Tablets were weighted accurately. The average weight was calculated and then crushed to obtain fine powder.A quantity of Tablet powder equivalent to about 10 mg Empagliflozin was transferred to 100 ml volumetricflask; 50 ml Methanol was added and sonicate for 10-15 min, volume was made up tothe mark with diluent to get 100 µg/ml for Empagliflozin and the solution filtered through 0.45µm nylon membrane filter. This solution was used at stock solution 1 ml of aliquot solution was pipetted out and transferred to a 10 ml volumetric flask. Then the volume made up to the mark with diluent to get sample solution containing 10 µg/ml of Empagliflozin. Solutions were analyzed for three times and % R.S.D. was determined.

5.13 Detection, Isolation and Characterization of Degradation Impurities of Empagliflozin and Dapagliflozin

Detection, Isolation and Characterization of Degradation impurities were carried out for both drug by using following method:



by using following chromatographic condition.:
An RP-HPLC system (make: Shimadzu, model- LC-2010) which was operated using a software, LC Solution, fitted with YMC ODS A C-18 (150mm x 4.6mm) Column and UV Detector (at 224 nm for Empagliflozin and 273 nm for Dapagliflozin) was used for the analysis. The mode was isocratic. A mixture (50:50) of Acetonitrile and Water was used as mobile phase with flow rate 0.5 ml/min for Empagliflozin and 0.7 ml/min for Dapagliflozin, respectively.

Isolation and Purification of all degradation Impurities were carried out by Waters semi-preparative HPLC with UV detector. All Isolated pure fractions were lyophilized using Lyofreeze lyophilizer.

Characterisation of all degradation impurities were carried out by Shimadzu IR-8400 S model with lab solutions software to recognize the functional groups present in the compounds. KBr was used as a dispersion medium to prepare the sample pellets. Mass Spectra were recorded on MS-ESI Mass Spectrometer using Empower 3.0 Software and ^{1}H NMR spectra of degradation Impurities were analysed in DMSO-d6 solvent on Bruker Avance Neo 400 MHz at K M Solutions, Ahmedabad.

CHAPTER 6

RESULTS AND DISCUSSION

6.RESULTS AND DWASCUSSION

6.1 MELTING POINT DETERMINATION

- Determination was done by Melting Point Apparatus.
- Model: VEEGO VMP-PM

Table No: 6.1 Melting point determination

Drug name	Observed Melting point	Standard Melting point
Empagliflozin	152-154ºC	151-153 ºC [50]
Dapagliflozin	66-68 º C	65-69º C [51]

6.2 SPECTRAL ANALYSWAS (INFRWERED SPECTRA SCANNING)

The infrared spectrum of Empagliflozin and Dapagliflozin were obtained in a Potassium Bromide drifted method, Shimadzu-8400 FT-IR spectrometer 4000-400 cm^{-1}.

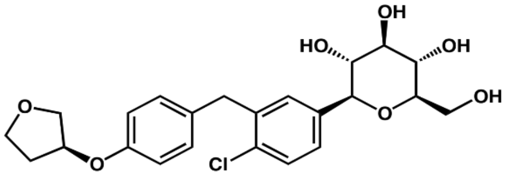


Figure No:6.1 Structure of Empagliflozin

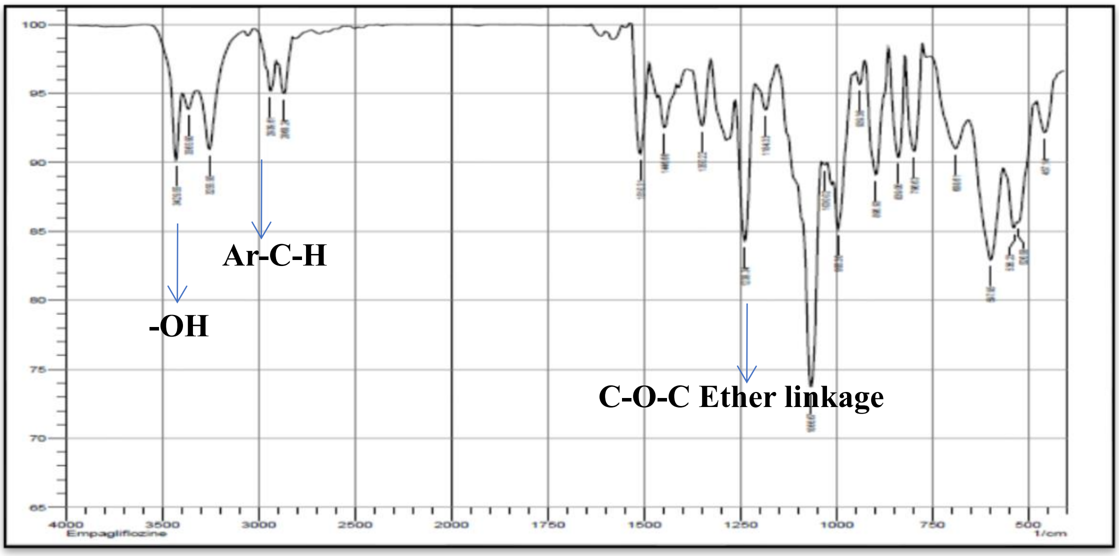


Figure No:6.2 IR Spectra of Empagliflozin

Table No: 6.2 Interpretation of IR for Empagliflozin

Functional Group	Standard Range (cm^{-1}) [33]	Observed value (cm^{-1})
-OH	3200-3600	3429.55
Ar- C-H	2850-2970	2939.61
C-O-C (Ether linkage)	1040-1250	1239.34

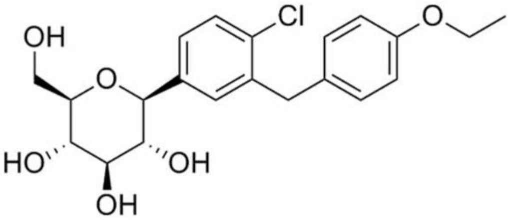


Figure No:6.3 Structure of Dapagliflozin

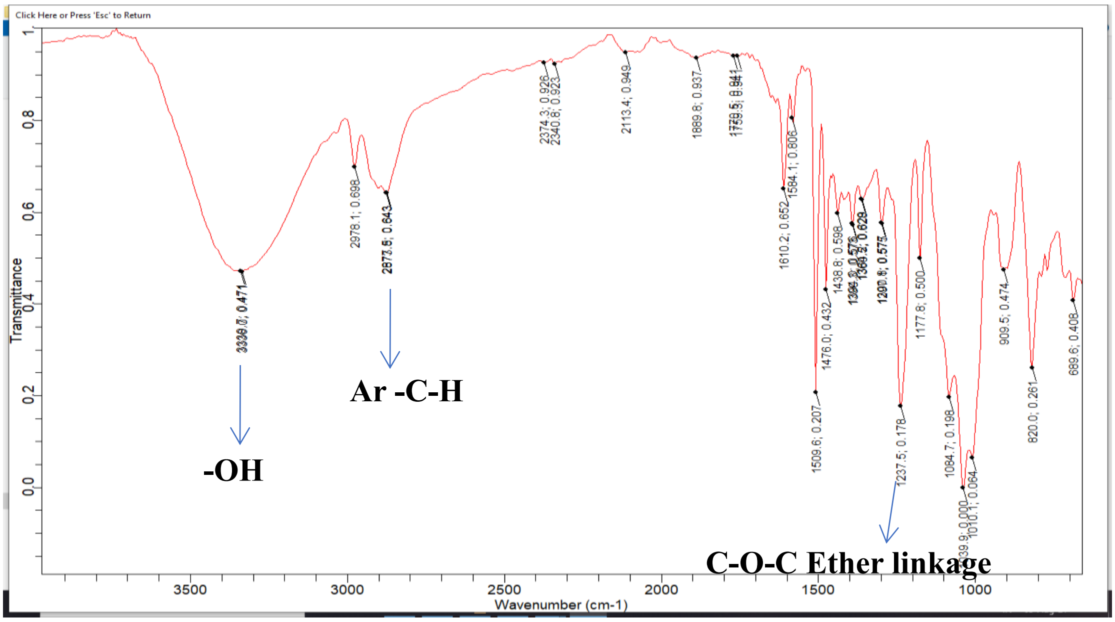


Figure No:6.4 IR Spectra of Dapagliflozin

Table No: 6.3 Interpretation of IR for Dapagliflozin

Functional Group	Standard Range (cm^{-1}) [33]	Observed value (cm$^{-1)}$
-OH	3200-3600	3330
- C-H	2850-2970	2873.6
C-O-C (Ether linkage)	1040-1250	1237.5

6.3 SOLUBILITY DETAILS OF DRUGS

Table No: 6.4 Observed solubility of drugs

Solvent	**Solubility**	
	Empagliflozin	**Dapagliflozin**
Water	Slightly soluble	Slightly soluble
Methanol	soluble	soluble
DMSO	Freely soluble	Freely soluble
Acetonitrile	soluble	Freely soluble

6.4 SELECTION OF WAVELENGTH

- In Methanol, Drug was given good absorbance. Therefore, Methanol was selected as solvent for further process.
- Model: Shimadzu UV-1800 double beam spectrophotometer connected to a computer loaded with UV probe 2.34 software.
- The spectra of Empagliflozin and Dapagliflozin were recorded between 200- 400 nm, and 224 nm and 273 nm were chosen as detection wavelengths for Empagliflozin and Dapagliflozin, respectively.

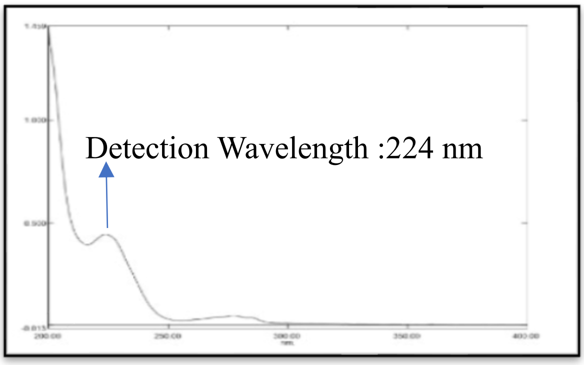


Figure No:6.5 UV Spectra of Empagliflozin

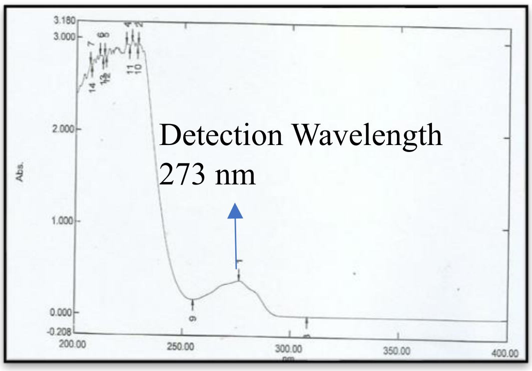


Figure No:6.6 UV Spectra of Dapagliflozin

6.5 MOBILE PHASE OPTIMIZATION TRIALS

6.5.1 Mobile phase optimization trials for Empagliflozin

Various ratios of mobile phase containing Acetonitrile: Water were tried in order to find the best conditions for the separation of drugs. It was found that Acetonitrile: Water (50:50 % v/v) with flow rate 0.5 ml/min for Empagliflozin and 0.7 ml/min for Dapagliflozin gives satisfactory result.

6.5.1.1 Trial 1

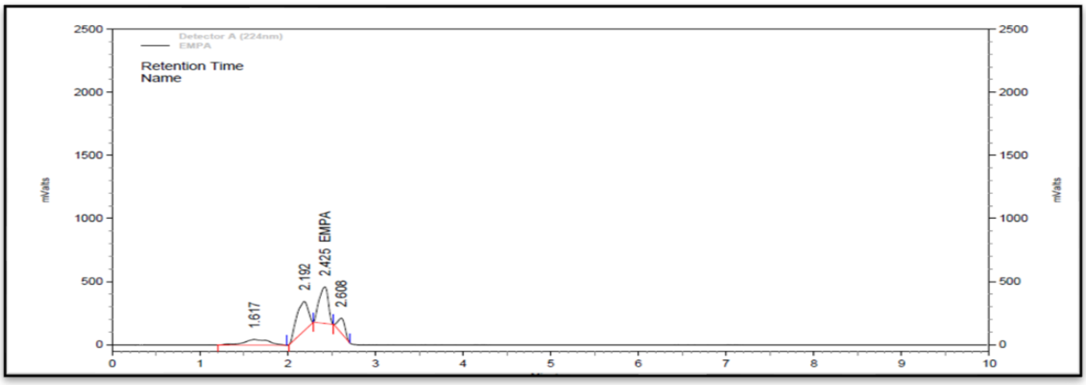


Figure No: 6.7 Trial 1 Chromatogram of Empagliflozin (100µg/ml)

Table No: 6.5 Observed result for Trial 1

Column	Mobile Phase	Flow Rate	Detection Wavelength	Retention time	Peak Description
YMC ODS A C-18 (150mmx4.6mm)	Acetonitrile: Water 60:40 (%v/v)	1 ml/min	224 nm	Not defined	Peak Splitting

6.5.1.2 Trial 2

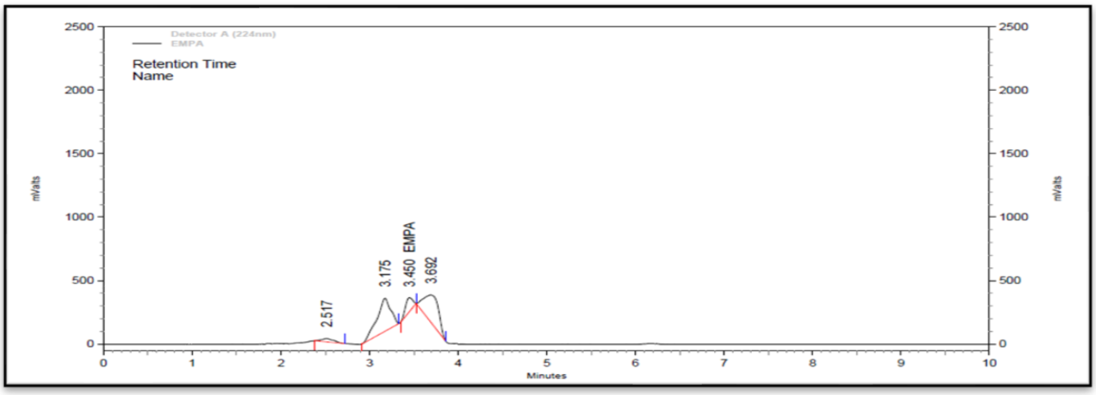


Figure No: 6.8 Trial 2 Chromatogram of Empagliflozin (100µg/ml)

Table No: 6.6 Observed result for Trial 2

Column	Mobile Phase	Flow Rate	Detection Wavelength	Retention time	Peak Description
YMC ODS A C-18 (150mmx4.6mm)	Acetonitrile: Water 60:40 (%v/v)	0.7 ml/min	224 nm	Not defined	Peak Splitting

6.5.1.3 Trial 3

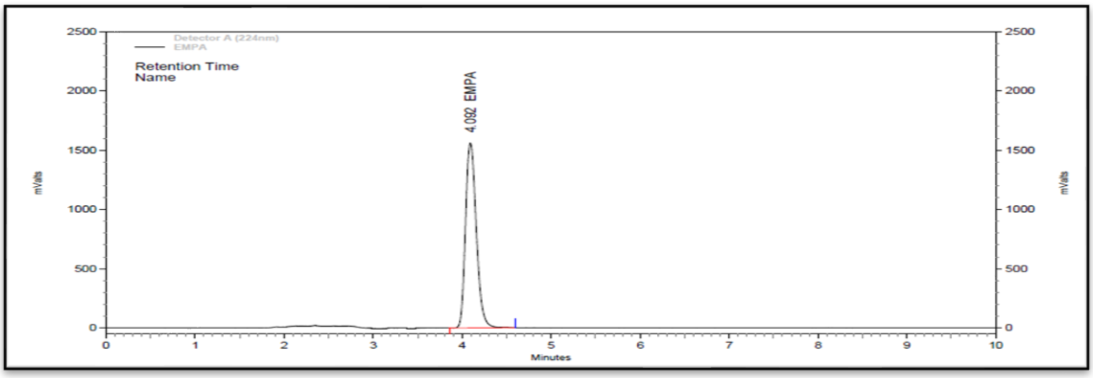


Figure No: 6.9 Trial 3 Chromatogram of Empagliflozin (100µg/ml)

Table No: 6.7 Observed result for Trial 3

Column	Mobile Phase	Flow Rate	Detection Wavelength	Retention time	Peak Description
YMC ODS A C-18 (150mmx4.6mm)	Acetonitrile: Water 50:50 (%v/v)	0.7 ml/min	224 nm	4.09 min	Short retention time

6.5.1.4 Trial 4

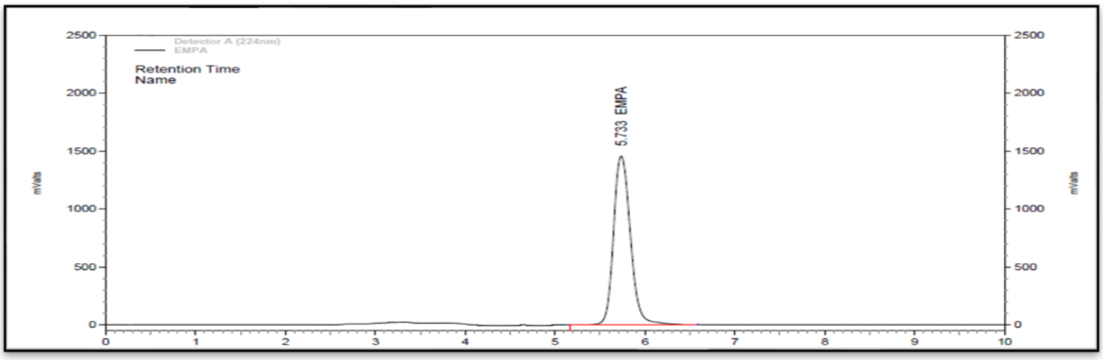


Figure No: 6.10 Optimized Chromatogram of Empagliflozin (100µg/ml)

Table No: 6.8 Observed result for Optimized Trial 4

Column	Mobile Phase	Flow Rate	Detection Wavelength	Retention time	Peak Description
YMC ODS A C-18 (150mmx4.6mm)	Acetonitrile: Water 50:50 (% v/v)	0.5 ml/min	224 nm	5.73 min	Satisfactory

Table No: 6.9 Optimization of HPLC chromatographic condition for Empagliflozin

Sr. No.	Chromatographic Parameter	Optimize Condition
1	Flow rate	0.5 ml/min
2	Detection Wavelength	224 nm
3	Mobile Phase composition	Acetonitrile: Water 50:50 (% v/v)
4	Column	YMC ODS A C-18 (150mmx4.6mm)
5	Column Temperature	40ºC
6	Injection volume	20 µl
7	Diluent	Methanol
8	Retention Time	5.73 min
9	Asymmetry factor	1.10
10	Theoretical Plates	4667

6.5.2 Mobile phase optimization trials for Dapagliflozin

6.5.2.1 Trial 1

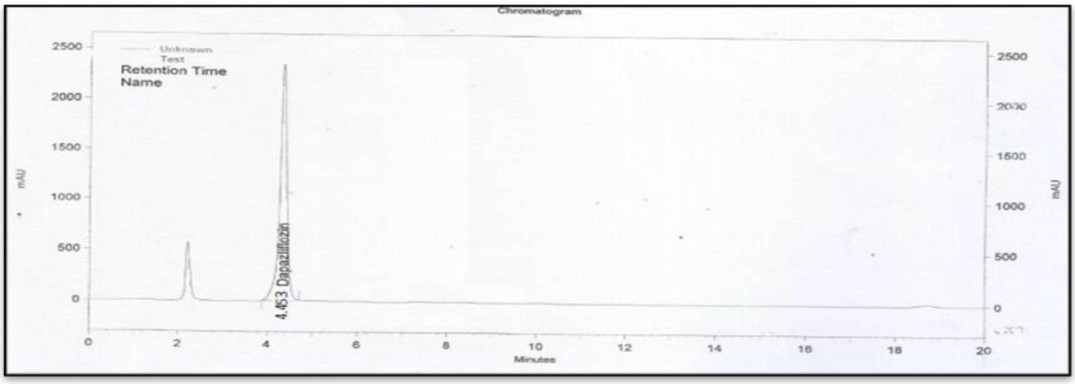


Figure No: 6.11 Trial 1 Chromatogram of Dapagliflozin (100µg/ml)

Table No: 6.10 Observed result for Trial 1

Column	Mobile Phase	Flow Rate	Detection Wavelength	Retention time	Peak Description
YMC ODS A C-18 (150mmx4.6mm)	Acetonitrile: Water 50:50 (% v/v)	1 ml/min	273 nm	4.45 min	Short Retention Time

6.5.2.2 Trial 2

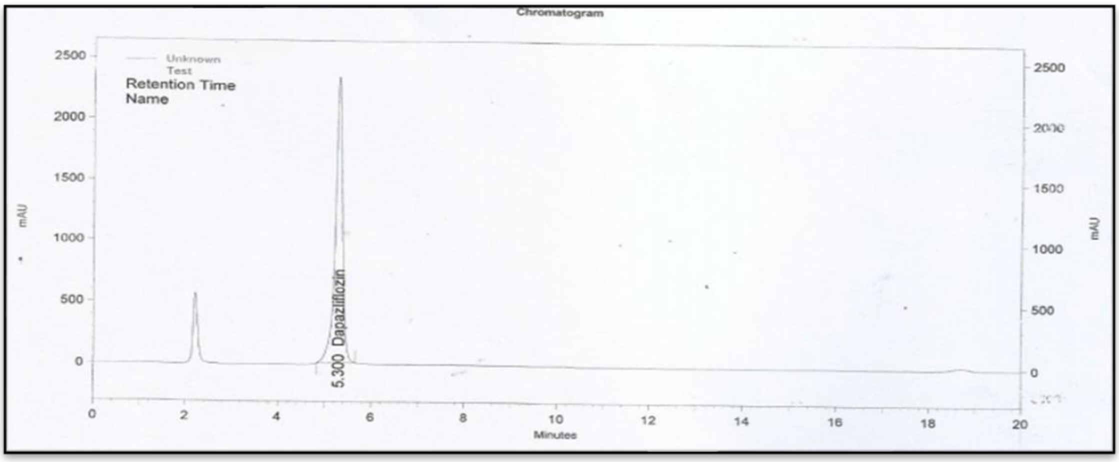


Figure No:6.12 Optimized Chromatogram of Dapagliflozin (100µg/ml)

Table No: 6.11 Observed result for Optimized Trial 2

Column	Mobile Phase	Flow Rate	Detection Wavelength	Retention time	Peak Description
YMC ODS A C-18 (150mmx4.6mm)	Acetonitrile: Water 50:50 (% v/v)	0.7 ml/min	273 nm	5.30 min	Satisfactory

Table No: 6.12 Optimization of HPLC chromatographic condition for Dapagliflozin

Sr. No.	Chromatographic Parameter	Optimize Condition
1	Flow rate	0.7 ml/min
2	Detection Wavelength	273 nm
3	Mobile Phase composition	Acetonitrile: Water 50:50 (% v/v)
4	Column	YMC ODS A C-18 (150mmx4.6mm)
5	Column Temperature	40ºC
6	Injection volume	20 µl
7	Diluent	Methanol
8	Retention Time	5.30 min
9	Asymmetry factor	1.08
10	Theoretical Plates	4932

6.6 IDENTIFICATION OF PEAKS OF API AND MARKETED FORMULATION

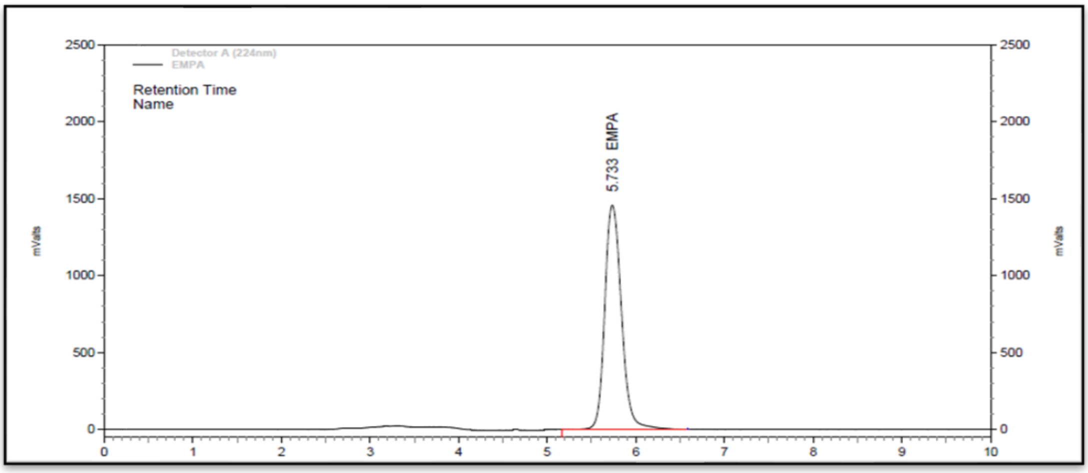


Figure No: 6.13 Optimized Chromatogram of Empagliflozin API (100µg/ml)

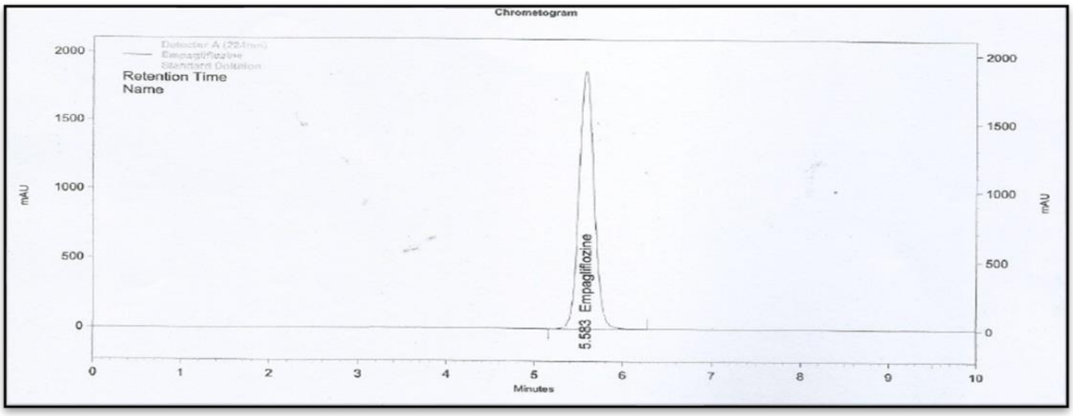


Figure No: 6.14 Optimized Chromatogram of Empagliflozin Tablet (100µg/ml)

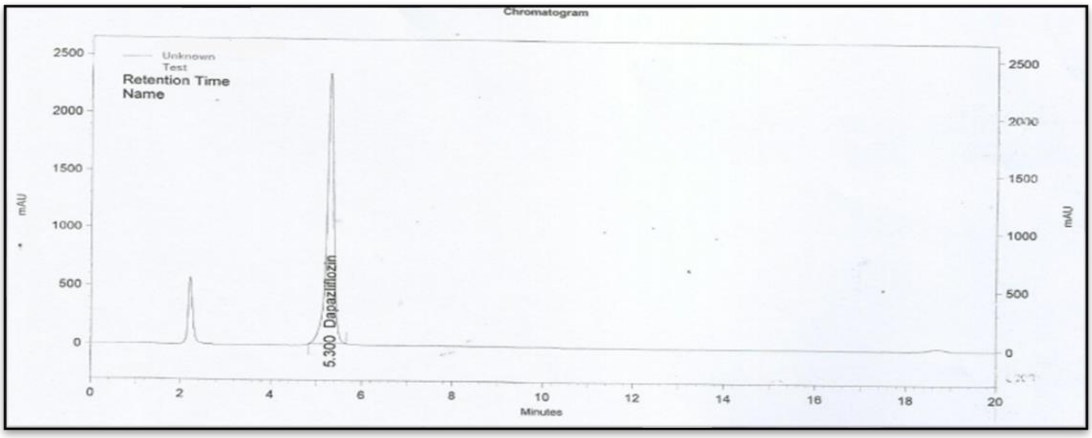


Figure No: 6.15 Optimized Chromatogram of Dapagliflozin API (100µg/ml)

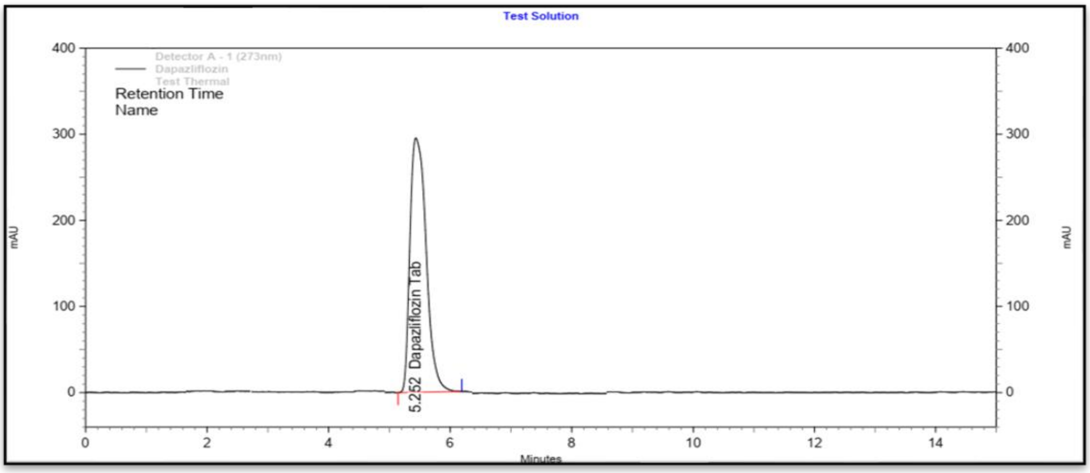


Figure No: 6.16 Optimized Chromatogram of Dapagliflozin Tablet (100µg/ml)

6.7 FORCED DEGRADATION STUDY

6.7.1 Forced Degradation Study of Empagliflozin in API.

6.7.1.1 Acid and Alkali Degradation in 0.1 N HCl and 0.1 N NaOH

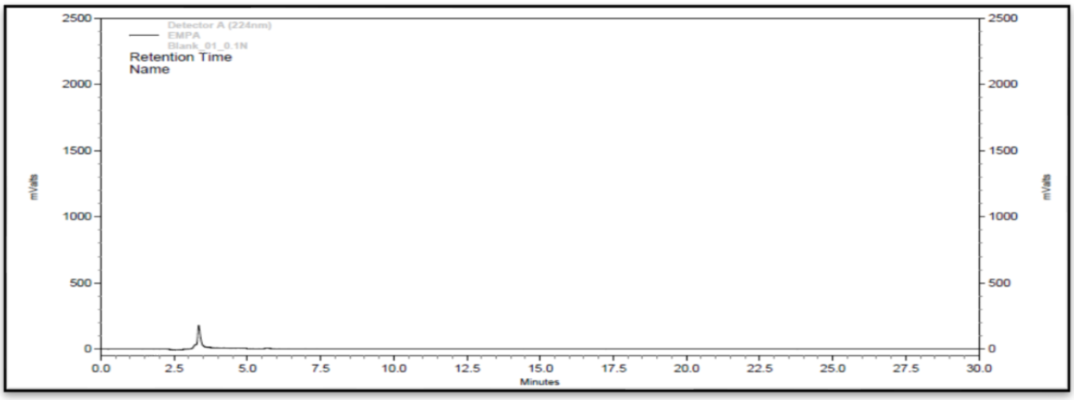


Figure No: 6.17 Chromatogram of 0.1 N Blank Solution

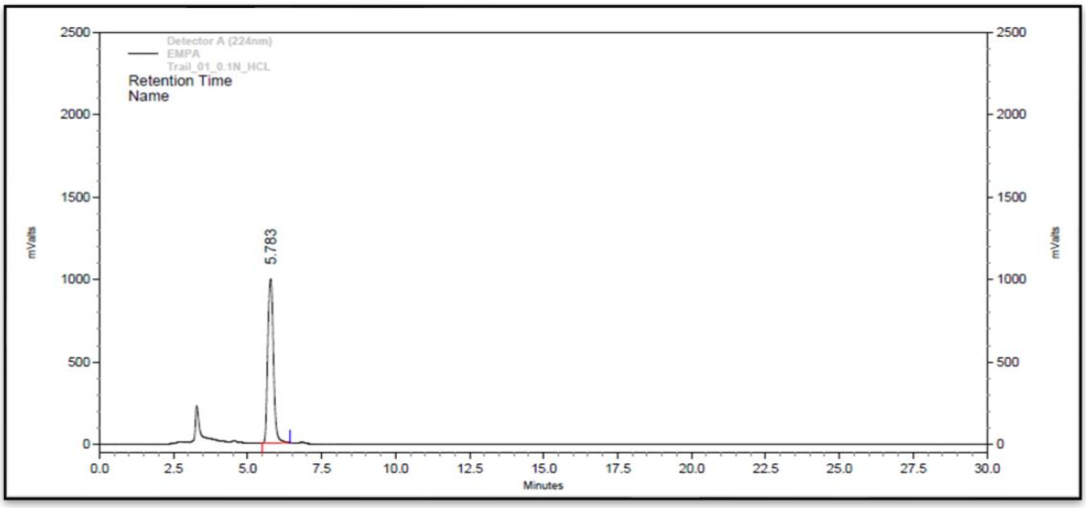


Figure No: 6.18 Chromatogram of acid degradation in 0.1 N HCl for 24 hr at 60ºC

Table No: 6.13 Result of acid degradation in 0.1 N HCl for 24 hr at 60ºC

Preparation of solution	Degradation condition	Observation
1 ml Stock Solution (1000 µg/ml) + 1 ml 0.1 N HCl, After 24 hr Add 1 ml 0.1 N NaOH and Dilute up to 10 ml with Mobile Phase to make final concentration 100µg/ml	In 0.1 N HCl At 60ºC for 24 hr	No Degradation

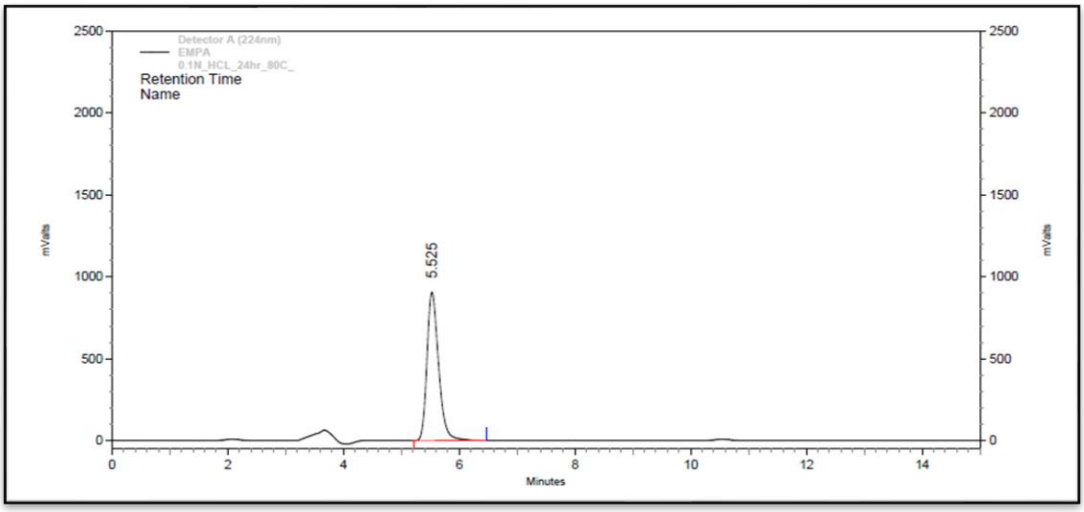


Figure No: 6.19 Chromatogram of acid degradation in 0.1 N HCl for 24 hr at 80ºC

Table No: 6.14 Result of acid degradation in 0.1 N HCl for 24 hr at 80ºC

Preparation of solution	**Degradation condition**	**Observation**
1 ml Stock Solution (1000 µg/ml) + 1 ml 0.1 N HCl, After 24 hr Add 1 ml 0.1 N NaOH and Dilute up to 10 ml with Mobile Phase to make final concentration 100µg/ml	In 0.1 N HCl At 80ºC for 24 hr	No Degradation

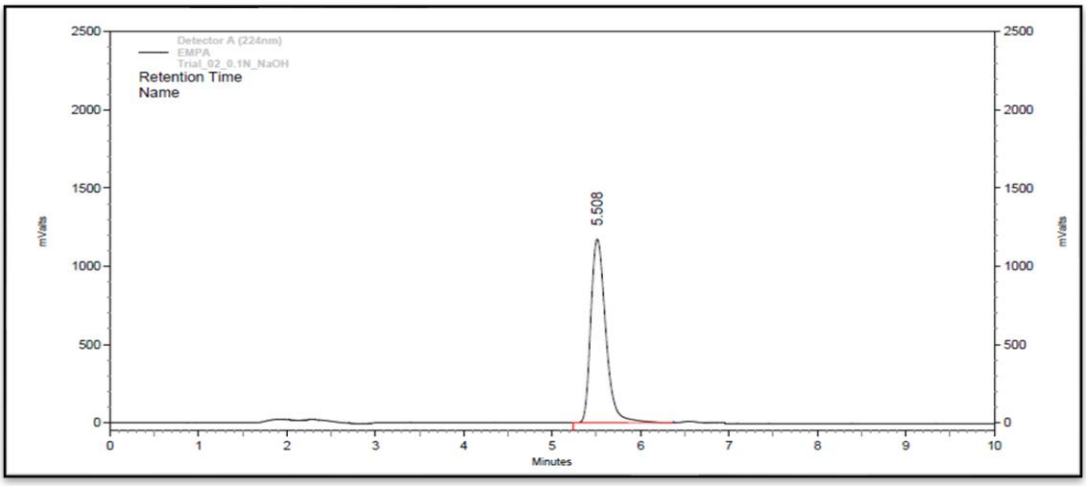


Figure No: 6.20 Chromatogram of alkali degradation in 0.1 N NaOH for 24 hr at 60ºC

Table No: 6.15 Result of alkali degradation in 0.1 N NaOH for 24 hr at 60ºC

Preparation of solution	Degradation condition	Observation
1 ml Stock Solution (1000 µg/ml) + 1 ml 0.1 N NaOH, After 24 hr Add 1 ml 0.1 N HCl and Dilute up to 10 ml with Mobile Phase to make final concentration 100µg/ml	In 0.1 N NaOH At 60ºC for 24 hr	No Degradation

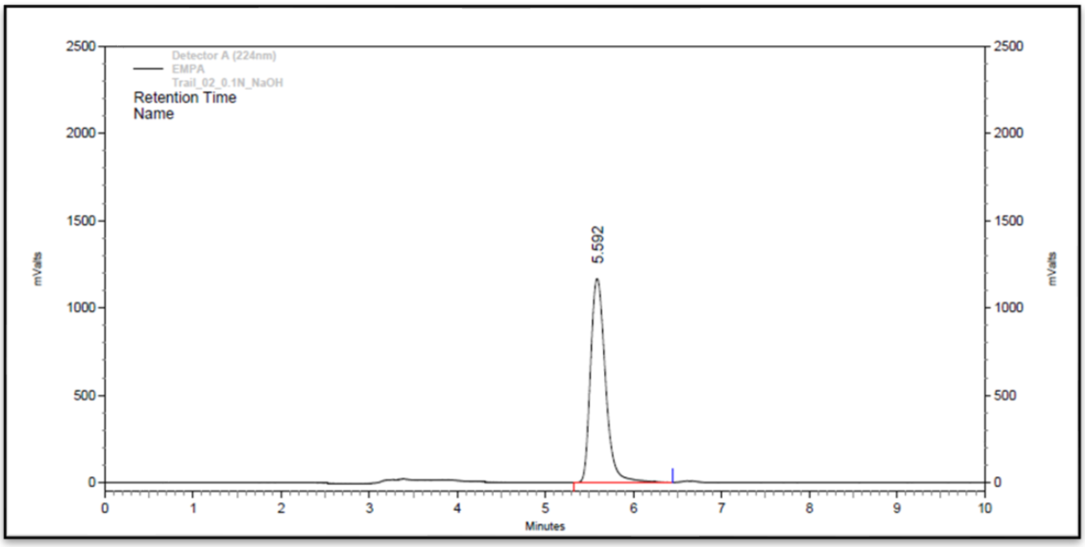


Figure No: 6.21 Chromatogram of alkali degradation in 0.1 N NaOH for 24 hr at 80ºC

Table No: 6.16 Result of alkali degradation in 0.1 N NaOH for 24 hr at 80ºC

Preparation of solution	Degradation condition	Observation
1 ml Stock Solution (1000 µg/ml) + 1 ml 0.1 N NaOH, After 24 hr Add 1 ml 0.1 N HCl and Dilute up to 10 ml with Mobile Phase to make final concentration 100µg/ml	In 0.1 N NaOH At 80ºC for 24 hr	No Degradation

6.7.1.2 Acid and Alkali Degradation in 1 N HCl and 1 N NaOH

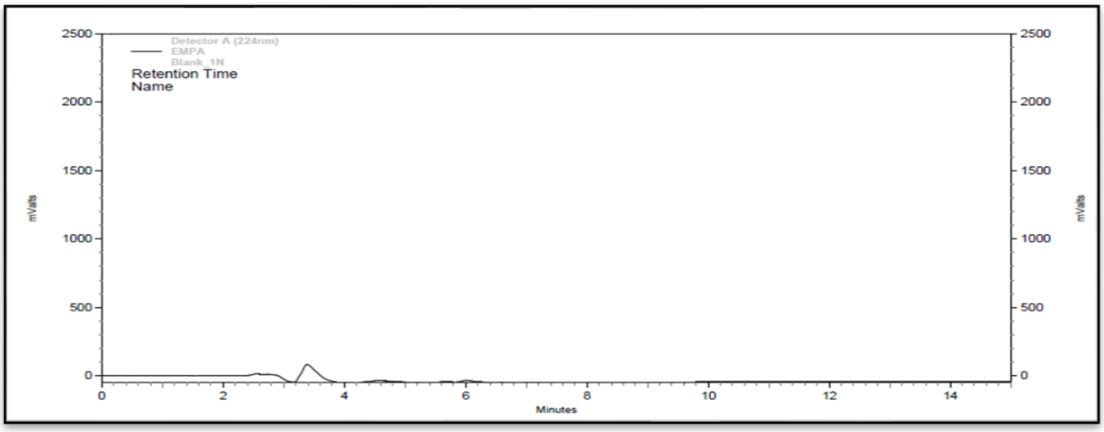


Figure No: 6.22 Chromatogram of 1 N Blank Solution

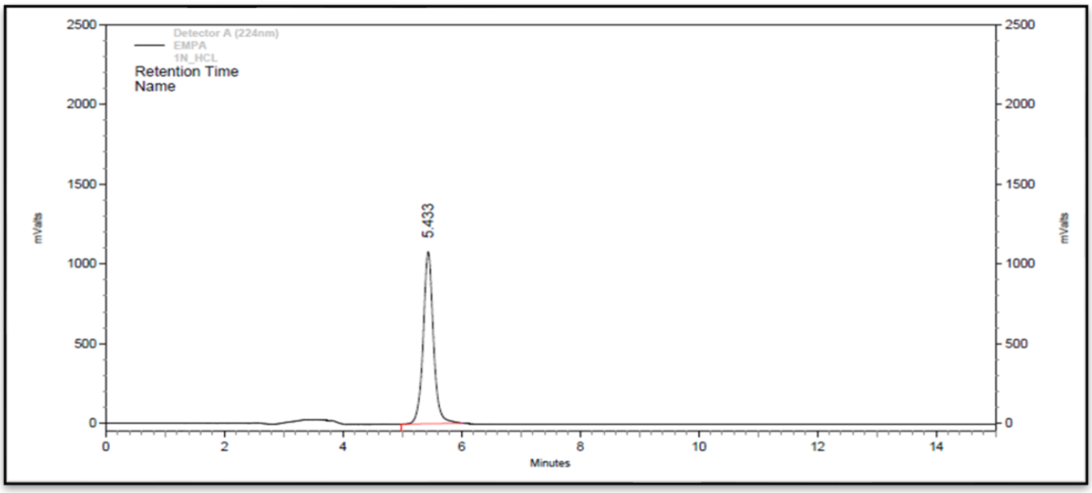


Figure No: 6.23 Chromatogram of acid degradation in 1 N HCl for 24 hr at 60ºC

Table No: 6.17 Result of acid degradation in 1 N HCl for 24 hr at 60ºC

Preparation of solution	Degradation condition	Observation
1 ml Stock Solution (1000 µg/ml) + 1 ml 1 N HCl, After 24 hr Add 1 ml 1 N NaOH and Dilute up to 10 ml with Mobile Phase to make final concentration 100µg/ml	In 1 N HCl At 60ºC for 24 hr	No Degradation

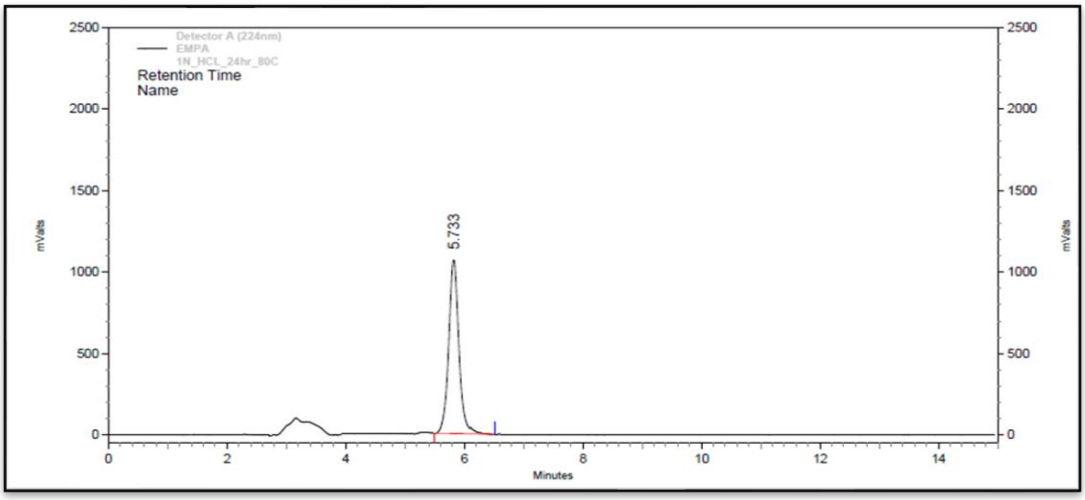


Figure No: 6.24 Chromatogram of acid degradation in 1 N HCl for 24 hr at 80ºC

Table No: 6.18 Result of acid degradation in 1 N HCl for 24 hr at 80ºC

Preparation of solution	Degradation condition	Observation
1 ml Stock Solution (1000 µg/ml) + 1 ml 1 N HCl, After 24 hr Add 1 ml 1 N NaOH and Dilute up to 10 ml with Mobile Phase to make final concentration 100µg/ml	In 1 N HCl At 80ºC for 24 hr	No Degradation

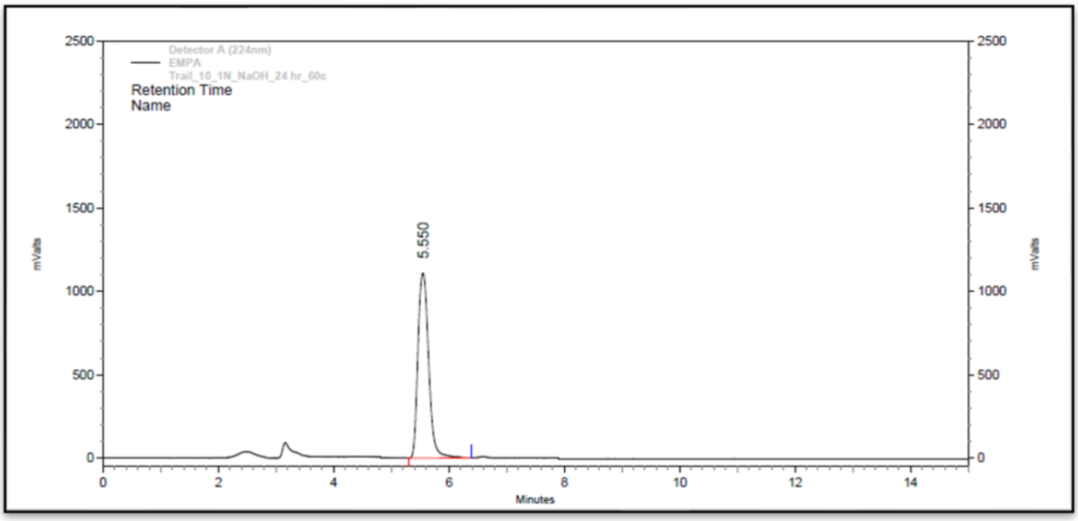


Figure No: 6.25 Chromatogram of alkali degradation in 1 N NaOH for 24 hr at 60ºC

Table No: 6.19 Result of alkali degradation in 1 N NaOH for 24 hr at 60ºC

Preparation of solution	Degradation condition	Observation
1 ml Stock Solution (1000 µg/ml) + 1 ml 1 N NaOH, After 24 hr Add 1 ml 1 N HCl and Dilute up to 10 ml with Mobile Phase to make final concentration 100µg/ml	In 1 N NaOH At 60ºC for 24 hr	No Degradation

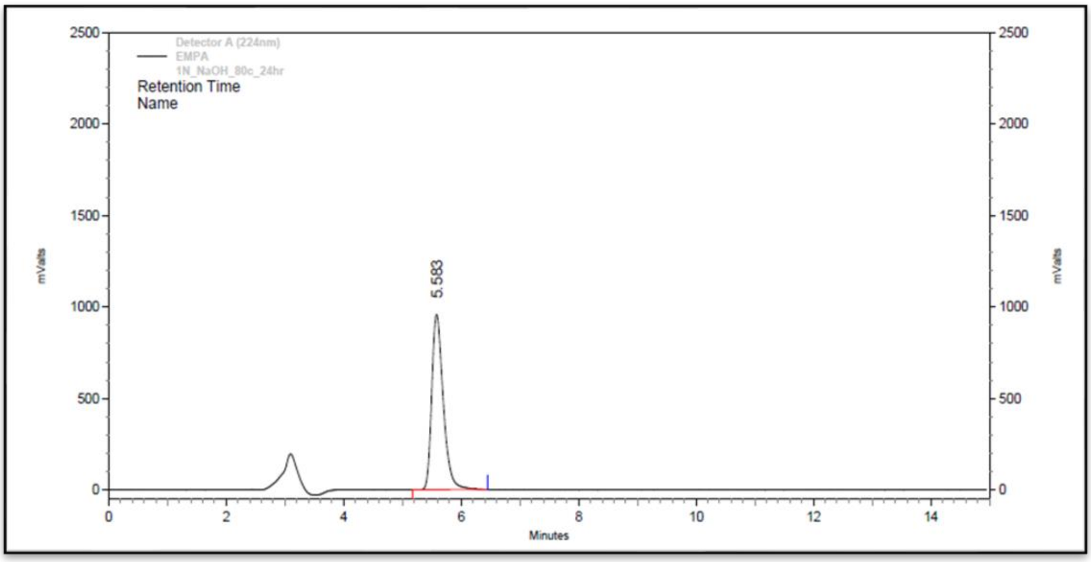


Figure No: 6.26 Chromatogram of alkali degradation in 1 N NaOH for 24 hr at 80ºC

Table No: 6.20 Result of alkali degradation in 1 N NaOH for 24 hr at 80ºC

Preparation of solution	Degradation condition	Observation
1 ml Stock Solution (1000 µg/ml) + 1 ml 1 N NaOH, After 24 hr Add 1 ml 1 N HCl and Dilute up to 10 ml with Mobile Phase to make final concentration 100µg/ml	In 1 N NaOH At 80ºC for 24 hr	No Degradation

6.7.1.3 Acid and Alkali Degradation in 2 N HCl and 2 N NaOH

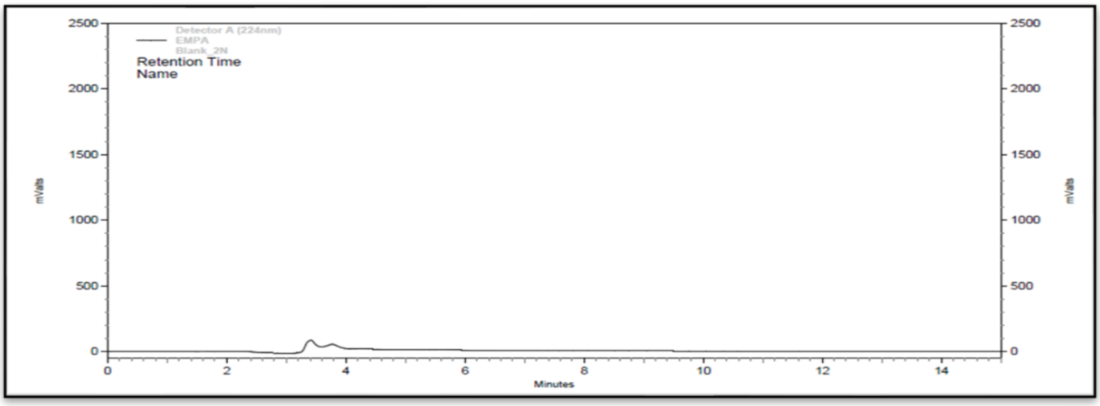


Figure No: 6.27 Chromatogram of 2 N Blank Solution

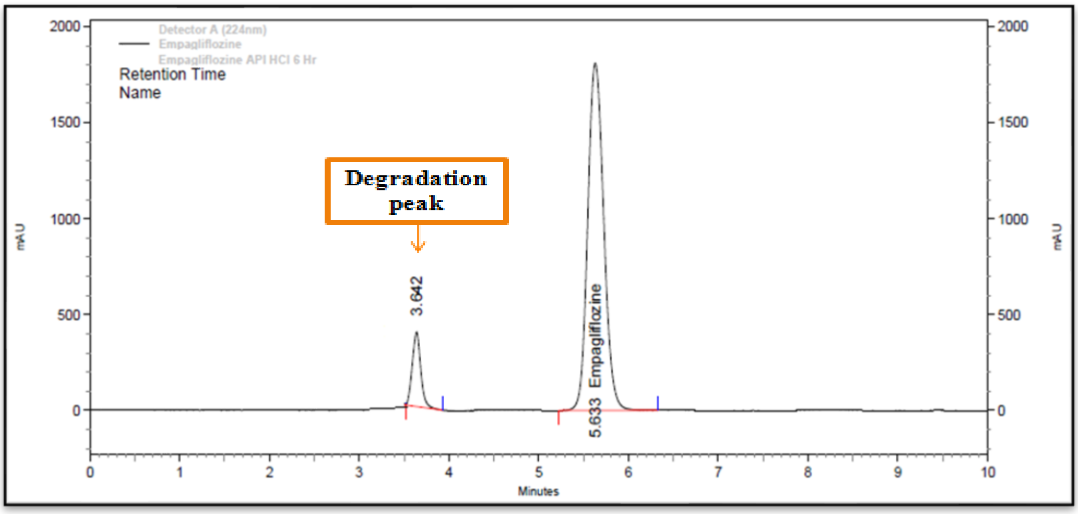


Figure No: 6.28 Chromatogram of acid degradation in 2 N HCl for 06 hr at 60ºC

Table No: 6.21 Result of acid degradation in 2 N HCl for 06 hr at 60ºC

Preparation of solution	Degradation condition	Observation
1 ml Stock Solution (1000 µg/ml) + 1 ml 2 N HCl, After 06 hr Add 1 ml 2 N NaOH and Dilute up to 10 ml with Mobile Phase to make final concentration 100µg/ml	In 2 N HCl At 60ºC for 06 hr	09.43 % degradation Observe at 3.64 min

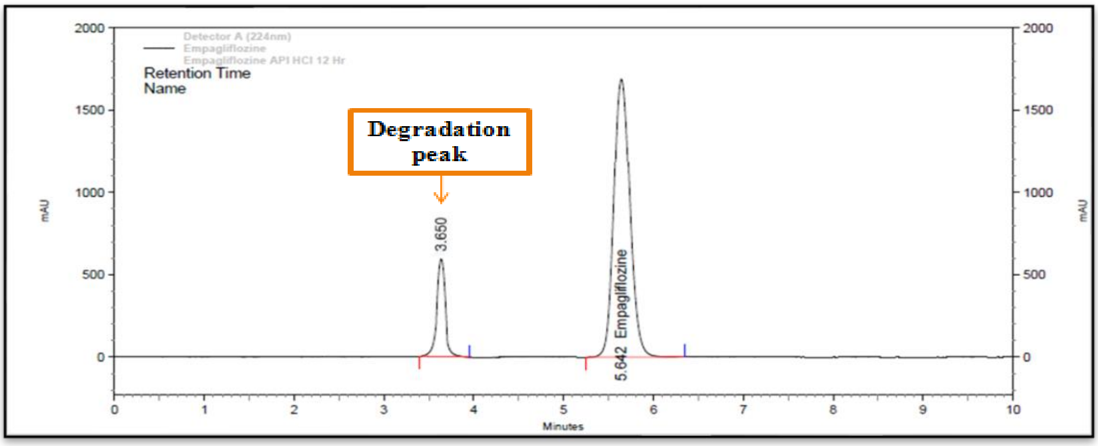


Figure No: 6.29 Chromatogram of Acid degradation in 2 N HCl for 12 hr at 60ºC

Table No: 6.22 Result of acid degradation in 2 N HCl for 12 hr at 60ºC

Preparation of solution	**Degradation condition**	**Observation**
1 ml Stock Solution (1000 µg/ml) + 1 ml 2 N HCl, After 12 hr Add 1 ml 2 N NaOH and Dilute up to 10 ml with Mobile Phase to make final concentration 100µg/ml	In 2 N HCl At 60ºC for 12 hr	17.91 % degradation Observe at 3.65 min

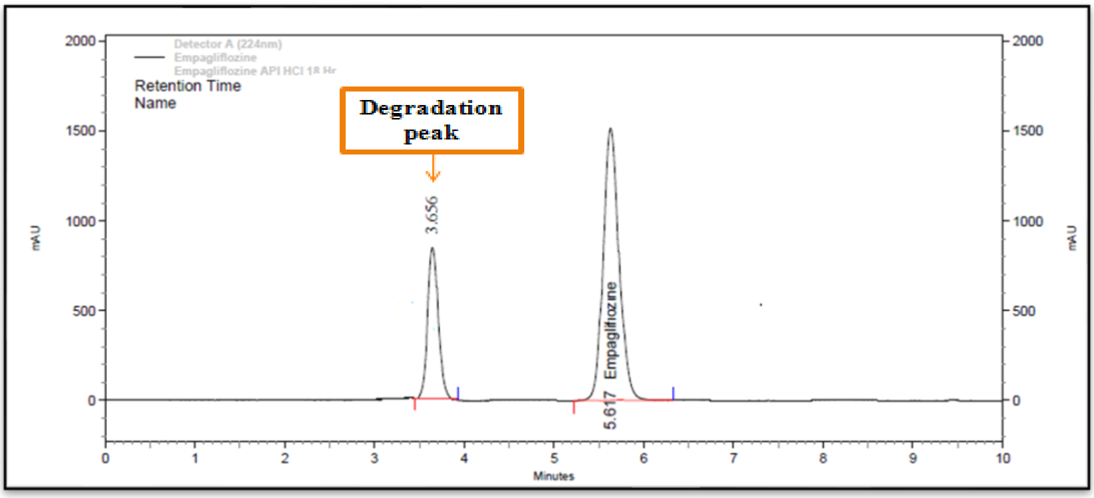


Figure No: 6.30 Chromatogram of acid degradation in 2 N HCl for 18 hr at 60ºC

Table No: 6.23 Result of acid degradation in 2 N HCl for 18 hr at 60°C

Preparation of solution	Degradation condition	Observation
1 ml Stock Solution (1000 µg/ml) + 1 ml 2 N HCl, After 18 hr Add 1 ml 2 N NaOH and Dilute up to 10 ml with Mobile Phase to make final concentration 100µg/ml	In 2 N HCl At 60°C for 18 hr	30.34 % degradation Observe at 3.65 min

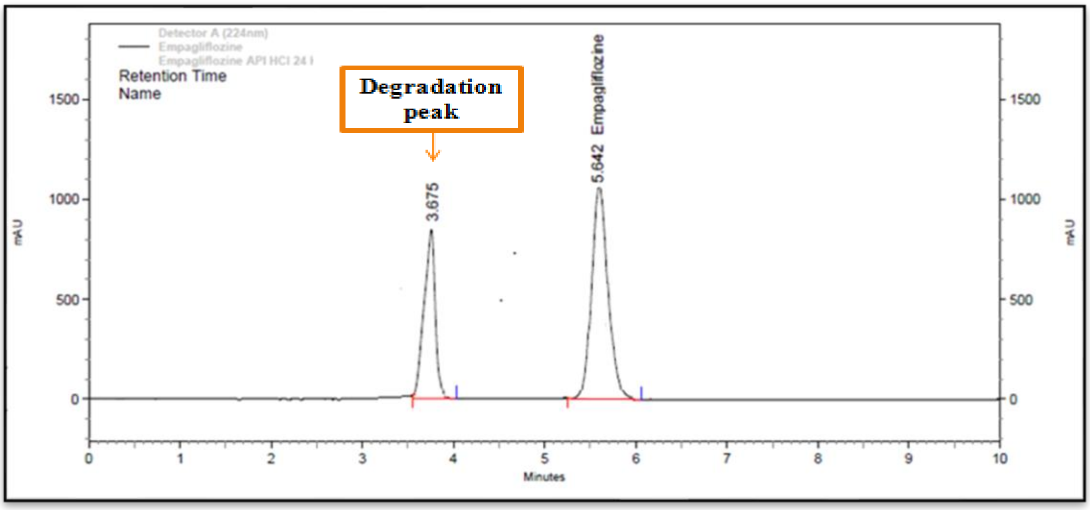


Figure No: 6.31 Chromatogram of acid degradation in 2 N HCl for 24 hr at 60°C

Table No: 6.24 Result of acid degradation in 2 N HCl for 24 hr at 60°C

Preparation of solution	Degradation condition	Observation
1 ml Stock Solution (1000 µg/ml) + 1 ml 2 N HCl, After 24 hr Add 1 ml 2 N NaOH and Dilute up to 10 ml with Mobile Phase to make final concentration 100µg/ml	In 2 N HCl At 60°C for 24 hr	48.54 % degradation Observe at 3.67 min

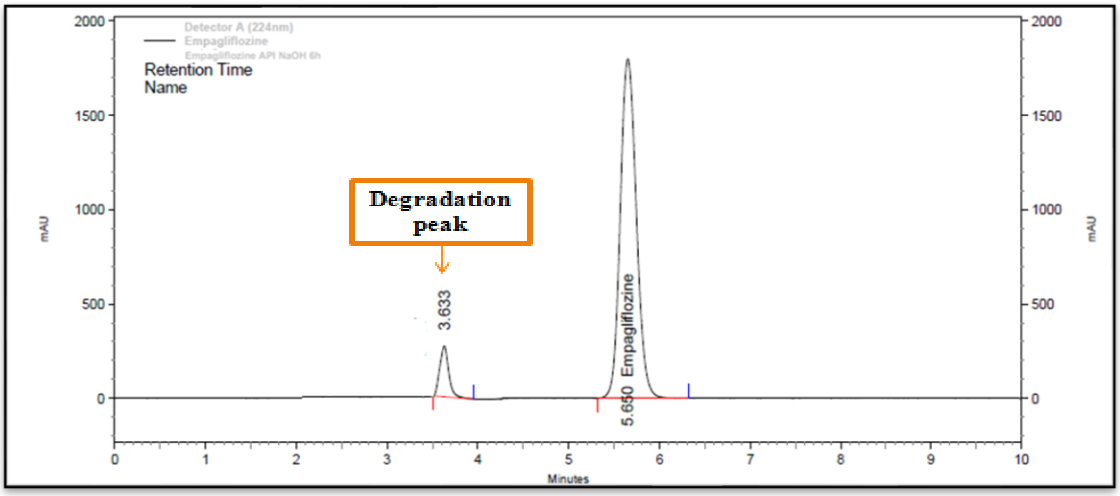


Figure No: 6.32 Chromatogram of alkali degradation in 2 N NaOH for 06 hr at 60ºC

Table No: 6.25 Result of alkali degradation in 2 N NaOH for 06 hr at 60ºC

Preparation of solution	Degradation condition	Observation
1 ml Stock Solution (1000 µg/ml) + 1 ml 2 N NaOH, After 06 hr Add 1 ml 2 N HCl and Dilute up to 10 ml with Mobile Phase to make final concentration 100µg/ml	In 2 N NaOH At 60ºC for 06 hr	08. 98 % degradation Observe at 3.63 min

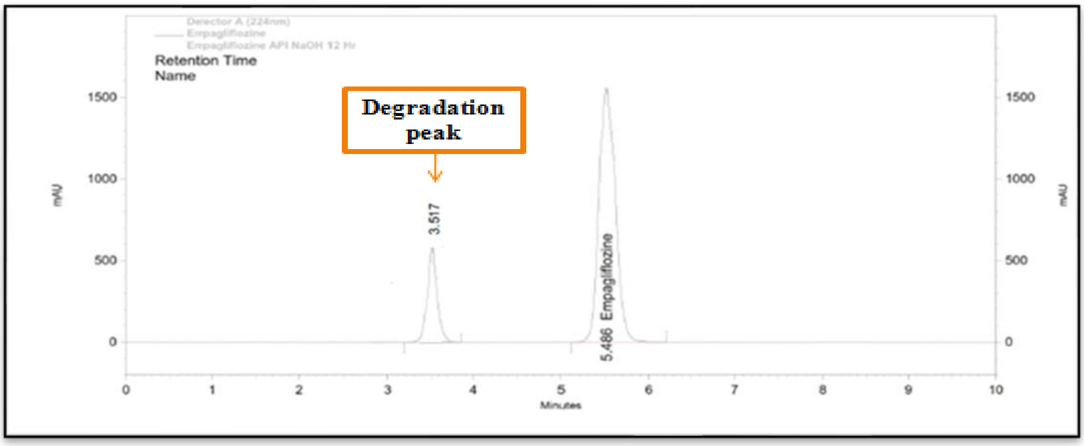


Figure No: 6.33 Chromatogram of alkali degradation in 2 N NaOH for 12 hr at 60ºC

Table No: 6.26 Result of alkali degradation in 2 N NaOH for 12 hr at 60ºC

Preparation of solution	Degradation condition	Observation
1 ml Stock Solution (1000 µg/ml) + 1 ml 2 N NaOH, After 12 hr Add 1 ml 2 N HCl and Dilute up to 10 ml with Mobile Phase to make final concentration 100µg/ml	In 2 N NaOH At 60ºC for 12 hr	15.67 % degradation Observe at 3.51 min

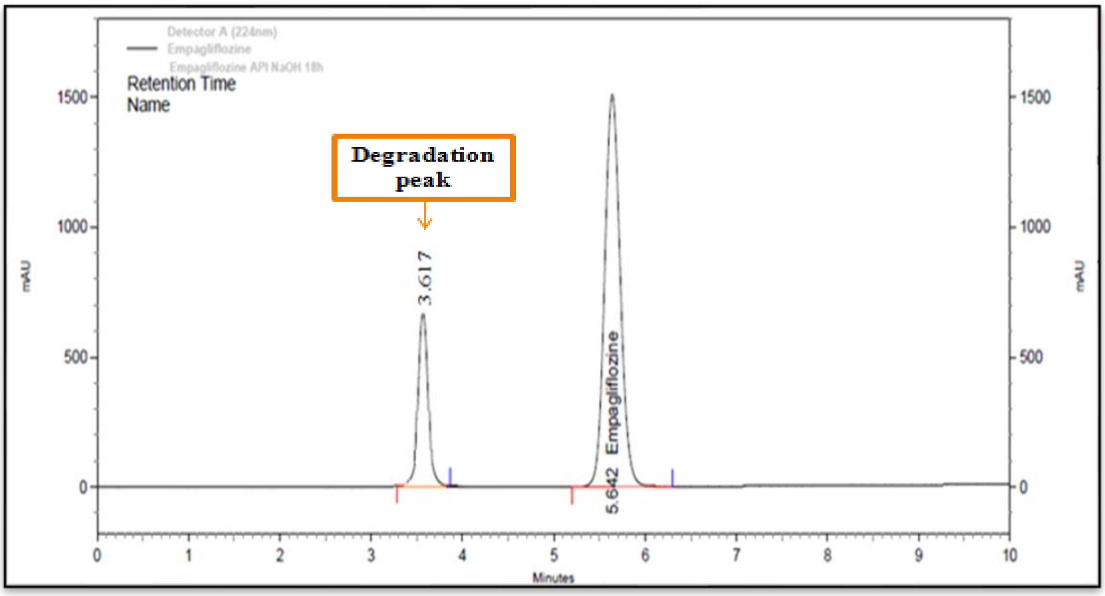


Figure No: 6.34 Chromatogram of alkali degradation in 2 N NaOH for 18 hr at 60ºC

Table No: 6.27 Result of alkali degradation in 2 N NaOH for 18 hr at 60ºC

Preparation of solution	Degradation condition	Observation
1 ml Stock Solution (1000 µg/ml) + 1 ml 2 N NaOH, After 18 hr Add 1 ml 2 N HCl and Dilute up to 10 ml with Mobile Phase to make final concentration 100µg/ml	In 2 N NaOH At 60ºC for 18 hr	23.43 % degradation Observe at 3.61 min

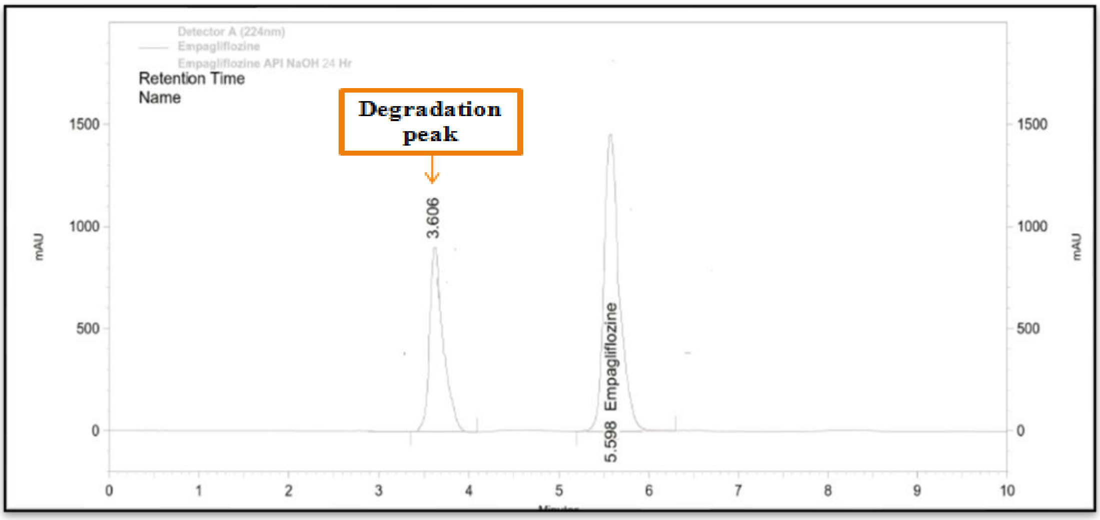


Figure No: 6.35 Chromatogram of alkali degradation in 2 N NaOH for 24 hr at 60ºC

Table No: 6.28 Result of alkali degradation in 2 N NaOH for 24 hr at 60ºC

Preparation of solution	Degradation condition	Observation
1 ml Stock Solution (1000 µg/ml) + 1 ml 2 N NaOH, After 24 hr Add 1 ml 2 N HCl and Dilute up to 10 ml with Mobile Phase to make final concentration 100µg/ml	In 2 N NaOH At 60ºC for 24 hr	31.79 % degradation Observe at 3.60 min

6.7.1.4 Oxidative Degradation in 3%, 20% and 30% H_2O_2

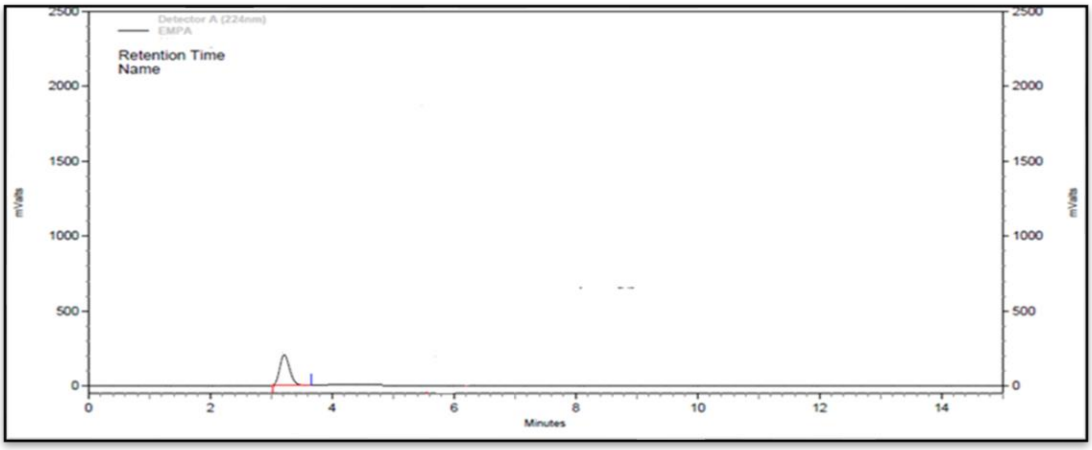


Figure No:6.36 Chromatogram of 3% H_2O_2 Blank Solution

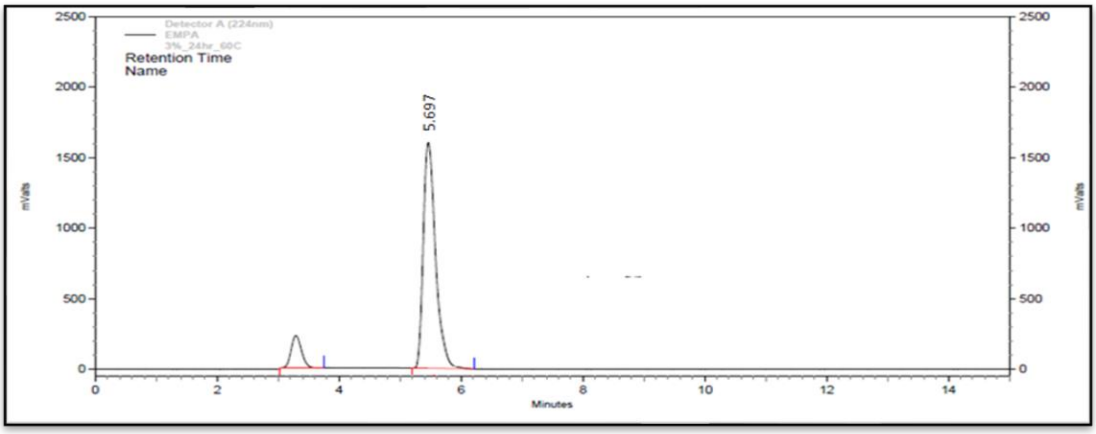


Figure No: 6.37 Chromatogram of Oxidative degradation in 3% H_2O_2 for 24 hr at 60ºC

Table No: 6.29 Result of Oxidative degradation in 3% H_2O_2 for 24 hr at 60ºC

Preparation of solution	Degradation condition	Observation
1 ml Stock Solution(1000 µg/ml) + 1 ml 3% H_2O_2, After 24 hr , Dilute up to 10 ml with Mobile Phase to make final concentration 100µg/ml	In 3% H_2O_2 At 60ºC for 24 hr	No Degradation

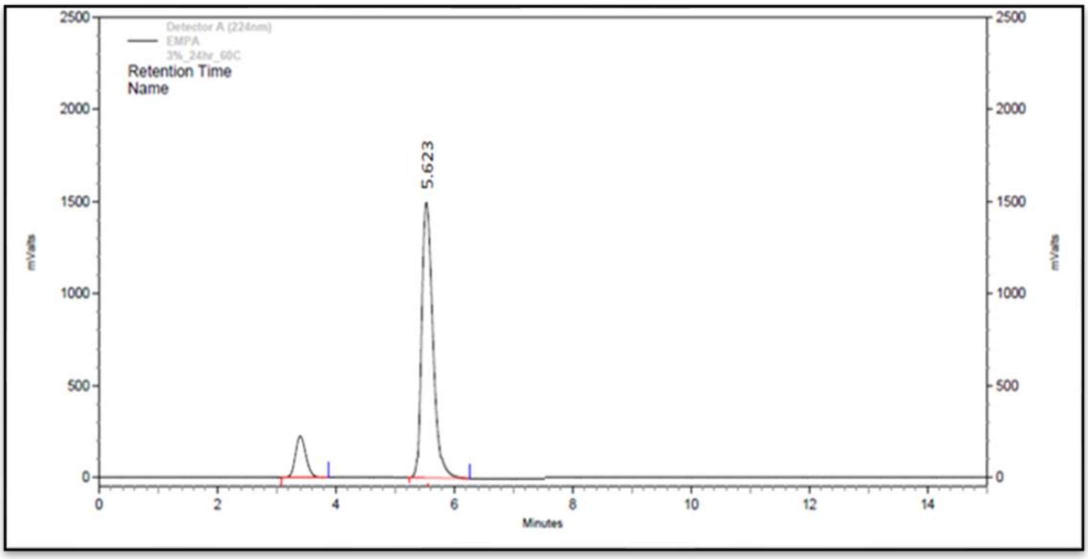


Figure No: 6.38 Chromatogram of Oxidative degradation in 3% H_2O_2 for 24 hr at 80ºC

Table No: 6.30 Result of Oxidative degradation in 3% H_2O_2 for 24 hr at 80ºC

Preparation of solution	Degradation condition	Observation
1 ml Stock Solution (1000 µg/ml) + 1 ml 3% H_2O_2, After 24 hr , Dilute up to 10 ml with Mobile Phase to make final concentration 100µg/ml	In 3% H_2O_2 At 80ºC for 24 hr	No Degradation

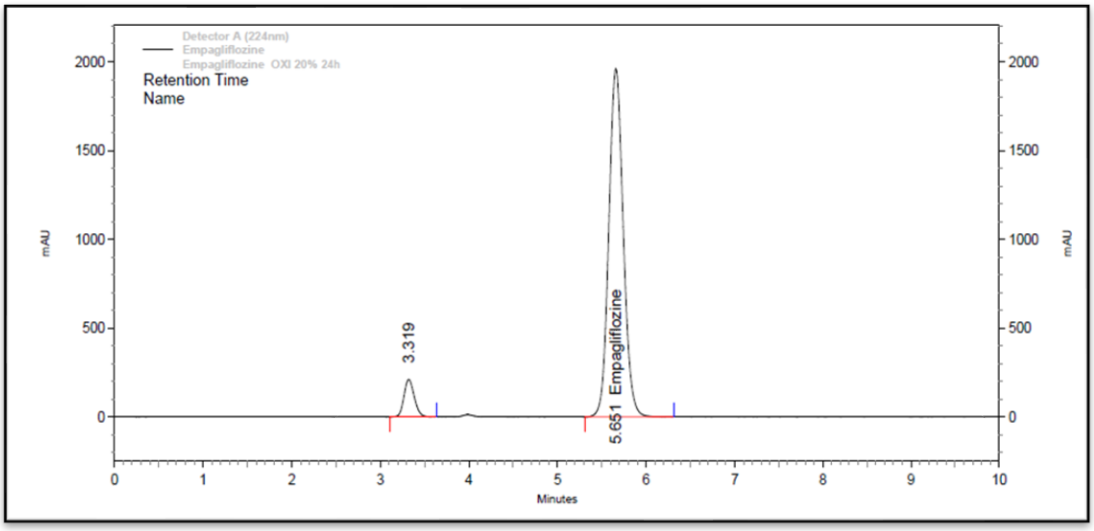


Figure No: 6.39 Chromatogram of Oxidative degradation in 20% H_2O_2 for 24 hr at 60ºC

Table No: 6.31 Result of Oxidative degradation in 20% H_2O_2 for 24 hr at 60ºC

Preparation of solution	Degradation condition	Observation
1 ml Stock Solution (1000 µg/ml) + 1 ml 20 % H_2O_2, After 24 hr , Dilute up to 10 ml with Mobile Phase to make final concentration 100µg/ml	In 20% H_2O_2 At 60ºC for 24 hr	No Degradation

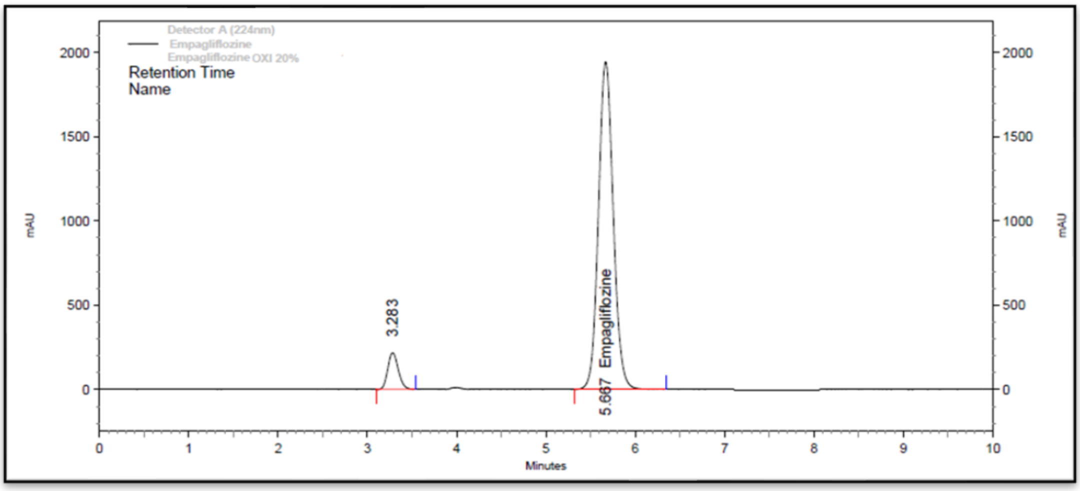


Figure No: 6.40 Chromatogram of Oxidative degradation in 20% H_2O_2 for 24 hr at 80ºC

Table No: 6.32 Result of Oxidative degradation in 20% H_2O_2 for 24 hr at 80ºC

Preparation of solution	**Degradation condition**	**Observation**
1 ml Stock Solution (1000 µg/ml) + 1 ml 20% H_2O_2, After 24 hr , Dilute up to 10 ml with Mobile Phase to make final concentration 100µg/ml	In 20% H_2O_2 At 80ºC for 24 hr	No Degradation

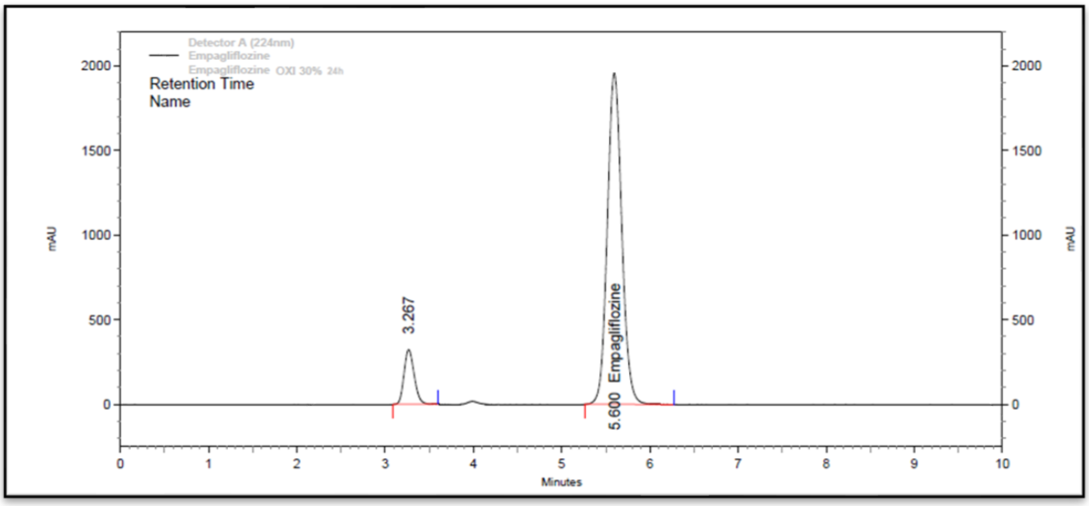


Figure No: 6.41 Chromatogram of Oxidative degradation in 30% H_2O_2 for 24hr at 60ºC

Table No: 6.33 Result of Oxidative degradation in 30% H_2O_2 for 24hr at 60°C

Preparation of solution	Degradation condition	Observation
1 ml Stock Solution (1000 µg/ml) + 1 ml 30% H_2O_2, After 24 hr , Dilute up to 10 ml with Mobile Phase to make final concentration 100µg/ml	In 30% H_2O_2 At 60°C for 24 hr	No Degradation

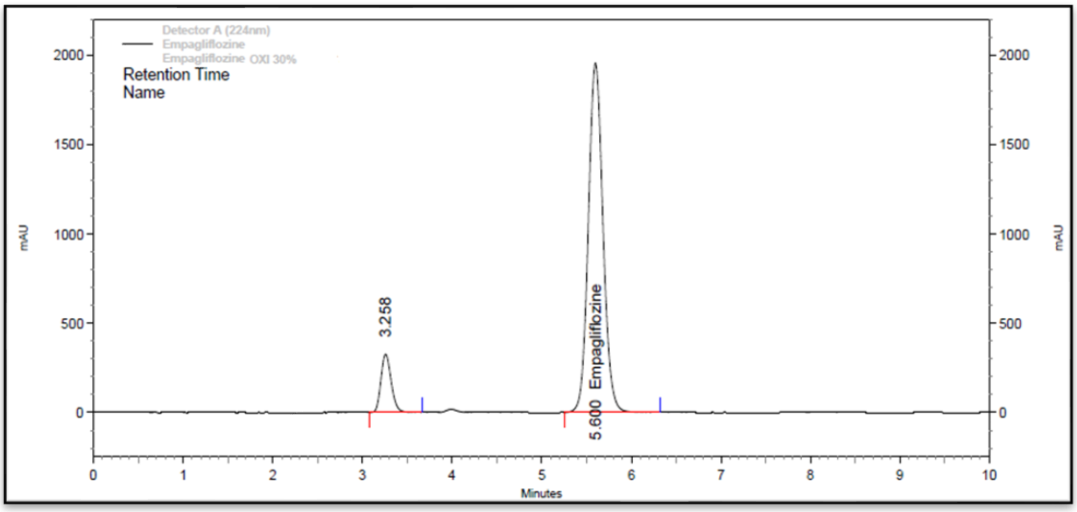


Figure No: 6.42 Chromatogram of Oxidative degradation in 30% H_2O_2 for 24 hr at 80°C

Table No: 6.34 Result of Oxidative degradation in 30% H_2O_2 for 24 hr at 80°C

Preparation of solution	Degradation condition	Observation
1 ml Stock Solution (1000 µg/ml) + 1 ml 30% H_2O_2, After 24 hr , Dilute up to 10 ml with Mobile Phase to make final concentration 100µg/ml	In 30% H_2O_2 At 80°C for 24 hr	No Degradation

6.7.1.5 Thermal Degradation

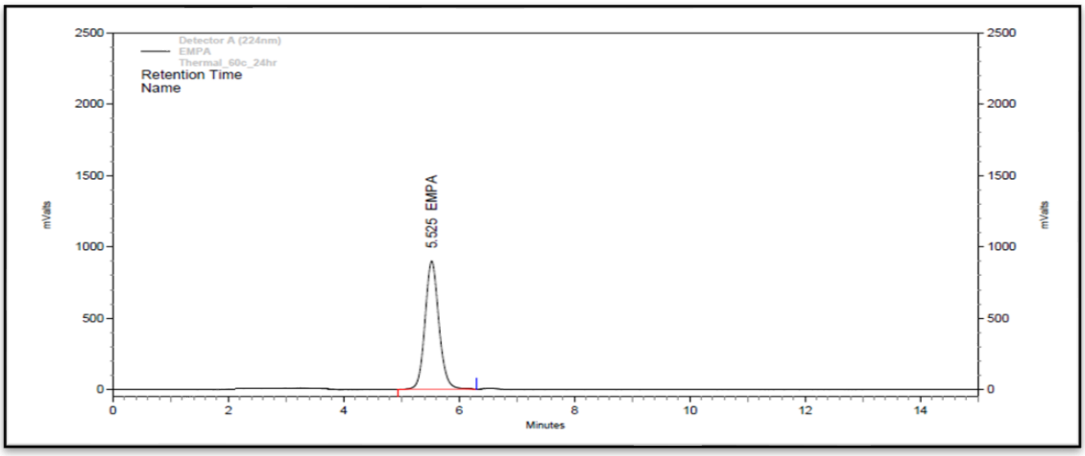


Figure No: 6.43 Chromatogram of Thermal degradation for 24 hr at 60ºC

Table No: 6.35 Result of Thermal degradation for 24 hr at 60ºC

Preparation of solution	**Degradation condition**	**Observation**
10 mg of Empagliflozin in dry state expose for given condition, Dilution made up to 10 ml with Methanol to get concentration 100 µg/ml	In Hot air Oven At 60ºC for 24 hr	No Degradation

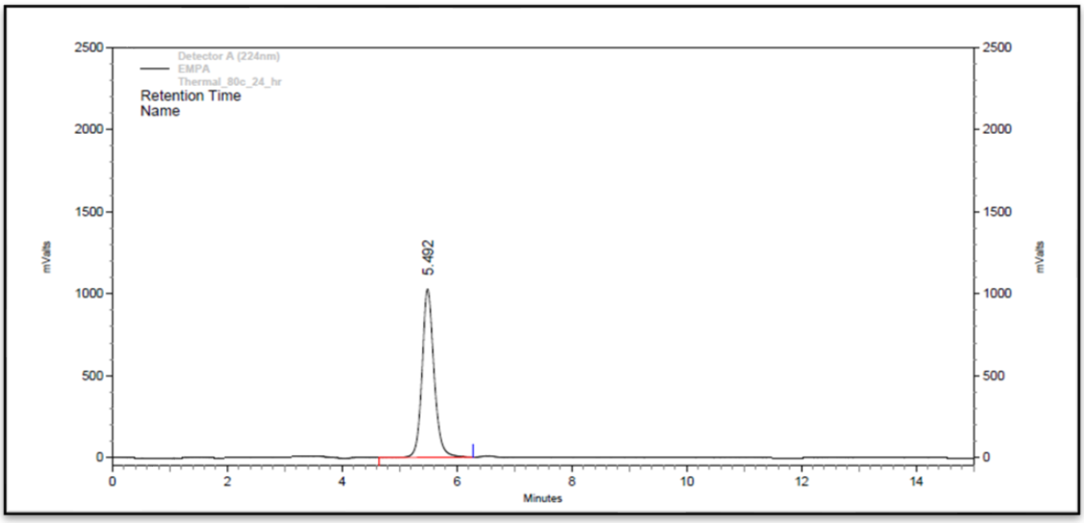


Figure No: 6.44 Chromatogram of Thermal degradation for 24 hr at 80ºC

Table No: 6.36 Result of Thermal degradation for 24 hr at 80ºC

Preparation of solution	Degradation condition	Observation
10 mg of Empagliflozin in dry state expose for given condition, Dilution made up to 10 ml with Methanol to get concentration 100 µg/ml	In Hot air Oven At 80ºC for 24 hr	No Degradation

6.7.1.6 Photo Degradation

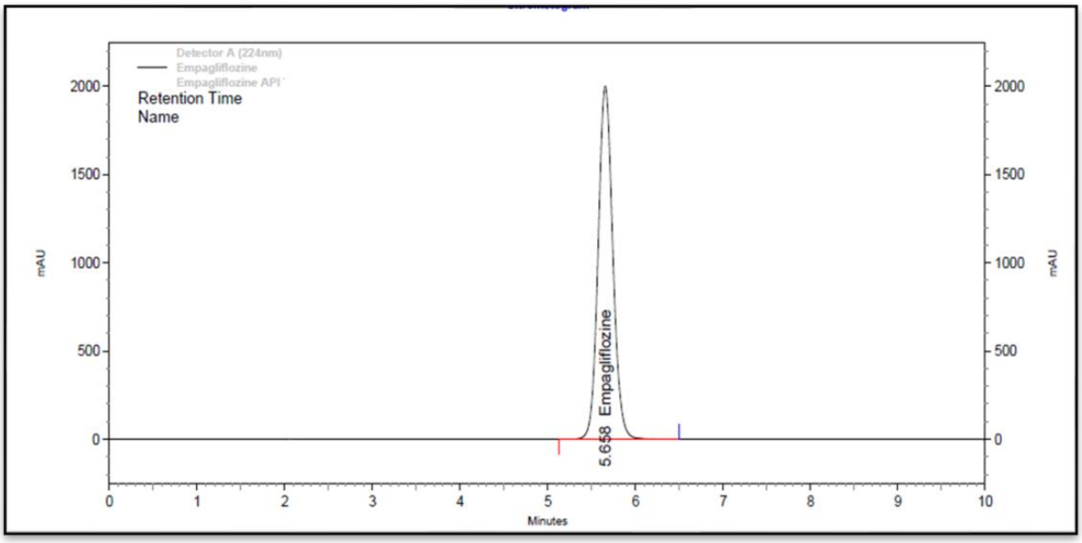


Figure No: 6.45 Chromatogram of Photo degradation for 7 days in UV Light

Table No: 6.37 Result of Photo degradation for 7 days in UV Light

Preparation of solution	Degradation condition	Observation
10 mg of Empagliflozin in dry state expose under UV Light for 7 days, Dilution made up to 10 ml with Methanol to get concentration 100 µg/ml	In UV Chamber For 7 Days	No Degradation

Table No: 6.38 Summery of Degradation study of Empagliflozin in API

Sr. No	Condition	Temperature (ºC)	Time duration for degradation	Retention time of Std peak (min)	Degradation observed Yes/No	% Degradation observed	Retention time of degradation peak (min)
1	0.1 N HCl	60	24 hr	5.78	No		-
		80	24 hr	5.52	No		-
2	0.1 N NaOH	60	24 hr	5.50	No		-
		80	24 hr	5.59	No		-
3	1 N HCl	60	24 hr	5.43	No		-
		80	24 hr	5.73	No		-
4	1 N NaOH	60	24 hr	5.55	No		-
		80	24 hr	5.58	No		-
5	2 N HCl	60	6 hr	5.63	Yes	09.43	3.64
			12 hr	5.64	Yes	17.91	3.65
			18 hr	5.61	Yes	30.34	3.65
			24 hr	5.64	Yes	48.54	3.67
6	2 N NaOH	60	6 hr	5.65	Yes	8.98	3.63
			12 hr	5.48	Yes	15.67	3.51
			18 hr	5.64	Yes	23.43	3.61
			24 hr	5.59	Yes	31.79	3.60
7	3% H_2O_2	60	24 hr	5.69	No	-	-
		80	24 hr	5.62	No	-	-
	20 % H_2O_2	60	24 hr	5.65	No	-	-
		80	24 hr	5.66	No	-	-

	30 % H_2O_2	60	24 hr	5.60	No	-	-
		80	24 hr	5.60	No	-	-
8	Thermal In Hot Air Oven	60	24 hr	5.52	No	-	-
		80	24 hr	5.49	No	-	-
9	Photo degradation Study	UV Light	7 Days	5.65	No	-	-

6.7.2 Forced Degradation Study of Empagliflozin in Marketed Formulation (JARDIANCE)

6.7.2.1 Acid and Alkali Degradation in 2 N HCl and 2 N NaOH

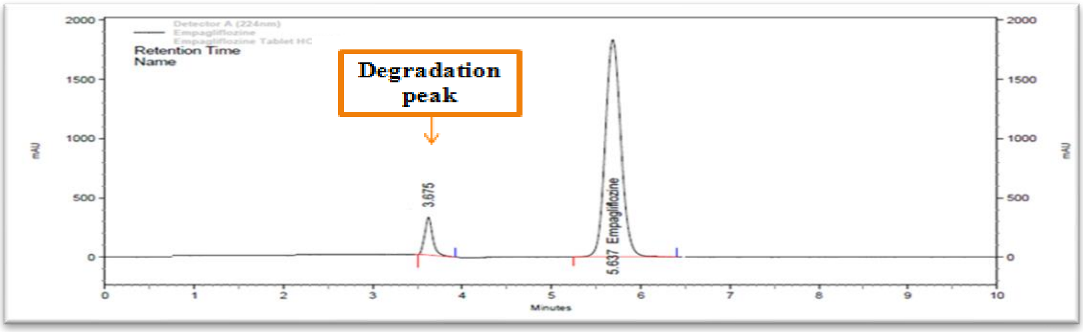


Figure No: 6.46 Chromatogram of Acid degradation in 2 N HCl for 6 hr at 60ºC

Table No: 6.39 Result of Acid degradation in 2 N HCl for 6 hr at 60ºC

Preparation of solution	Degradation condition	Observation
1 ml Sample Stock Solution(1000 µg/ml) + 1 ml 2 N HCl, After 6 hr Add 1 ml 2 N NaOH and Dilute up to 10 ml with Mobile Phase to make final concentration 100µg/ml	In 2 N HCl At 60ºC for 6 hr	Total 08.87% degradation Observe at 3.67 min

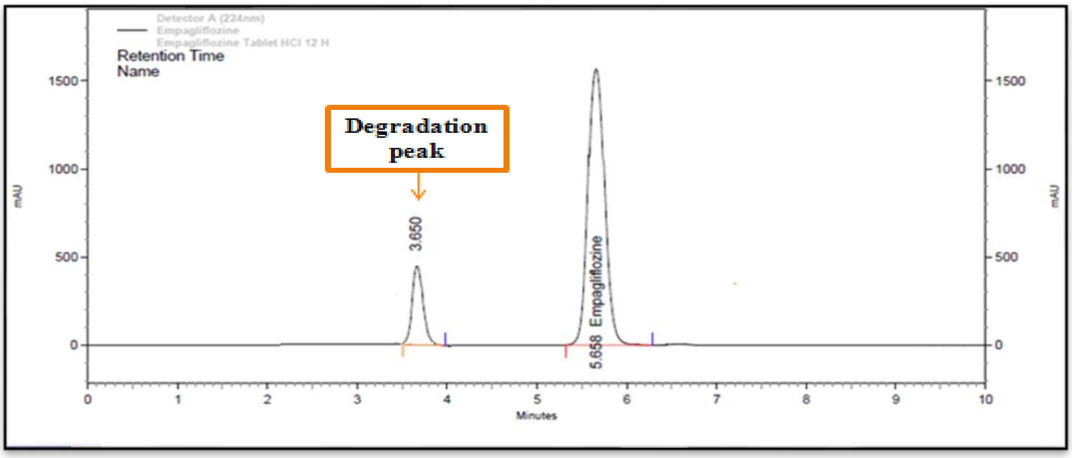


Figure No: 6.47 Chromatogram of Acid degradation in 2 N HCl for 12 hr at 60°C

Table No: 6.40 Result of Acid degradation in 2 N HCl for 12 hr at 60°C

Preparation of solution	Degradation condition	Observation
1 ml Sample Stock Solution (1000 µg/ml) + 1 ml 2 N HCl, After 12 hr Add 1 ml 2 N NaOH and Dilute up to 10 ml with Mobile Phase to make final concentration 100µg/ml	In 2 N HCl At 60°C for 12 hr	15.12 % degradation Observe at 3.65 min

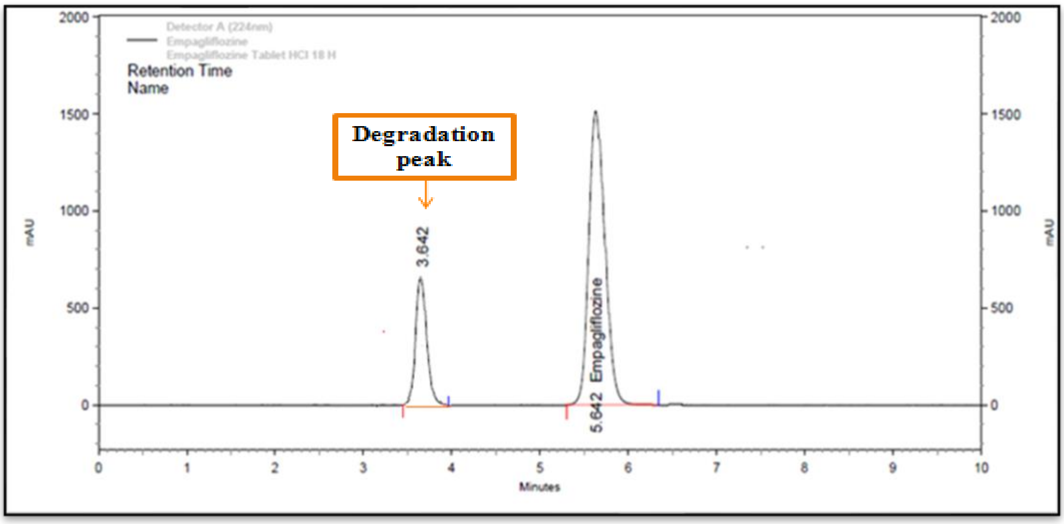


Figure No: 6.48 Chromatogram of Acid degradation in 2 N HCl for 18 hr at 60°C

Table No: 6.41 Result of Acid degradation in 2 N HCl for 18 hr at 60°C

Preparation of solution	Degradation condition	Observation
1 ml Sample Stock Solution(1000 µg/ml) + 1 ml 2 N HCl, After 18 hr Add 1 ml 2 N NaOH and Dilute up to 10 ml with Mobile Phase to make final concentration 100µg/ml	In 2 N HCl At 60°C for 18 hr	26.67 % degradation Observe at 3.64 min

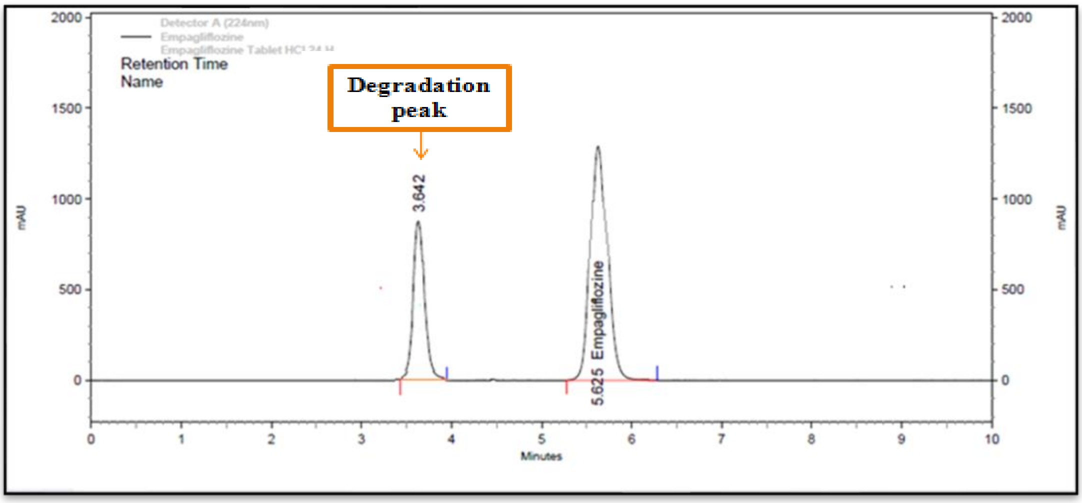


Figure No: 6.49 Chromatogram of Acid degradation in 2 N HCl for 24 hr at 60°C

Table No: 6.42 Result of Acid degradation in 2 N HCl for 24 hr at 60°C

Preparation of solution	Degradation condition	Observation
1 ml Sample Stock Solution(1000 µg/ml) + 1 ml 2 N HCl, After 24 hr Add 1 ml 2 N NaOH and Dilute up to 10 ml with Mobile Phase to make final concentration 100µg/ml	In 2 N HCl At 60°C for 24 hr	38.45 % degradation Observe at 3.64 min

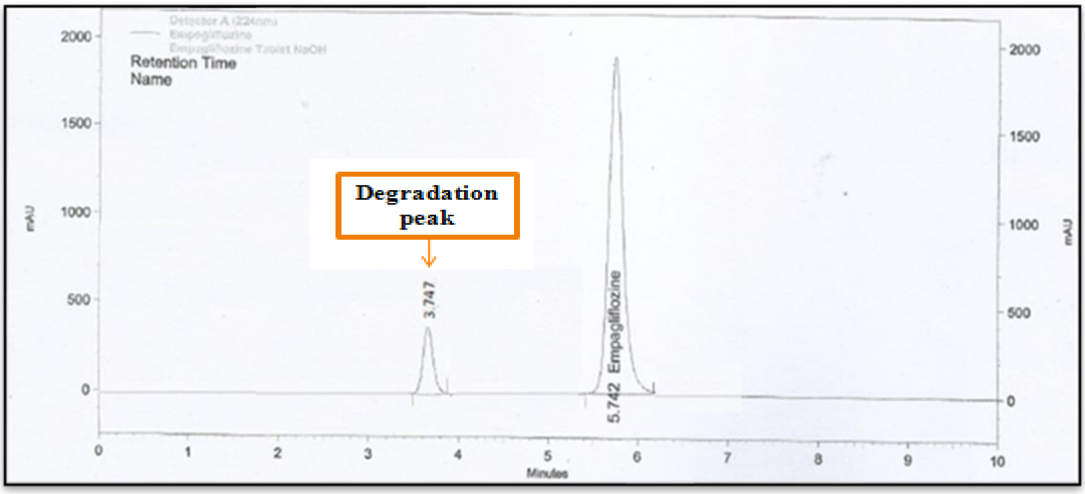


Figure No: 6.50 Chromatogram of Alkali degradation in 2 N NaOH for 06 hr at 60°C

Table No: 6.43 Result of Alkali degradation in 2 NaOH for 6 hr at 60°C

Preparation of solution	**Degradation condition**	**Observation**
1 ml Sample Stock Solution(1000 µg/ml) + 1 ml 2 N NaOH, After 06hr Add 1 ml 2 N HCl and Dilute up to 10 ml with Mobile Phase to make final concentration 100µg/ml	In 2 N NaOH At 60°C for 06 hr	06.49 % degradation Observe at 3.74 min

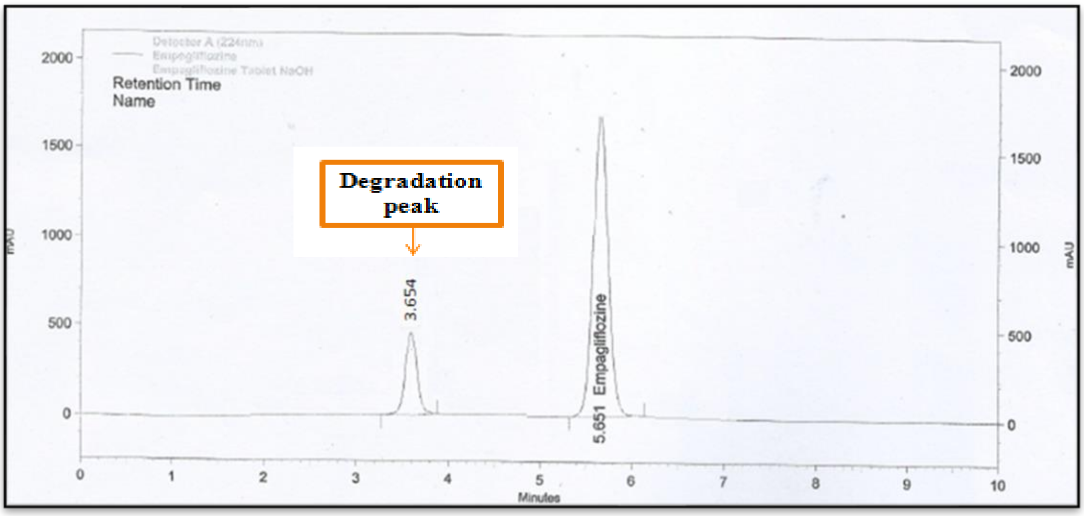


Figure No: 6.51 Chromatogram of Alkali degradation in 2 N NaOH for 12 hr at 60°C

Table No: 6.44 Result of Alkali degradation in 2 NaOH for 12 hr at 60°C

Preparation of solution	Degradation condition	Observation
1 ml Sample Stock Solution(1000 µg/ml) + 1 ml 2 N NaOH, After 12hr Add 1 ml 2 N HCl and Dilute up to 10 ml with Mobile Phase to make final concentration 100µg/ml	In 2 N NaOH At 60°C for 12 hr	12.67 % degradation Observe at 3.65 min

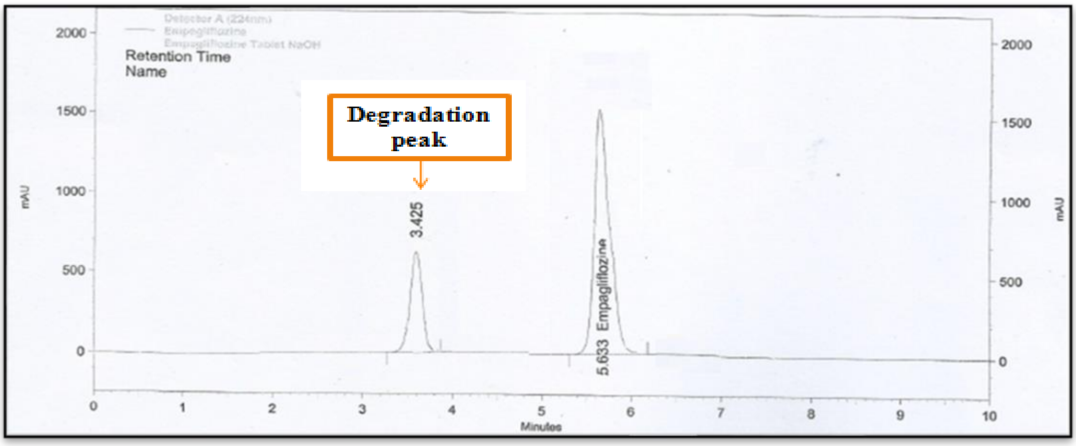


Figure No: 6.52 Chromatogram of Alkali degradation in 2 N NaOH for 18 hr at 60°C

Table No: 6.45 Result of Alkali degradation in 2 NaOH for 18 hr at 60°C

Preparation of solution	Degradation condition	Observation
1 ml Sample Stock Solution(1000 µg/ml) + 1 ml 2 N NaOH, After 18hr Add 1 ml 2 N HCl and Dilute up to 10 ml with Mobile Phase to make final concentration 100µg/ml	In 2 N NaOH At 60°C for 18 hr	21.35 % degradation Observe at 3.42 min

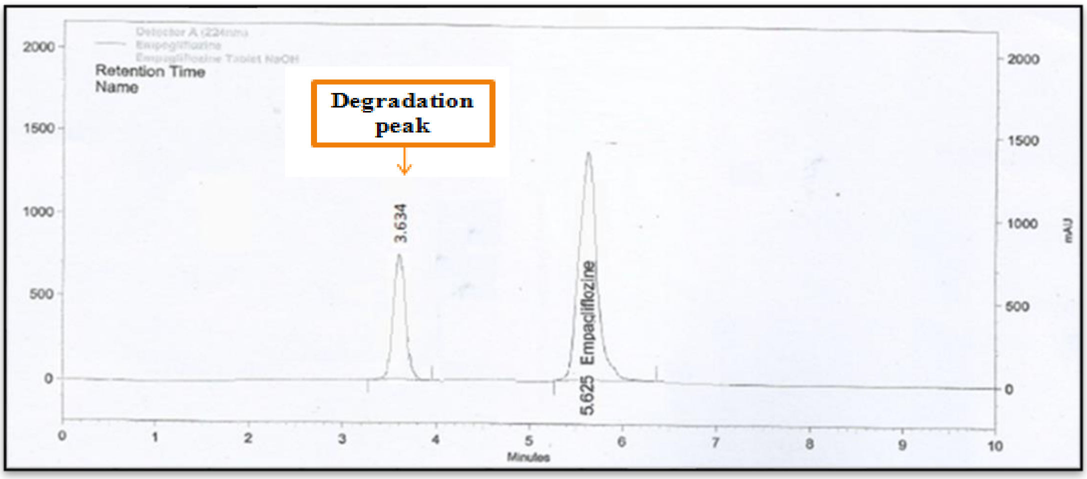


Figure No: 6.53 Chromatogram of Alkali degradation in 2 N NaOH for 24 hr at 60°C

Table No: 6.46 Result of Alkali degradation in 2 NaOH for 24 hr at 60°C

Preparation of solution	Degradation condition	Observation
1 ml Sample Stock Solution (1000 µg/ml) + 1 ml 2 N NaOH, After 24hr Add 1 ml 2 N HCl and Dilute up to 10 ml with Mobile Phase to make final concentration 100µg/ml	In 2 N NaOH At 60°C for 24 hr	30.76 % degradation Observe at 3.63 min

6.7.2.2 Oxidative Degradation in 20% and 30 % H_2O_2

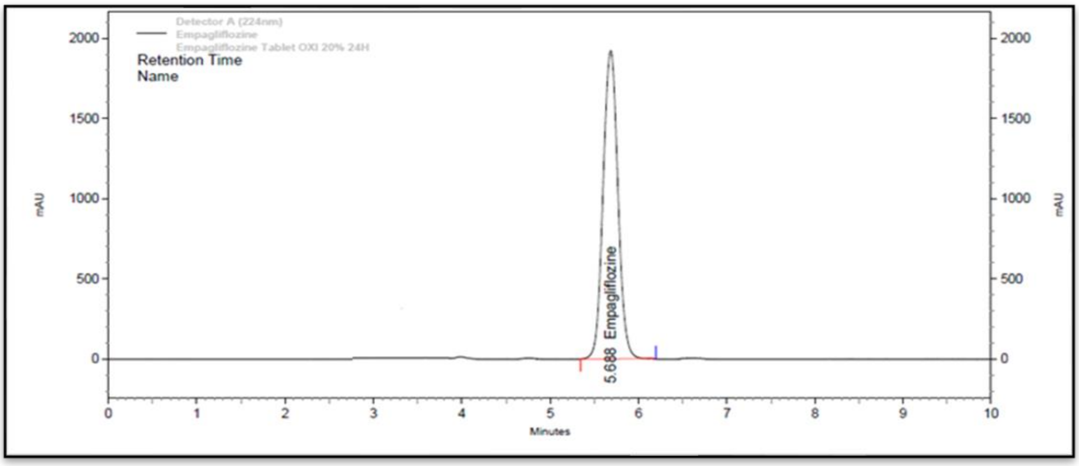


Figure No: 6.54 Chromatogram of Oxidative degradation in 20% H_2O_2 for 24 hr at 60°C

Table No: 6.47 Result of Oxidative degradation in 20% H_2O_2 for 24 hr at 60ºC

Preparation of solution	Degradation condition	Observation
1 ml Sample Stock Solution(1000 µg/ml) + 1 ml 20 % H_2O_2, After 24 hr , Dilute up to 10 ml with Mobile Phase to make final concentration 100µg/ml	In 20% H_2O_2 At 60ºC for 24 hr	No Degradation

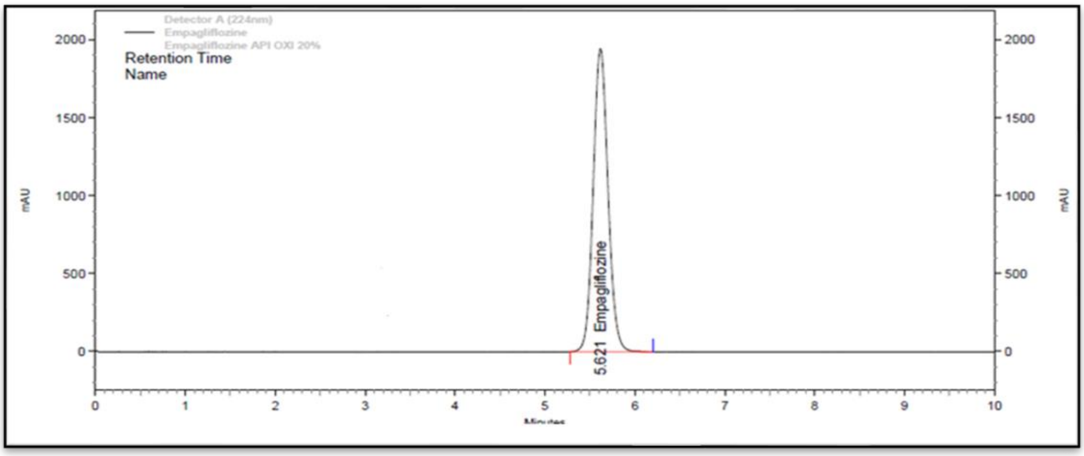


Figure No: 6.55 Chromatogram of Oxidative degradation in 20% H_2O_2 for 24 hr at 80ºC

Table No: 6.48 Result of Oxidative degradation in 20% H_2O_2 for 24 hr at 80ºC

Preparation of solution	Degradation condition	Observation
1 ml Sample Stock Solution(1000 µg/ml) + 1 ml 20% H_2O_2, After 24 hr , Dilute up to 10 ml with Mobile Phase to make final concentration 100µg/ml	In 20% H_2O_2 At 80ºC for 24 hr	No Degradation

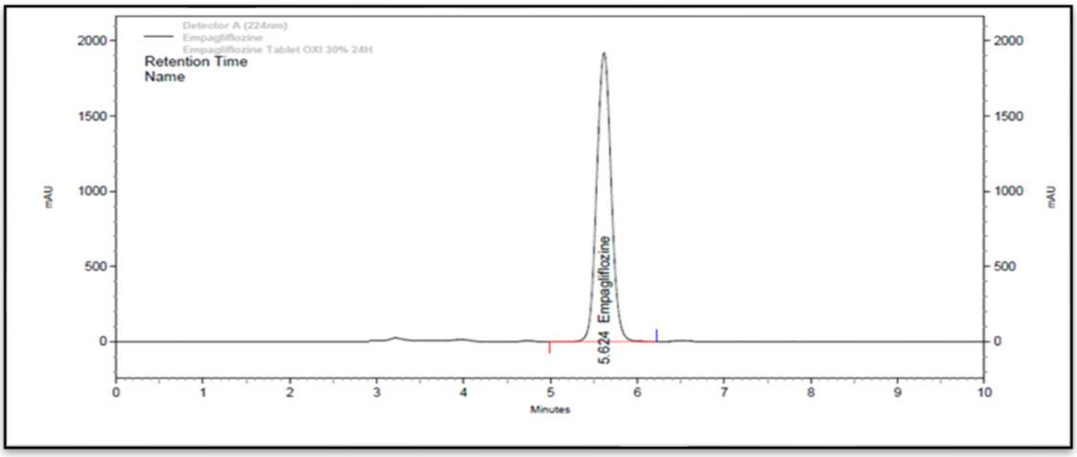


Figure No: 6.56 Chromatogram of Oxidative degradation in 30% H_2O_2 for 24 hr at 60ºC

Table No: 6.49 Result of Oxidative degradation in 30% H_2O_2 for 24 hr at 60ºC

Preparation of solution	**Degradation condition**	**Observation**
1 ml sample Stock Solution(1000 µg/ml) + 1 ml 30% H_2O_2, After 24 hr , Dilute up to 10 ml with Mobile Phase to make final concentration 100µg/ml	In 30% H_2O_2 At 60ºC for 24 hr	No Degradation

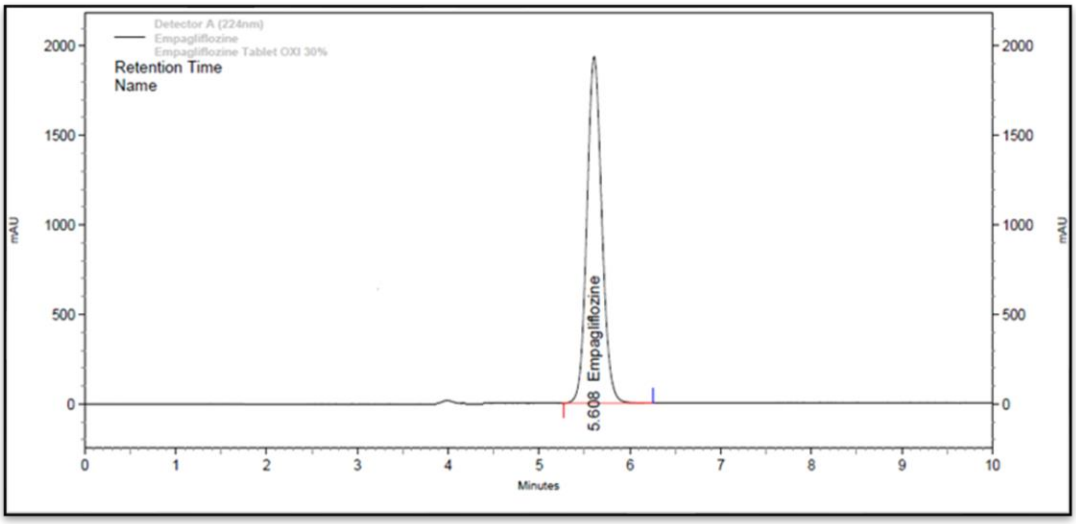


Figure No: 6.57 Chromatogram of Oxidative degradation in 30% H_2O_2 for 24 hr at 80ºC

Table No: 6.50 Result of Oxidative degradation in 30% H_2O_2 for 24 hr at 80ºC

Preparation of solution	Degradation condition	Observation
1 ml sample Stock Solution(1000 µg/ml) + 1 ml 30% H_2O_2, After 24 hr , Dilute up to 10 ml with Mobile Phase to make final concentration 100µg/ml	In 30% H_2O_2 At 80ºC for 24 hr	No Degradation

6.7.2.3 Thermal Degradation

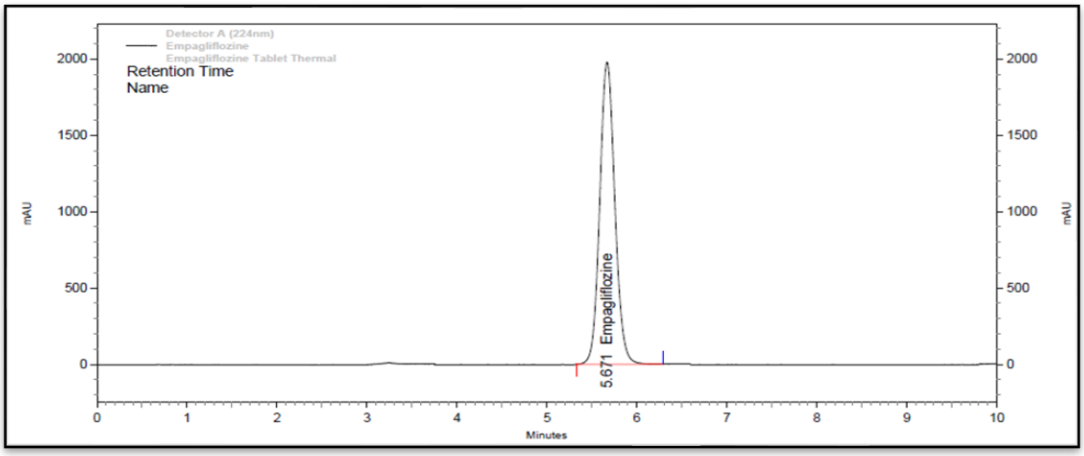


Figure No: 6.58 Chromatogram of Thermal degradation for 24 hr at 80ºC

Table No: 6.51 Result of Thermal degradation for 24 hr at 80ºC

Preparation of solution	Degradation condition	Observation
Powder of Tablet equivalent to 10 mg of Empagliflozin in dry state expose for given condition, Dilution made up to 10 ml with Methanol to get concentration 100 µg/ml	In Hot air Oven At 80ºC for 24 hr	No Degradation

6.7.2.4 Photo Degradation

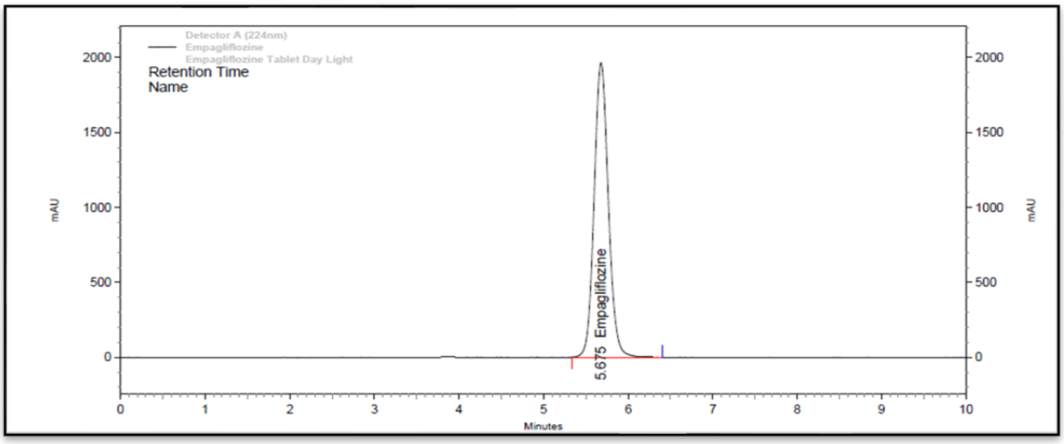


Figure No: 6.59 Chromatogram of Photo degradation for 7 days in UV Light

Table No: 6.52 Result of Photo degradation for 7 days in UV Light

Preparation of solution	**Degradation condition**	**Observation**
Powder of Tablet equivalent to 10 mg of Empagliflozin in dry state expose under UV Light for 7 days, Dilution made with Methanol up to 10 ml to get concentration 100 µg/ml	In UV Chamber For 7 Days	No Degradation

Table No: 6.53 Summary of Degradation study of Empagliflozin Marketed in Formulation

Sr. No	Condition	Temperature (ºC)	Time duration for degradation	Retention time of Std peak (min)	Degradation observed Yes/No	% Degradation observed	Retention time of degradation peak (min)
1	2 N HCl	60	6 hr	5.63	Yes	8.87	3.67
			12 hr	5.65	Yes	15.12	3.65
			18 hr	5.64	Yes	26.67	3.64
			24 hr	5.62	Yes	38.45	3.64
2	2 N NaOH	60	6 hr	5.74	Yes	06.49	3.74
			12 hr	5.65	Yes	12.67	3.65
			18 hr	5.63	Yes	21.35	3.42
			24 hr	5.62	Yes	30.76	3.63
3	20 % H_2O_2	60	24 hr	5.68	No	-	-
		80	24 hr	5.62	No	-	-
	30 % H_2O_2	60	24 hr	5.62	No	-	-
		80	24 hr	5.60	No	-	-
4	Thermal In Hot Air Oven	80	24 hr	5.67	No	-	-
5	Photo degradation Study	UV Light	7 Days	5.67	No	-	-

6.7.3 Forced Degradation Study of Dapagliflozin in API.

6.7.3.1 Acid and Alkali Degradation in 0.1 N HCl and 0.1 N NaOH

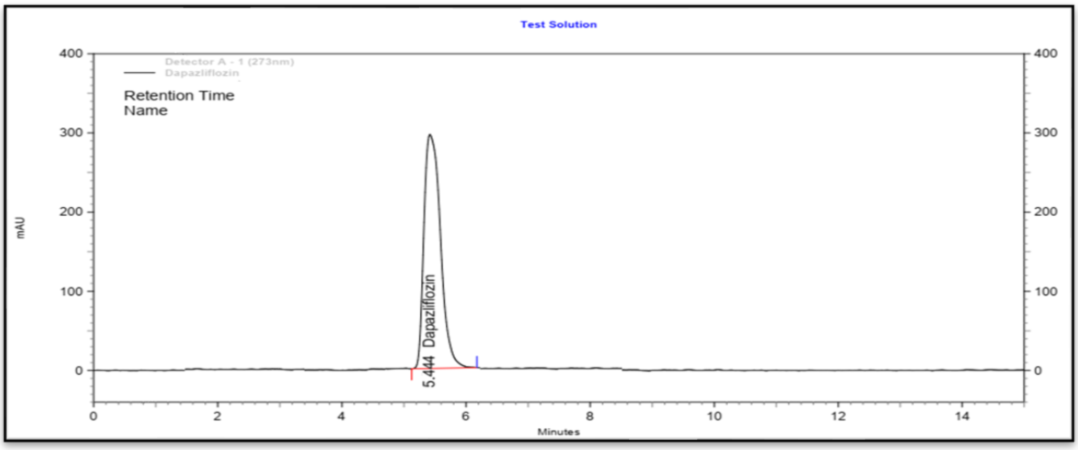


Figure No: 6.60 Chromatogram of Acid degradation in 0.1 N HCl for 24 hr at 80°C

Table No: 6.54 Result of Acid degradation in 0.1 N HCl for 24 hr at 80°C

Preparation of solution	Degradation condition	Observation
1 ml Stock Solution (1000 µg/ml) + 1 ml 0.1 N HCl, After 24 hr Add 1 ml 0.1 N NaOH and Dilute up to 10 ml with Mobile Phase to make final concentration 100µg/ml	In 0.1 N HCl At 80°C for 24 hr	No Degradation

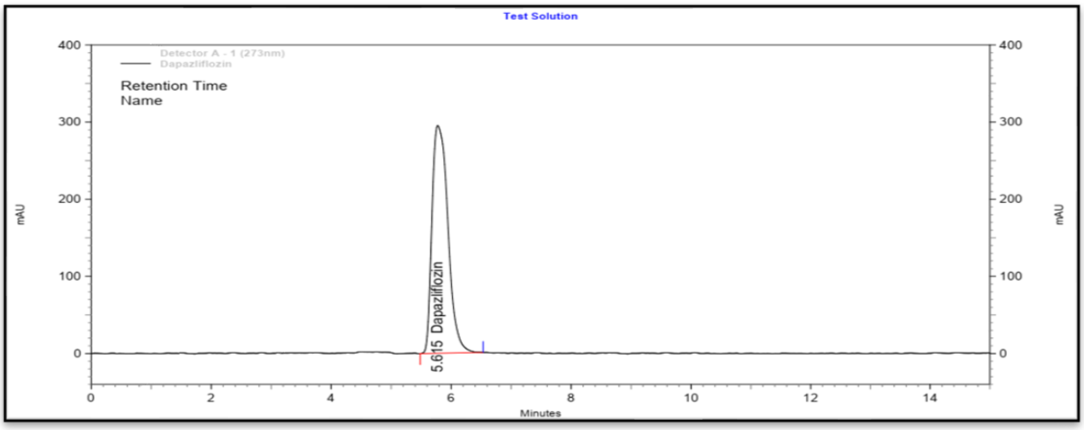


Figure No: 6.61 Chromatogram of Alkali degradation in 0.1 N NaOH for 24hr at 80°C

Table No: 6.55 Result of Acid degradation in 0.1 N NaOH for 24 hr at 80ºC

Preparation of solution	Degradation condition	Observation
1 ml Stock Solution(1000 µg/ml) + 1 ml 0.1 N NaOH, After 24 hr Add 1 ml 0.1 N HCl and Dilute up to 10 ml with Mobile Phase to make final concentration 100µg/ml	In 0.1 N NaOH At 80ºC for 24 hr	No Degradation

6.7.3.2 Acid and Alkali Degradation in 1 N HCl and 1 N NaOH

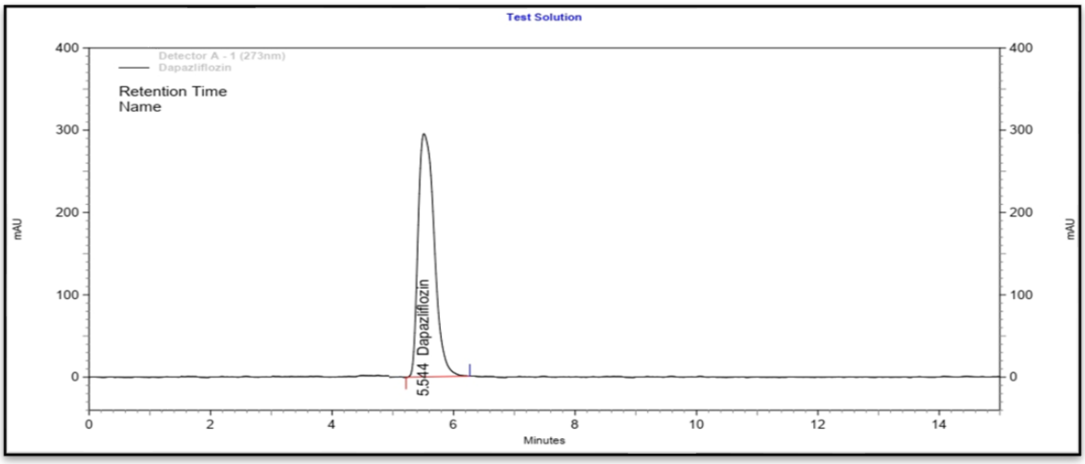


Figure No: 6.62 Chromatogram of Acid degradation in 1 N HCl for 24 hr at 80ºC

Table No: 6.56 Result of Acid degradation in 1 N HCl for 24 hr at 80ºC

Preparation of solution	Degradation condition	Observation
1 ml Stock Solution (1000 µg/ml) + 1 ml 1 N HCl, After 24 hr Add 1 ml 1 N NaOH and Dilute up to 10 ml with Mobile Phase to make final concentration 100µg/ml	In 1 N HCl At 80ºC for 24 hr	No Degradation

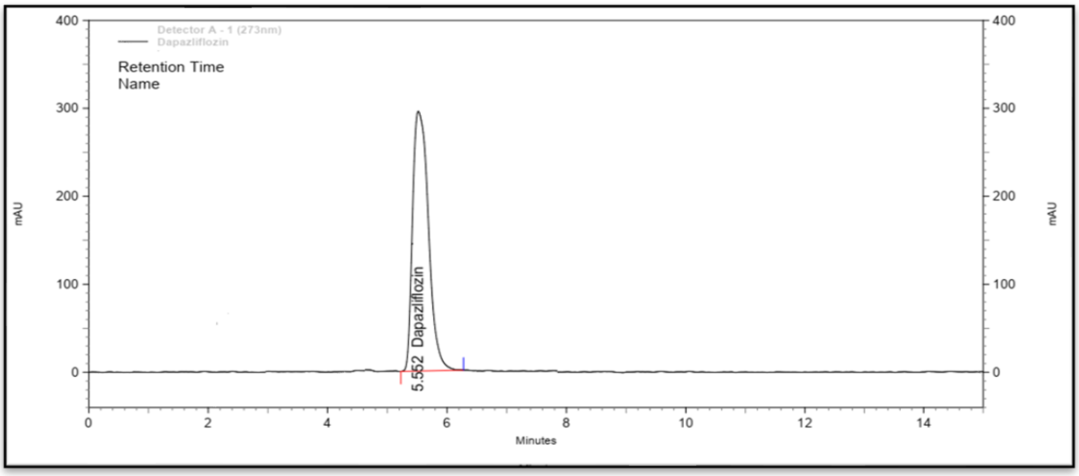


Figure No: 6.63 Chromatogram of Alkali degradation in 1 N NaOH for 24 hr at 80ºC

Table No: 6.57 Result of Alkali degradation in 1 N NaOH for 24 hr at 80ºC

Preparation of solution	Degradation condition	Observation
1 ml Stock Solution (1000 µg/ml) + 1 ml 1 N NaOH, After 24 hr Add 1 ml 1 N HCl and Dilute up to 10 ml with Mobile Phase to make final concentration 100µg/ml	In 1 N NaOH At 80ºC for 24 hr	No Degradation

6.7.3.3 Acid and Alkali Degradation in 2 N HCl and 2 N NaOH

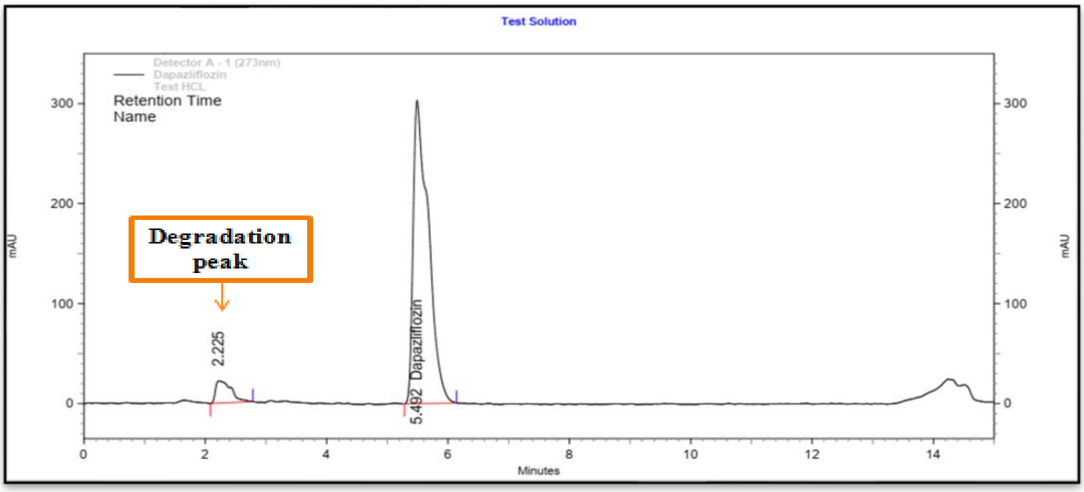


Figure No: 6.64 Chromatogram of Acid degradation in 2 N HCl for 6 hr at 60ºC

Table No: 6.58 Result of Acid degradation in 2 N HCl for 6 hr at 60ºC

Preparation of solution	Degradation condition	Observation
1 ml Stock Solution(1000 µg/ml) + 1 ml 2 N HCl, After 6 hr Add 1 ml 2 N NaOH and Dilute up to 10 ml with Mobile Phase to make final concentration 100µg/ml	In 2 N HCl At 60ºC for 6 hr	12.45 % degradation Observe at 2.22 min

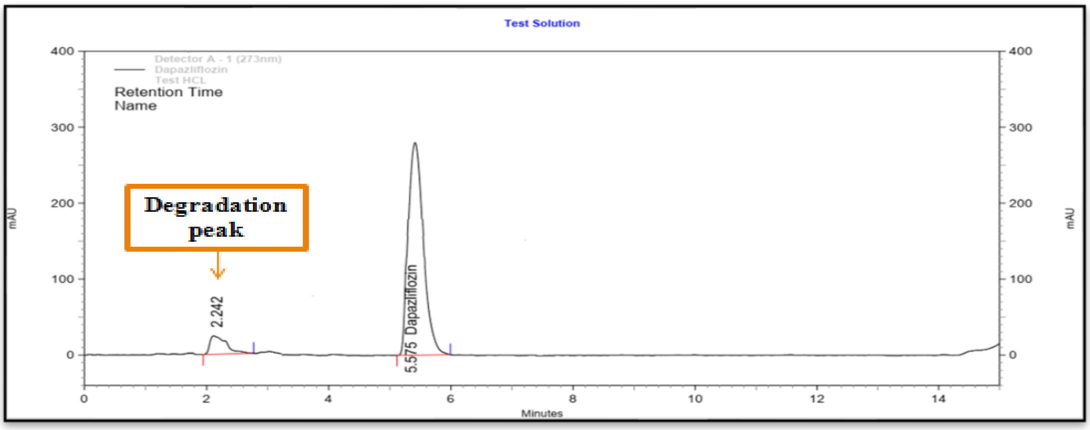


Figure No: 6.65 Chromatogram of Acid degradation in 2 N HCl for 12 hr at 60ºC

Table No: 6.59 Result of Acid degradation in 2 N HCl for 12 hr at 60ºC

Preparation of solution	Degradation condition	Observation
1 ml Stock Solution(1000 µg/ml) + 1 ml 2 N HCl, After 12 hr Add 1 ml 2 N NaOH and Dilute up to 10 ml with Mobile Phase to make final concentration 100µg/ml	In 2 N HCl At 60ºC for 12 hr	17.76 % degradation Observe at 2.24 min

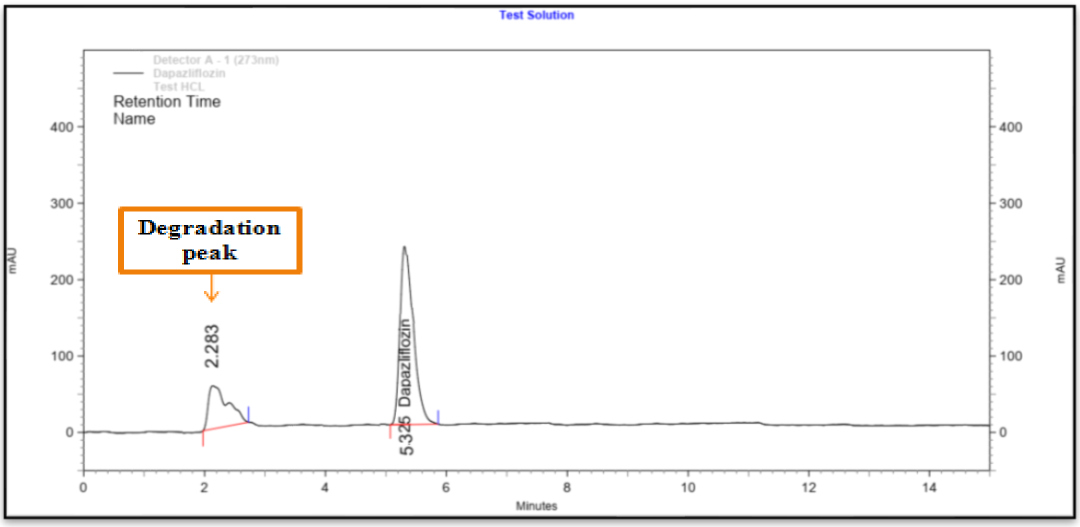


Figure No: 6.66 Chromatogram of Acid degradation in 2 N HCl for 18 hr at 60ºC

Table No: 6.60 Result of Acid degradation in 2 N HCl for 18 hr at 60ºC

Preparation of solution	Degradation condition	Observation
1 ml Stock Solution (1000 µg/ml) + 1 ml 2 N HCl, After 18 hr Add 1 ml 2 N NaOH and Dilute up to 10 ml with Mobile Phase to make final concentration 100µg/ml	In 2 N HCl At 60ºC for 18 hr	26.84 % degradation Observe at 2.28 min

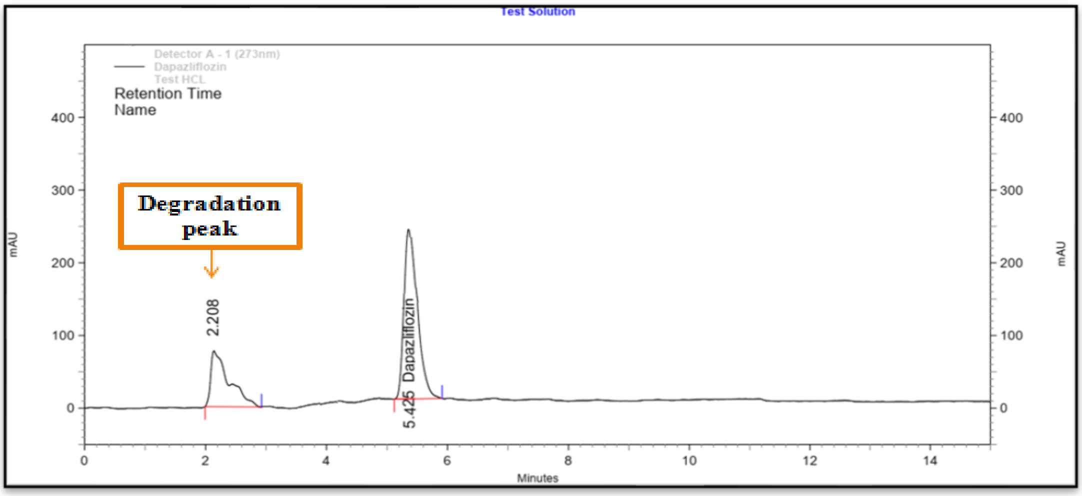


Figure No: 6.67 Chromatogram of Acid degradation in 2 N HCl for 24 hr at 60ºC

Table No: 6.61 Result of Acid degradation in 2 N HCl for 24 hr at 60ºC

Preparation of solution	Degradation condition	Observation
1 ml Stock Solution(1000 µg/ml) + 1 ml 2 N HCl, After 24 hr Add 1 ml 2 N NaOH and Dilute up to 10 ml with Mobile Phase to make final concentration 100µg/ml	In 2 N HCl At 60ºC for 24 hr	41.56 % degradation Observe at 2.20 min

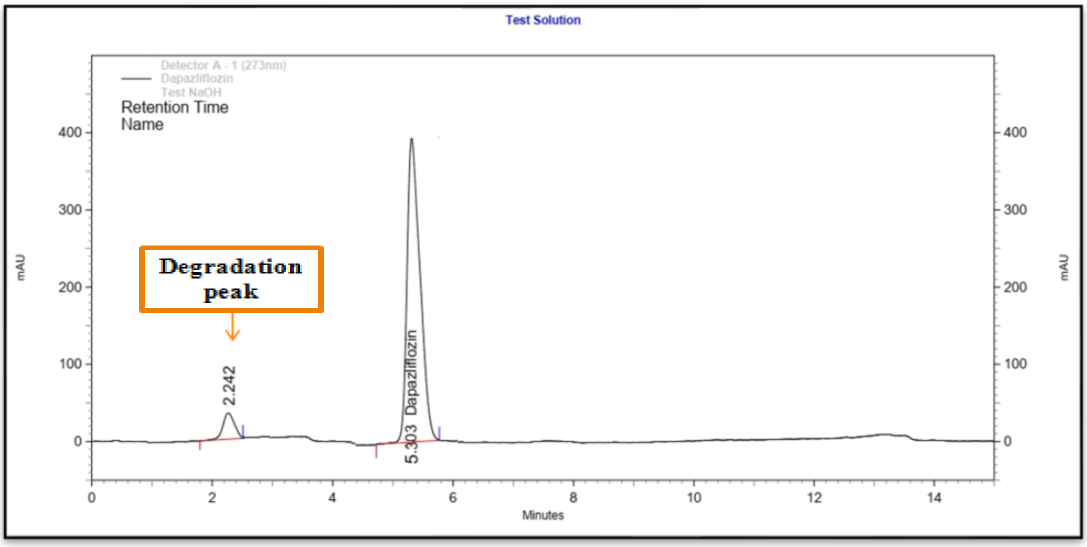


Figure No: 6.68 Chromatogram of Acid degradation in 2 N NaOH for 6 hr at 60ºC

Table No: 6.62 Result of Acid degradation in 2 N NaOH for 6 hr at 60ºC

Preparation of solution	Degradation condition	Observation
1 ml Stock Solution (1000 µg/ml) + 1 ml 2 N NaOH, After 06 hr Add 1 ml 2 N HCl and Dilute up to 10 ml with Mobile Phase to make final concentration 100µg/ml	In 2 N NaOH At 60ºC for 06 hr	11.33% degradation Observe at 2.24 min

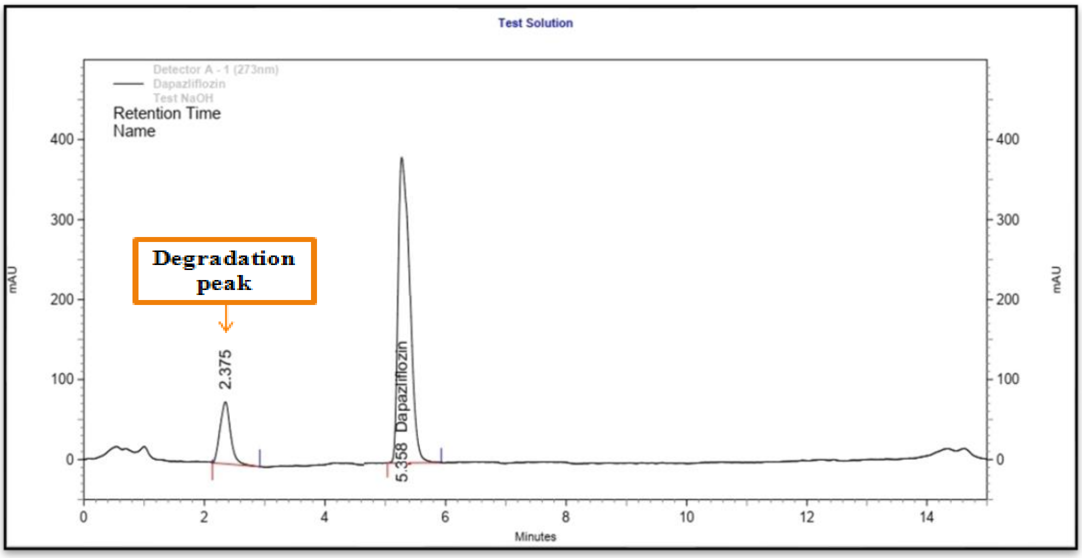


Figure No: 6.69 Chromatogram of Acid degradation in 2 N NaOH for 12 hr at 60ºC

Table No: 6.63 Result of Acid degradation in 2 N NaOH for 12 hr at 60ºC

Preparation of solution	Degradation condition	Observation
1 ml Stock Solution(1000 µg/ml) + 1 ml 2 N NaOH, After 12 hr Add 1 ml 2 N HCl and Dilute up to 10 ml with Mobile Phase to make final concentration 100µg/ml	In 2 N NaOH At 60ºC for 12 hr	25.23 % degradation Observe at 2.37 min

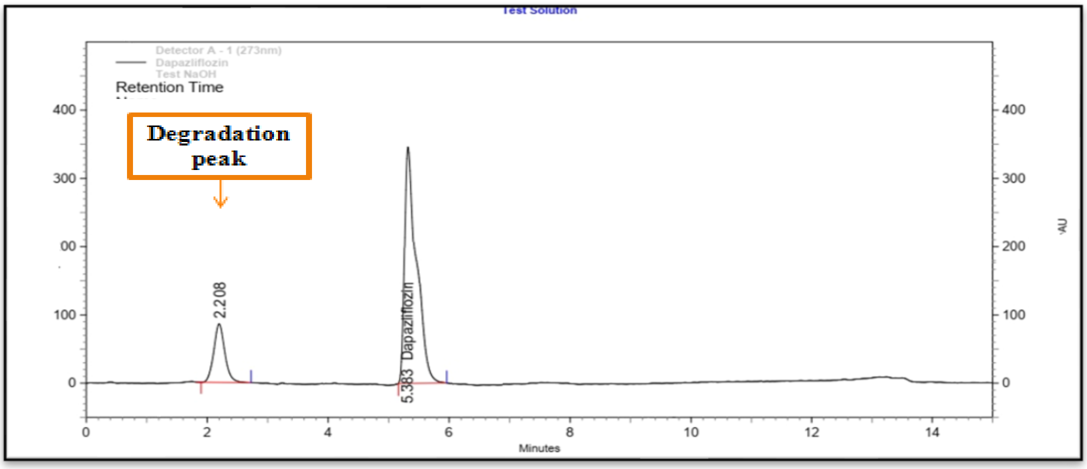


Figure No: 6.70 Chromatogram of Acid degradation in 2 N NaOH for 18 hr at 60ºC

Table No: 6.64 Result of Acid degradation in 2 N NaOH for 18 hr at 60ºC

Preparation of solution	Degradation condition	Observation
1 ml Stock Solution (1000 µg/ml) + 1 ml 2 N NaOH, After 18 hr Add 1 ml 2 N HCl and Dilute up to 10 ml with Mobile Phase to make final concentration 100µg/ml	In 2 N NaOH At 60ºC for 18 hr	34.65 % degradation Observe at 2.20 min

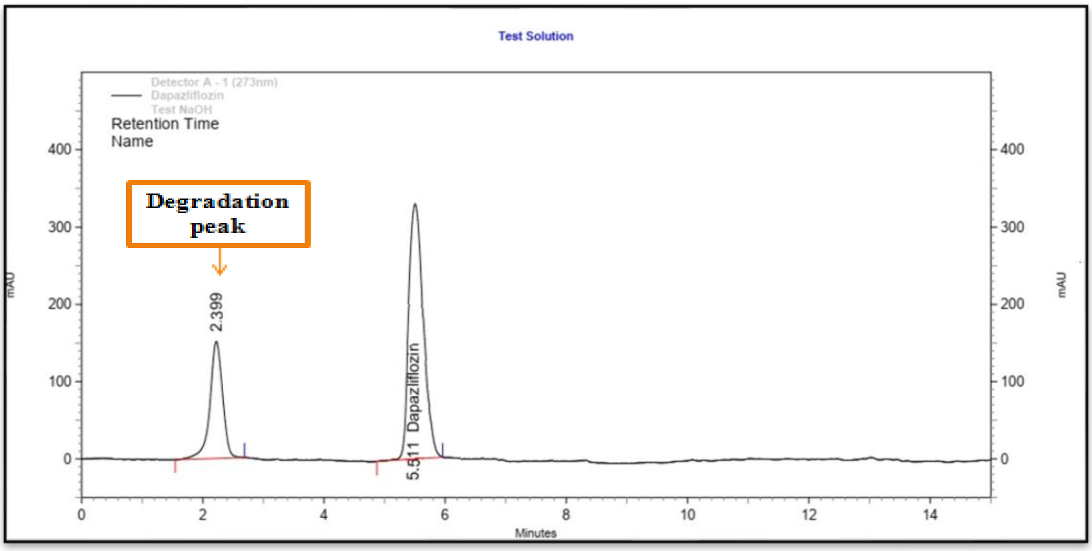


Figure No: 6.71 Chromatogram of Acid degradation in 2 N NaOH for 24 hr at 60ºC

Table No: 6.65 Result of Acid degradation in 2 N NaOH for 24 hr at 60ºC

Preparation of solution	Degradation condition	Observation
1 ml Stock Solution (1000 µg/ml) + 1 ml 2 N NaOH, After 24 hr Add 1 ml 2 N HCl and Dilute up to 10 ml with Mobile Phase to make final concentration 100µg/ml	In 2 N NaOH At 60ºC for 24 hr	43.84 % degradation Observe at 2.39 min

6.7.3.4 Oxidative Degradation in 20% and 30 % H_2O_2

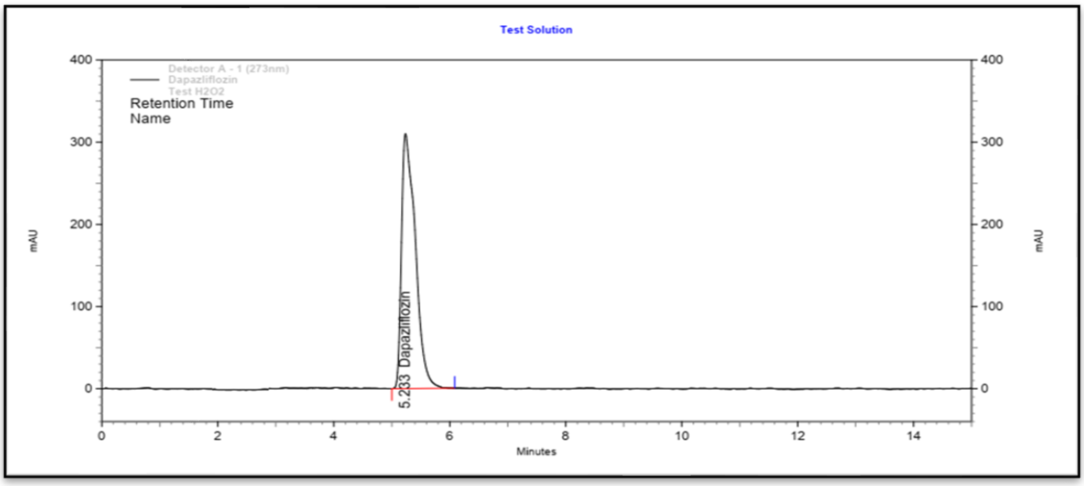


Figure No: 6.72 Chromatogram of Oxidative degradation in 20% H_2O_2 for 24 hr at 60ºC

Table No: 6.66 Result of Oxidative degradation in 20% H_2O_2 for 24 hr at 60ºC

Preparation of solution	Degradation condition	Observation
1 ml Stock Solution(1000 µg/ml) + 1 ml 20% H_2O_2, After 24 hr , Dilute up to 10 ml with Mobile Phase to make final concentration 100µg/ml	In 20% H_2O_2 At 60ºC for 24 hr	No degradation

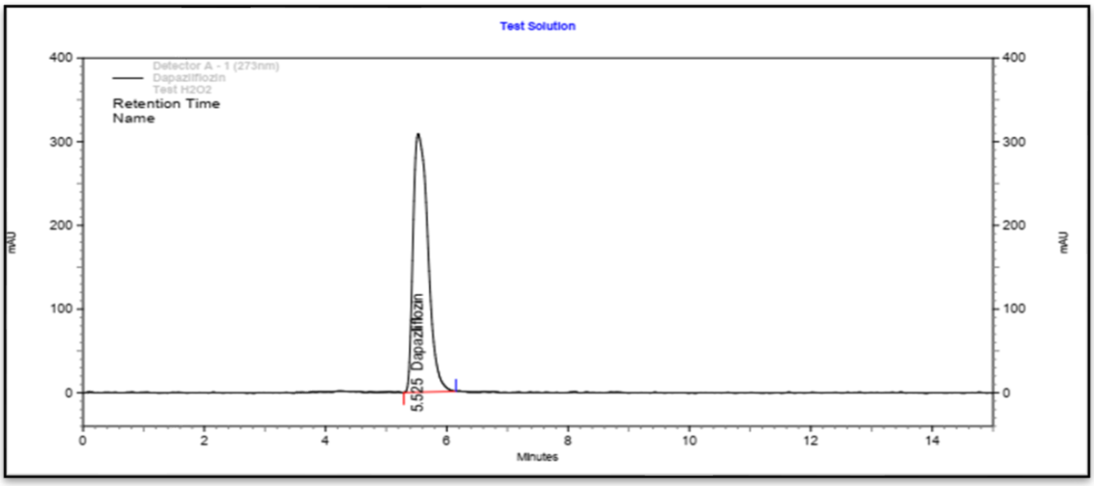


Figure No: 6.73 Chromatogram of Oxidative degradation in 30% H_2O_2 for 24 hr at 60ºC

Table No: 6.67 Result of Oxidative degradation in 30% H_2O_2 for 24 hr at 60ºC

Preparation of solution	Degradation condition	Observation
1 ml Stock Solution(1000 µg/ml) + 1 ml 30% H_2O_2, After 24 hr , Dilute up to 10 ml with Mobile Phase to make final concentration 100µg/ml	In 30% H_2O_2 At 60ºC for 24 hr	No degradation

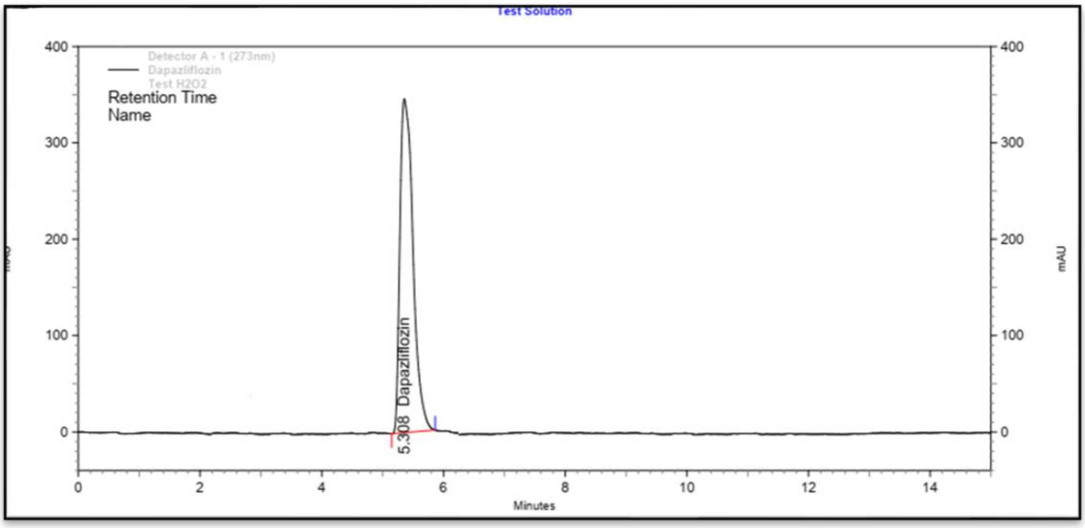


Figure No: 6.74 Chromatogram of Oxidative degradation in 30% H_2O_2 for 24 hr at 80ºC

Table No: 6.68 Result of Oxidative degradation in 30% H_2O_2 for 24 hr at 80ºC

Preparation of solution	Degradation condition	Observation
1 ml Stock Solution(1000 µg/ml) + 1 ml 30% H_2O_2, After 24 hr , Dilute up to 10 ml with Mobile Phase to make final concentration 100µg/ml	In 30% H_2O_2 At 80ºC for 24 hr	No degradation

6.7.3.5 Thermal Degradation

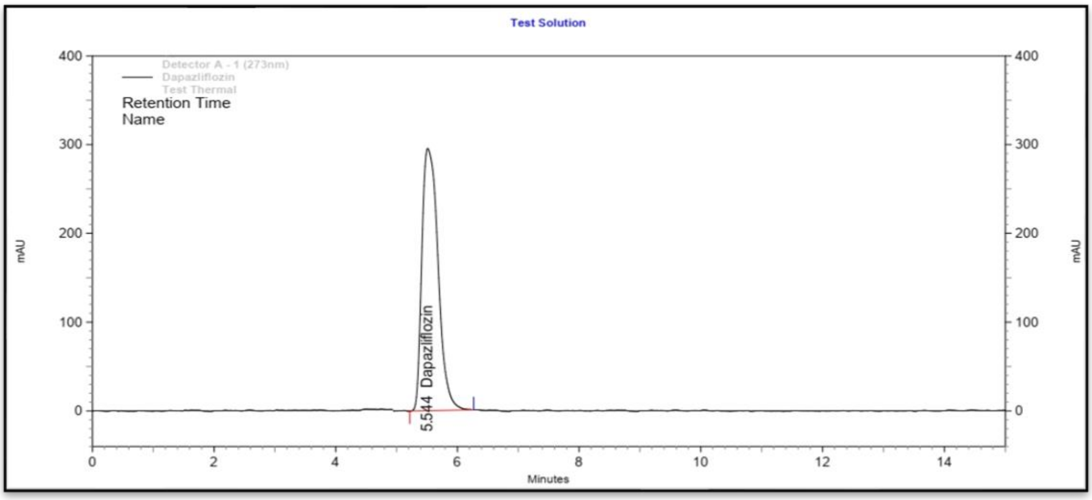


Figure No: 6.75 Chromatogram of Thermal degradation for 24 hr at 60ºC

Table No: 6.69 Result of Thermal degradation for 24 hr at 60ºC

Preparation of solution	Degradation condition	Observation
10 mg Dapagliflozin in dry state expose for given condition, Dilution made up to 10 ml with Methanol to get concentration 100 µg/ml	In Hot air Oven At 60ºC for 24 hr	No Degradation

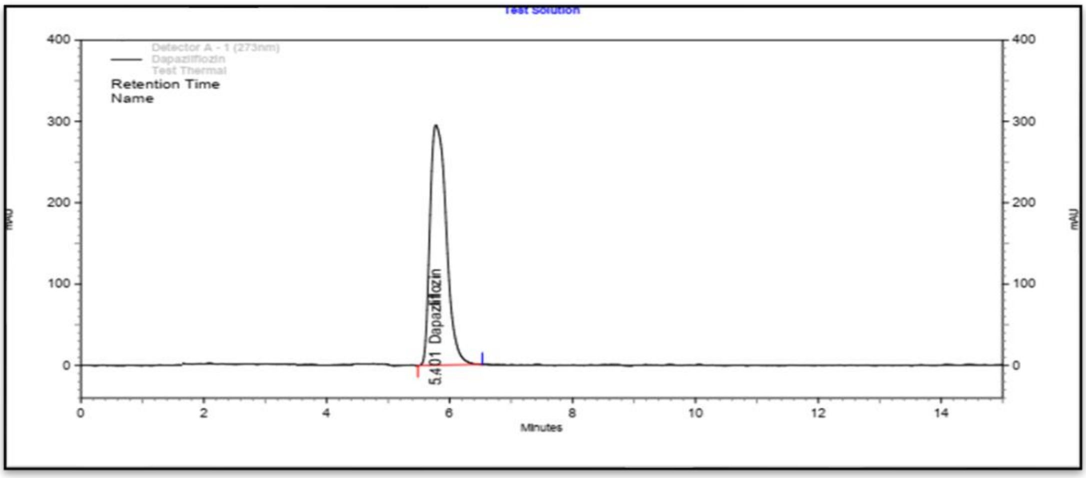


Figure No: 6.76 Chromatogram of Thermal degradation for 24 hr at 80ºC

Table No: 6.70 Result of Thermal degradation for 24 hr at 80ºC

Preparation of solution	Degradation condition	Observation
10 mg of Dapagliflozin in dry state expose for given condition, Dilution made up to 10 ml with Methanol to get concentration 100 µg/ml	In Hot air Oven At 80ºC for 24 hr	No Degradation

6.7.3.6 Photo Degradation

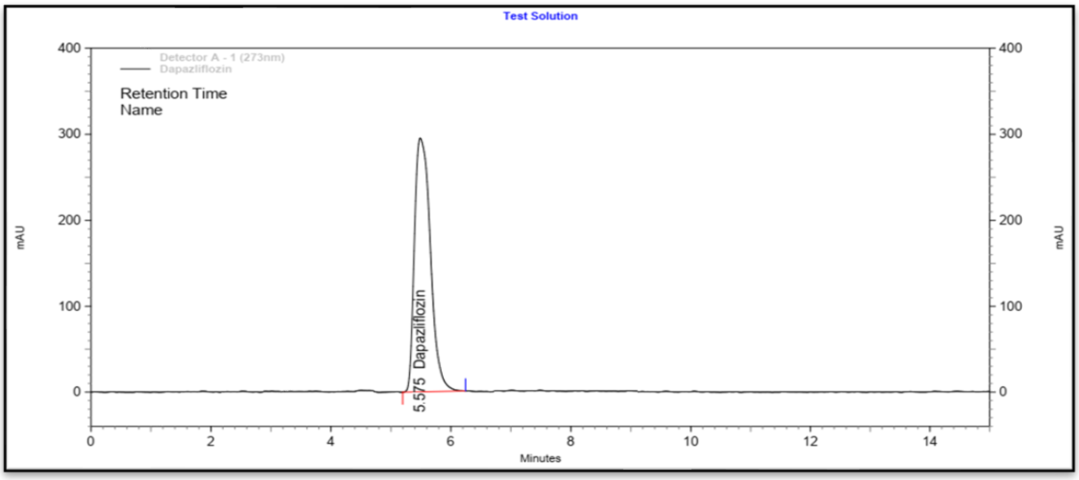


Figure No: 6.77 Chromatogram of Photo degradation for 7 days in UV Light

Table No: 6.71 Result of Photo degradation for 7 days in UV Light

Preparation of solution	Degradation condition	Observation
10 mg of Dapagliflozin in dry state expose under UV Light for 7 days, Dilution made up to 10 ml with Methanol to get concentration 100 µg/ml	In UV Chamber For 7 Days	No Degradation

Table No: 6.72 Summary of Degradation study of Dapagliflozin in API

Sr. No	Condition	Temperature (ºC)	Time duration for degradation	Retention time of Std peak (min)	Degradation observed Yes/No	% Degradation observed	Retention time of degradation peak (min)
1.	0.1 N HCl	80	24 hr	5.44	No	-	-
2.	1 N HCl	80	24 hr	5.54	No	-	-
3	2 N HCl	60	6 hr	5.49	Yes	12.45	2.25
			12 hr	5.57	Yes	17.76	2.24
			18 hr	5.32	Yes	26.84	2.28
			24 hr	5.42	Yes	41.56	2.20
4	0.1 N NaOH	80	24 hr	5.61	No	-	-
5	1 N NaOH	80	24 hr	5.55	No	-	-
6	2 N NaOH	60	6 hr	5.30	Yes	11.33	2.24
			12 hr	5.35	Yes	25.23	2.37
			18 hr	5.38	Yes	34.65	2.20
			24 hr	5.51	Yes	43.84	2.39
7	20 % H_2O_2	60	24 hr	5.23	No	-	-
		80	24 hr	5.54	No	-	-
	30 % H_2O_2	60	24 hr	5.52	No	-	-
		80	24 hr	5.30	No	-	-
8		60	24 hr	5.54	No	-	-

	Thermal In Hot Air Oven	80	24 hr	5.40	No	-	-
9	Photo degradation Study	UV Light	7 Days	5.57	No	-	-

6.7.4 Forced Degradation Study of Dapagliflozin in Marketed Formulation (DAPARYL 10)

6.7.4.1 Acid and Alkali Degradation in 2 N HCl and 2 N NaOH

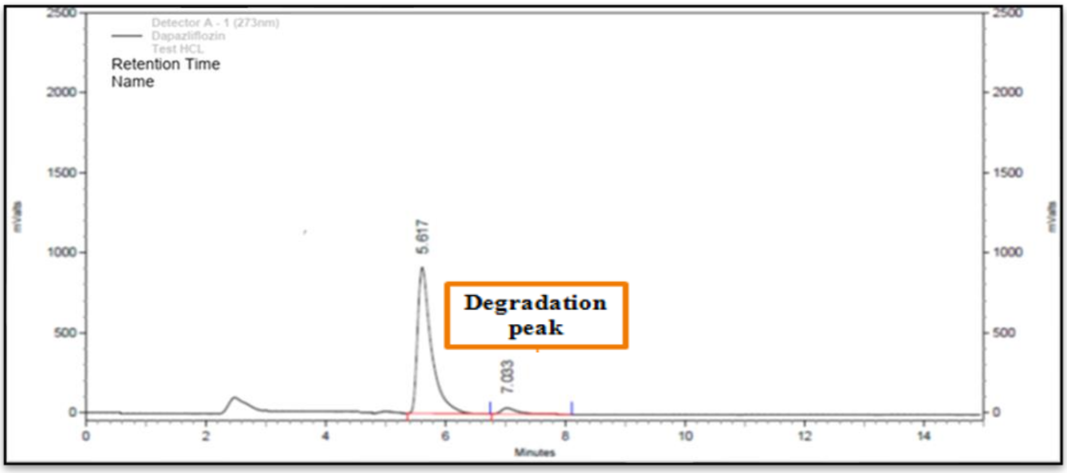


Figure No: 6.78 Chromatogram of Acid degradation in 2 N HCl for 6 hr at 60ºC

Table No: 6.73 Result of Acid degradation in 2 N HCl for 6 hr at 60ºC

Preparation of solution	Degradation condition	Observation
1 ml Stock Solution (1000 µg/ml) + 1 ml 2 N HCl, After 6 hr Add 1 ml 2 N NaOH and Dilute up to 10 ml with Mobile Phase to make final concentration 100µg/ml	In 2 N HCl At 60ºC for 6 hr	4.73 % degradation Observe at 7.03 min

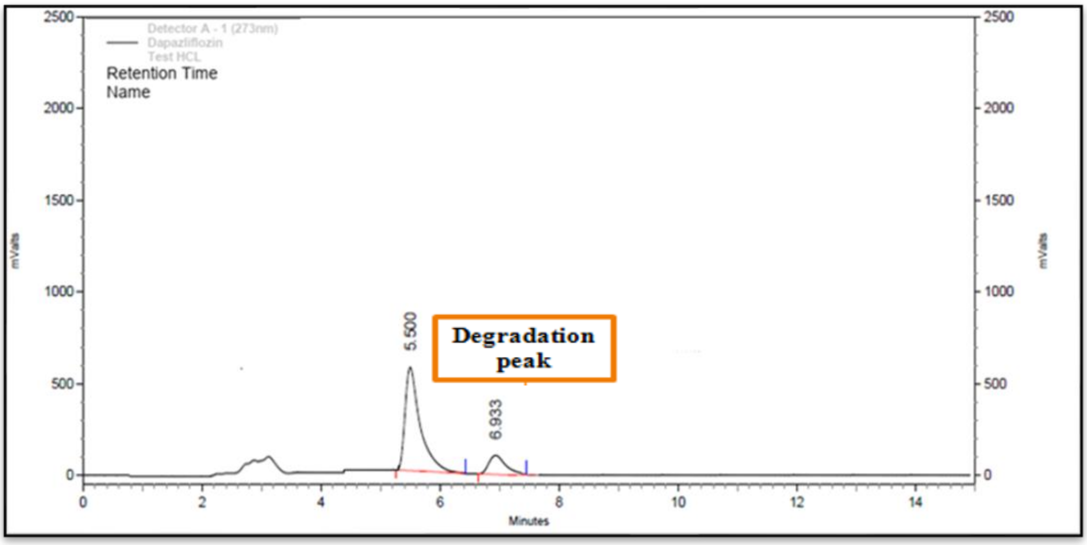


Figure No: 6.79 Chromatogram of Acid degradation in 2 N HCl for 12 hr at 60ºC

Table No: 6.74 Result of Acid degradation in 2 N HCl for 12 hr at 60ºC

Preparation of solution	Degradation condition	Observation
1 ml Stock Solution (1000 µg/ml) + 1 ml 2 N HCl, After 12 hr Add 1 ml 2 N NaOH and Dilute up to 10 ml with Mobile Phase to make final concentration 100µg/ml	In 2 N HCl At 60ºC for 12 hr	Total 18.05% degradation Observe at 6.93 min

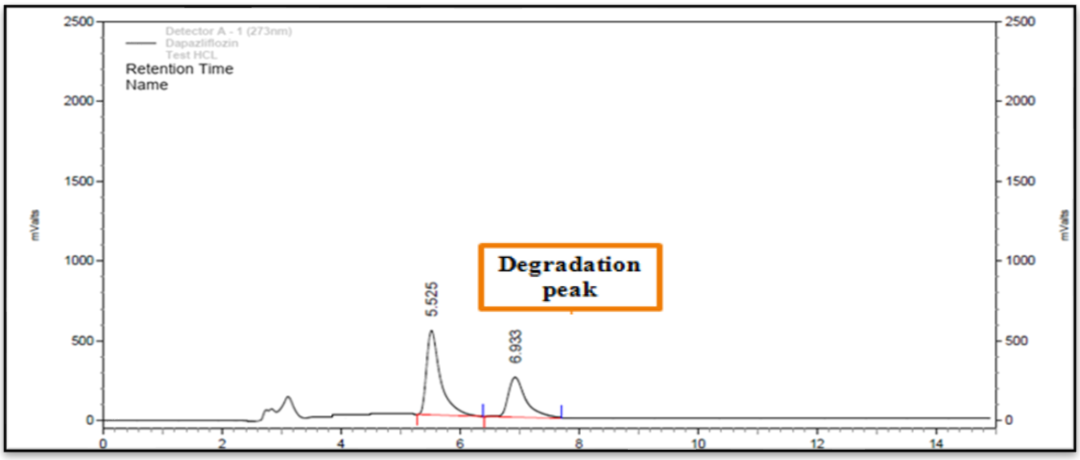


Figure No: 6.80 Chromatogram of Acid degradation in 2 N HCl for 18 hr at 60ºC

Table No: 6.75 Result of Acid degradation in 2 N HCl for 18 hr at 60ºC

Preparation of solution	Degradation condition	Observation
1 ml Stock Solution (1000 µg/ml) + 1 ml 2 N HCl, After 18 hr Add 1 ml 2 N NaOH and Dilute up to 10 ml with Mobile Phase to make final concentration 100µg/ml	In 2 N HCl At 60ºC for 18 hr	37.91 % degradation Observe at 6.93 min

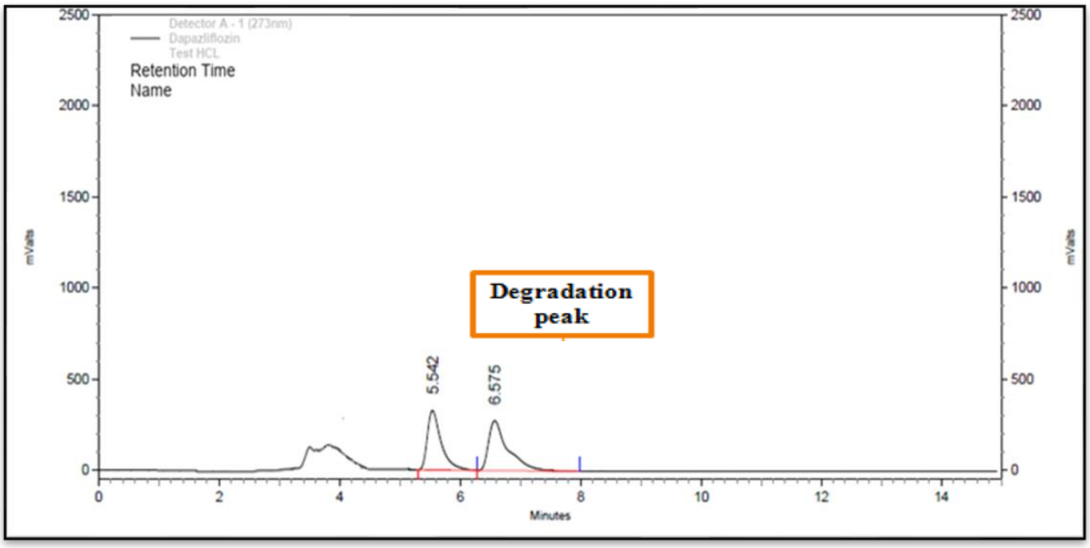


Figure No: 6.81 Chromatogram of Acid degradation in 2 N HCl for 24 hr at 60ºC

Table No: 6.76 Result of Acid degradation in 2 N HCl for 24 hr at 60ºC

Preparation of solution	Degradation condition	Observation
1 ml Stock Solution (1000 µg/ml) + 1 ml 2 N HCl, After 24 hr Add 1 ml 2 N NaOH and Dilute up to 10 ml with Mobile Phase to make final concentration 100µg/ml	In 2 N HCl At 60ºC for 24 hr	52.77 % degradation Observe at 6.57 min

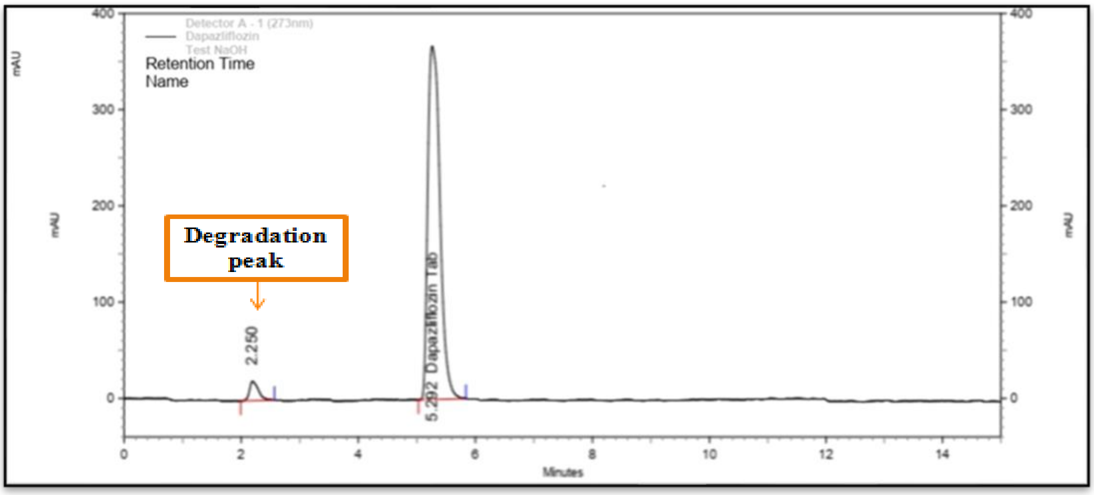


Figure No: 6.82 Chromatogram of Alkali degradation in 2 N NaOH for 6 hr at 60ºC

Table No: 6.77 Result of Alkali degradation in 2 N NaOH for 6 hr at 60ºC

Preparation of solution	Degradation condition	Observation
1 ml Sample Stock Solution (1000 µg/ml) + 1 ml 2 N NaOH, After 06hr Add 1 ml 2 N HCl and Dilute up to 10 ml with Mobile Phase to make final concentration 100µg/ml	In 2 N NaOH At 60ºC for 06 hr	07.54 % degradation Observe at 2.25 min

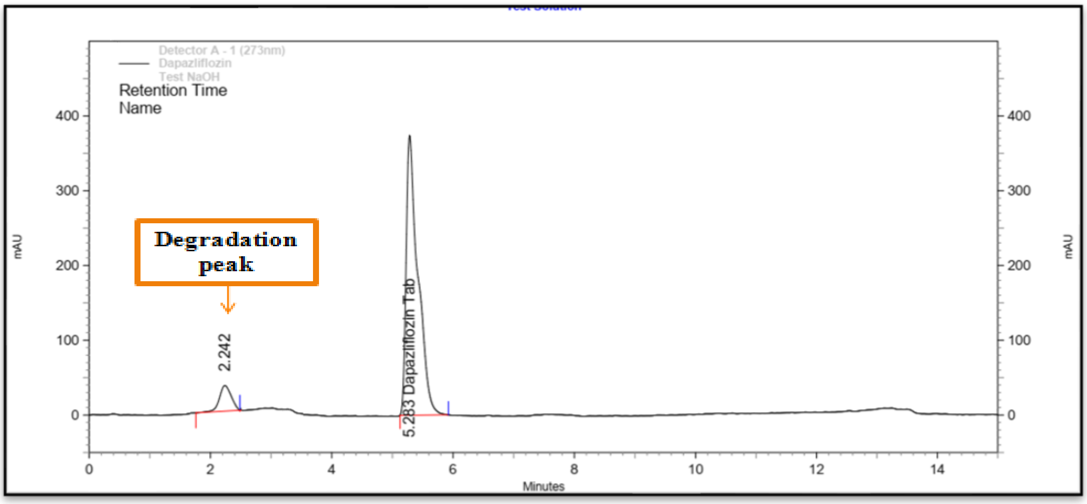


Figure No: 6.83 Chromatogram of Alkali degradation in 2 N NaOH for 12 hr at 60ºC

Table No: 6.78 Result of Alkali degradation in 2 N NaOH for 12 hr at 60ºC

Preparation of solution	Degradation condition	Observation
1 ml Sample Stock Solution(1000 µg/ml) + 1 ml 2 N NaOH, After 12hr Add 1 ml 2 N HCl and Dilute up to 10 ml with Mobile Phase to make final concentration 100µg/ml	In 2 N NaOH At 60ºC for 12 hr	17.32 % degradation Observe at 2.24 min

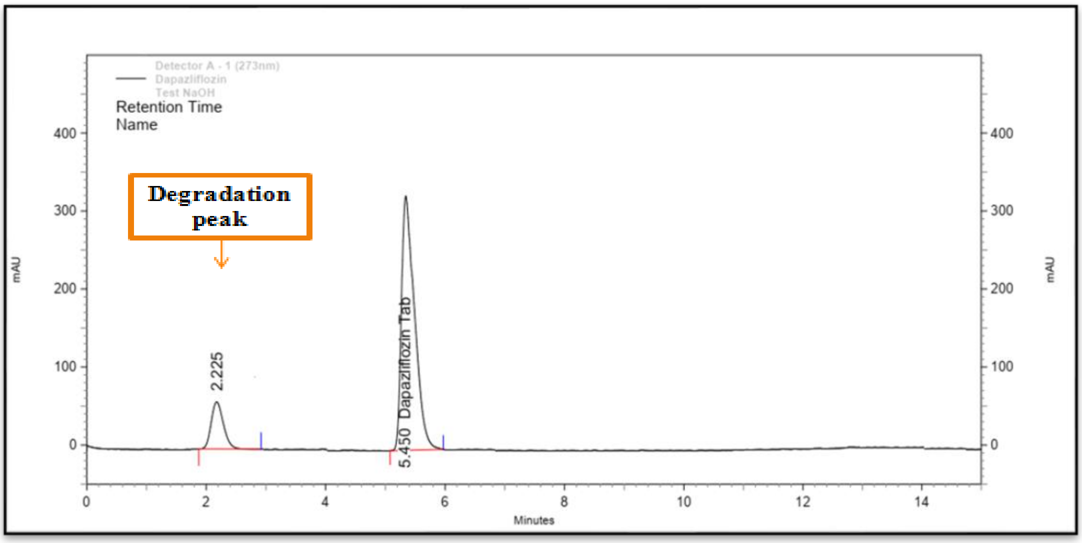


Figure No: 6.84 Chromatogram of Alkali degradation in 2 N NaOH for 18 hr at 60ºC

Table No: 6.79 Result of Alkali degradation in 2 N NaOH for 18 hr at 60ºC

Preparation of solution	Degradation condition	Observation
1 ml Sample Stock Solution(1000 µg/ml) + 1 ml 2 N NaOH, After 18hr Add 1 ml 2 N HCl and Dilute up to 10 ml with Mobile Phase to make final concentration 100µg/ml	In 2 N NaOH At 60ºC for 18 hr	25.87 % degradation Observe at 2.22 min

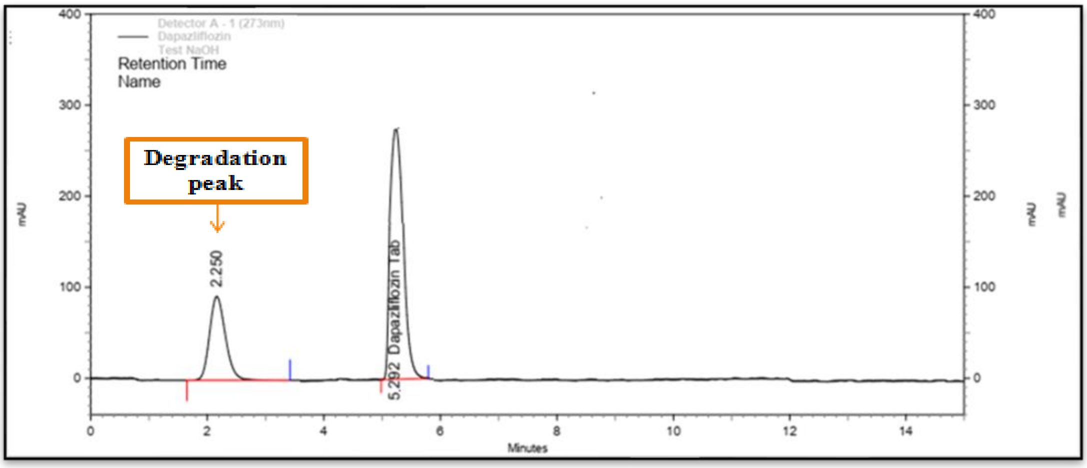


Figure No: 6.85 Chromatogram of Alkali degradation in 2 N NaOH for 24 hr at 60°C

Table No: 6.80 Result of Alkali degradation in 2 N NaOH for 24 hr at 60°C

Preparation of solution	Degradation condition	Observation
1 ml Sample Stock Solution (1000 µg/ml) + 1 ml 2 N NaOH, After 24hr Add 1 ml 2 N HCl and Dilute up to 10 ml with Mobile Phase to make final concentration 100µg/ml	In 2 N NaOH At 60°C for 24 hr	45.43 % degradation Observe at 2.25 min

6.7.4.2 Oxidative Degradation 20%, 30% H_2O_2

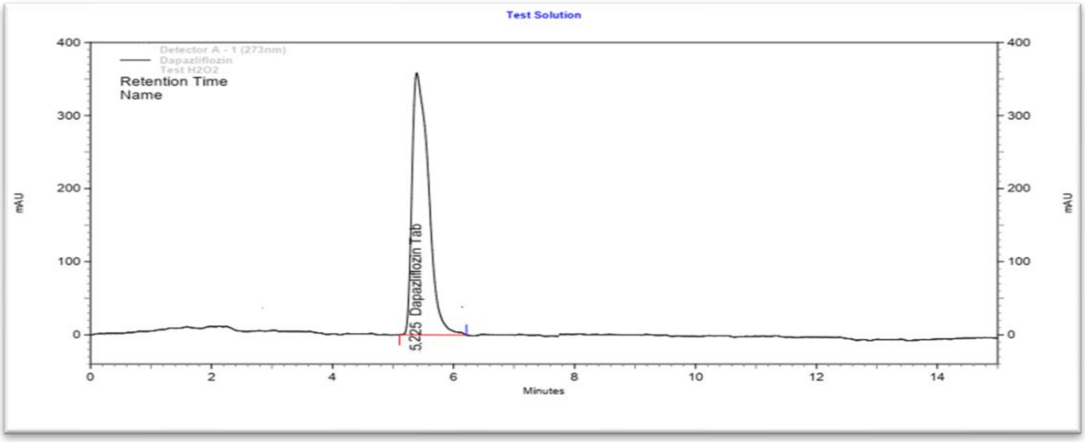


Figure No: 6.86 Chromatogram of Oxidative degradation in 20 % H_2O_2 for 24 hr at 60°C

Table No: 6. 81 Result of Oxidative degradation in 20 % H_2O_2 for 24 hr at 60ºC

Preparation of solution	Degradation condition	Observation
1 ml Sample Stock Solution (1000 µg/ml) + 1 ml 20 % H_2O_2, After 24 hr , Dilute up to 10 ml with Mobile Phase to make final concentration 100µg/ml	In 20% H_2O_2 At 60ºC for 24 hr	No Degradation

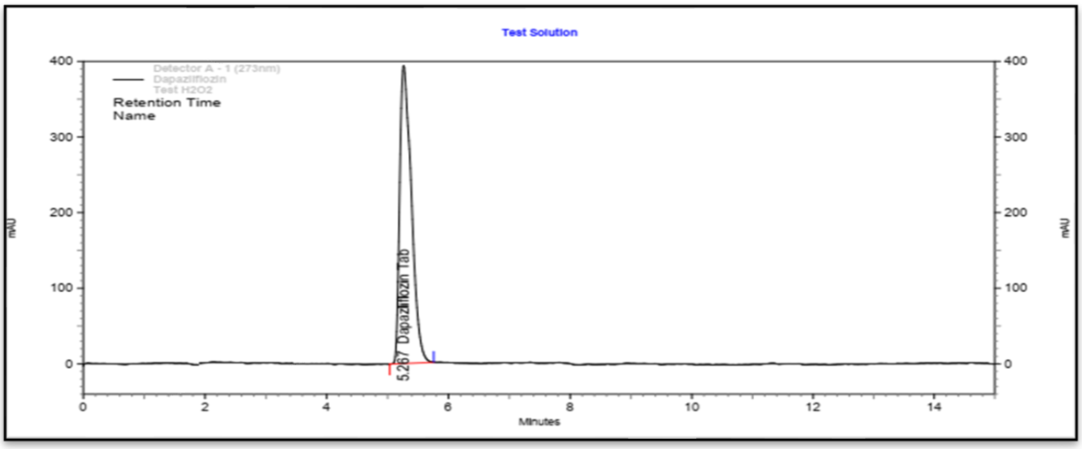


Figure No: 6.87 Chromatogram of Oxidative degradation in 20 % H_2O_2 for 24 hr at 80ºC

Table No: 6.82 Result of Oxidative degradation in 20 % H_2O_2 for 24 hr at 80ºC

Preparation of solution	Degradation condition	Observation
1 ml Sample Stock Solution (1000 µg/ml) + 1 ml 20% H_2O_2, After 24 hr , Dilute up to 10 ml with Mobile Phase to make final concentration 100µg/ml	In 20% H_2O_2 At 80ºC for 24 hr	No Degradation

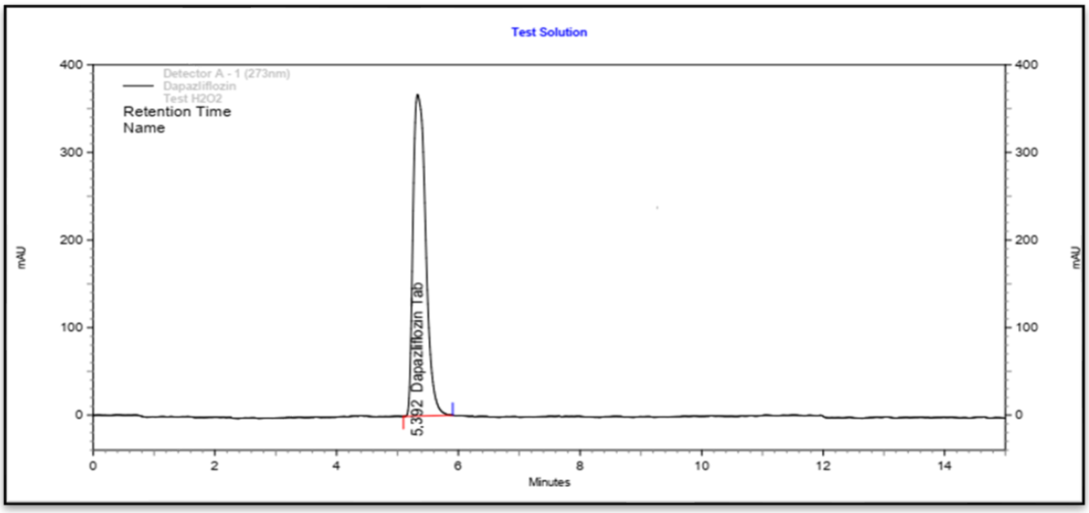


Figure No: 6.88 Chromatogram of Oxidative degradation in 30 % H_2O_2 for 24 hr at 60ºC

Table No: 6.83 Result of Oxidative degradation in 30 % H_2O_2 for 24 hr at 60ºC

Preparation of solution	**Degradation condition**	**Observation**
1 ml sample Stock Solution (1000 µg/ml) + 1 ml 30% H_2O_2, After 24 hr , Dilute up to 10 ml with Mobile Phase to make final concentration 100µg/ml	In 30% H_2O_2 At 60ºC for 24 hr	No Degradation

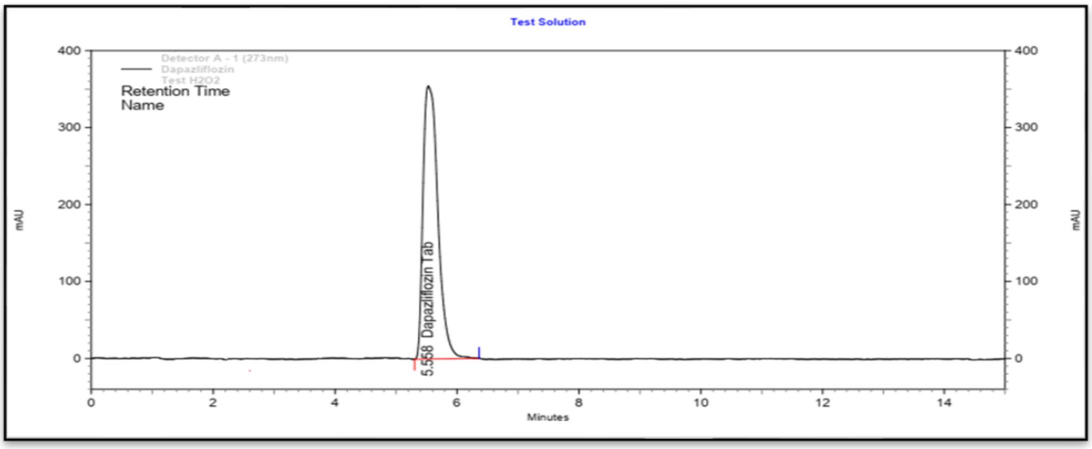


Figure No: 6.89 Chromatogram of Oxidative degradation in 30 % H_2O_2 for 24 hr at 80ºC

Table No: 6.84 Result of Oxidative degradation in 30 % H_2O_2 for 24 hr at 80ºC

Preparation of solution	Degradation condition	Observation
1 ml sample Stock Solution (1000 µg/ml) + 1 ml 30% H_2O_2, After 24 hr , Dilute up to 10 ml with Mobile Phase to make final concentration 100µg/ml	In 30% H_2O_2 At 80ºC for 24 hr	No Degradation

6.7.4.3 Thermal Degradation

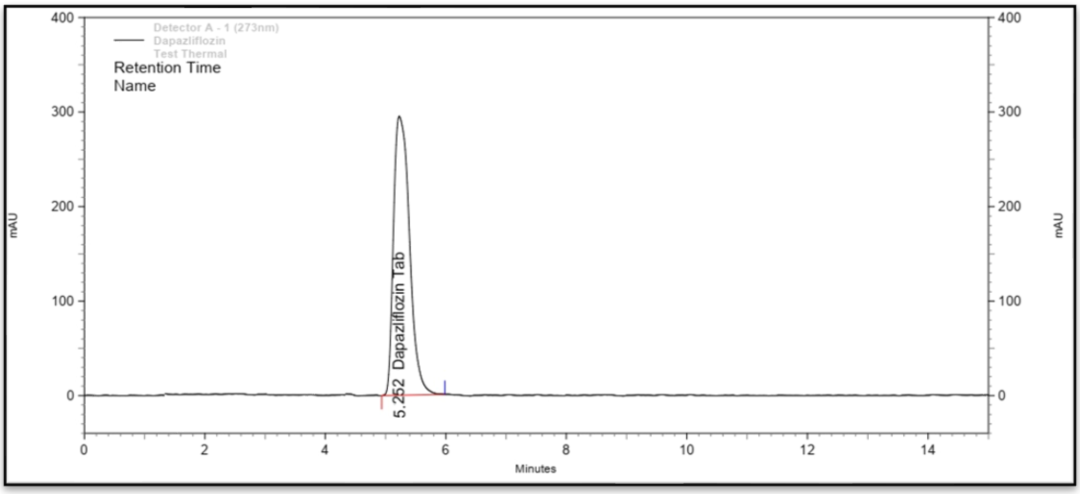


Figure No: 6.90 Chromatogram of Thermal degradation for 24 hr at 80ºC

Table No: 6.85 Result of Thermal degradation for 24 hr at 80ºC

Preparation of solution	Degradation condition	Observation
Powder of Tablet equivalent to 10 mg of Dapagliflozin in dry state expose for given condition, Dilution made up to 10 ml with Methanol to get concentration 100 µg/ml	In Hot air Oven At 80ºC for 24 hr	No Degradation

6.7.4.4 Photo Degradation

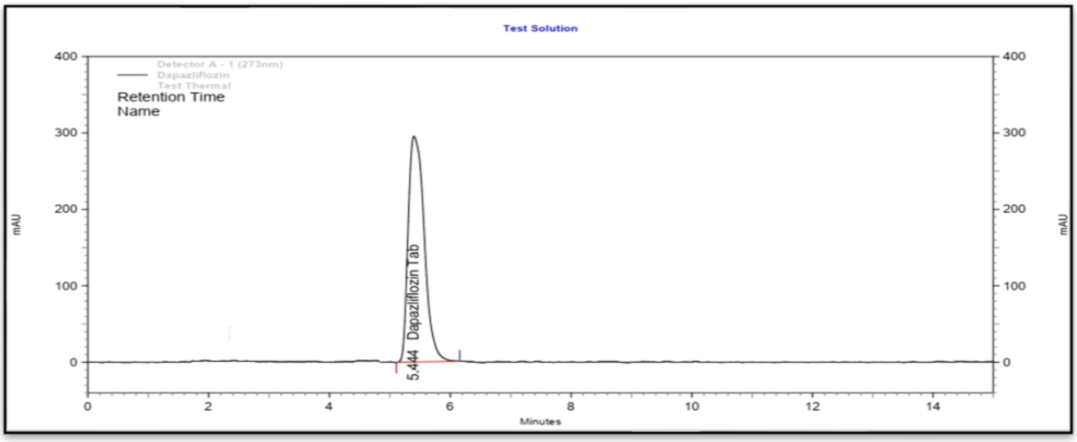


Figure No: 6.91 Chromatogram of Photo degradation for 7 days in UV Light

Table No: 6.86 Result of Photo degradation for 7 days in UV Light

Preparation of solution	Degradation condition	Observation
Powder of Tablet equivalent to 10 mg of Dapagliflozin in dry state expose under UV Light for 7 days, Dilution made with Methanol up to 10 ml to get concentration 100 µg/ml	In UV Chamber For 7 Days	No Degradation

Table No: 6.87 Summary of degradation study of Dapagliflozin in Marketed Formulation

Sr. No	Condition	Temperature (ºC)	Time duration for degradation	Retention time of Std peak (min)	Degradation observed Yes/No	% Degradation observed	Retention time of degradation peak (min)
1	2 N HCl	60	6 hr	5.35	Yes	4.73	7.00
			12 hr	5.32	Yes	18.05	6.93
			18 hr	5.33	Yes	37.91	6.93
			24 hr	5.35	Yes	52.77	6.57
2	2 N NaOH	60	6 hr	5.29	Yes	7.54	2.25
			12 hr	5.28	Yes	17.3	2.24
			18 hr	5.45	Yes	25.87	2.22
			24 hr	5.29	Yes	45.43	2.25
3	20 % H_2O_2	60	24 hr	5.22	No	-	-
		80	24 hr	4.26	No	-	-
	30 % H_2O_2	60	24 hr	5.39	No	-	-
		80	24 hr	5.55	No	-	-
4	Thermal In Hot Air Oven	80	24 hr	5.25	No	-	-
5	Photo degradation Study	UV Light	7 Days	5.44	No	-	-

6.8 METHOD VELIDATION

6.8.1 Method Validation of Empagliflozin

6.8.1.1 System suitability

The system suitability was evaluated by six replicates of Empagliflozin (100 µg/ml) were injected into chromatographic system and from the chromatograms %RSD, theoretical plates and peak symmetry were calculated.

Table No: 6.88 System suitability (n=6)

Parameters	Observed Values	Specification
Retention Time (min.)	5.63 ± 0.01	-
Peak area ± SD	2241258 ± 5862.32	-
% RSD	0.26	Less than 2
Theoretical plates	5583 ± 7.63	More than 2000
Tailing Factor	1.08 ± 0.02	Less than 2

6.8.1.2 Linearity and Range

Linearity in the concentration range was 50-150 µg/ml for Empagliflozin. Correlation coefficient (r^2) for calibration curve of Empagliflozin was found to be 0.999.

Table No: 6.89 Calibration Data for Empagliflozin (n=6)

Sr. No.	Concentration (µg/ml)	Peak area ± SD	% RSD
1	50	1143819 ± 4306	0.37
2	75	1674571± 7764	0.46
3	100	2230669 ± 8360	0.37
4	125	2767003± 7992	0.28
5	150	3350642 ± 8799	0.26

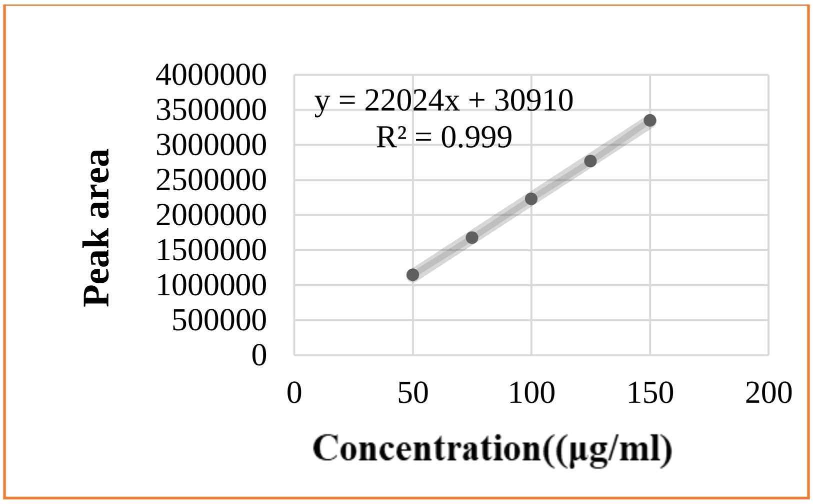


Figure No: 6.92 Calibration Curve for Empagliflozin

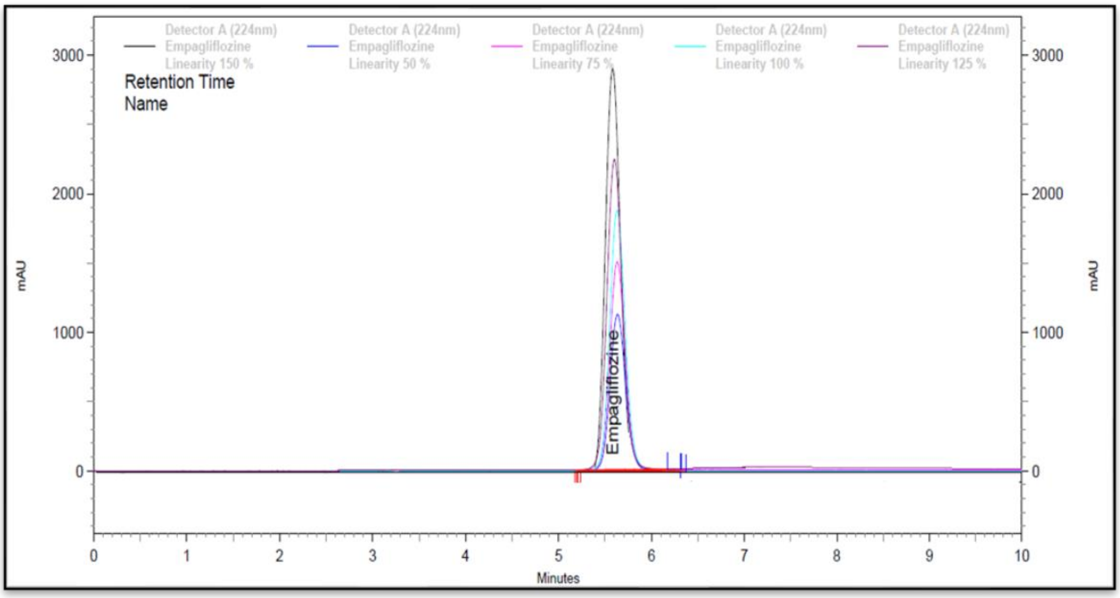


Figure No: 6.93 Chromatogram of Linearity for Empagliflozin (50-150 µg/ml)

Table No: 6.90 Linearity data

Sr. no.	Parameter	Value
1	Wavelength	224 nm
2	Regression equation	y = 22024X+30910
3	Linearity range(µg/ml)	50-150
4	Slope (m)	22024
5	Intercept (c)	30910
6	Correlation coefficient (r^2)	0.999

6.8.1.3 Precision

The result that the precision % RSD was less than 2 indicates that the proposed method was acceptable.

Table No: 6.91 Intraday precision data (n=3)

Theoretical Concentration (µg/ml)	Peak area	Peak area ± SD	%RSD
50	1146724	1147043 ± 3032	0.26
	1143752		
	1147652		
100	2236574	2238621 ± 4373	0.19
	2243643		
	2235647		
150	3350642	3357335 ± 7020	0.20
	3356721		
	3364643		

Table No: 6.92 Interday precision data (n=3)

Concentration (µg/ml)	Peak area	Peak area ± SD	%RSD
50	1147656	1149356 ± 6807	0.59
	1156853		
	1143561		
100	2257854	2248009 ± 9194	0.40
	2239645		
	2246527		
150	3348742	3364304 ± 15032	0.44
	3365424		
	3378745		

Table No: 6.93 Repeatability data (n=6)

Conc. (µg/ml)	Sample No.	Peak area
100	**1**	2243542
	2	2230669
	3	2232876
	4	2228432
	5	2220543
	6	2239453
Peak area ± SD		2232586 ± 8164
% RSD		0.36

6.8.1.4 Accuracy

Accuracy of the method was determined by recovery study from synthetic mixture at three levels (50%, 100% and 150%) of standard addition. The recovery for Empagliflozin was found to be in the range of 99.76-100.56 %

Table No: 6.94 Recovery data

Spiking Level (%)	Conc. of Formulation (µg/ml)	Conc. of Standar Spiking (µg/ml)	Total Conc. (µg/ml)	Conc. of Formulation Found (µg/ml) Mean ± SD	Total Conc. of Empagliflozin found (µg/ml) Mean ± SD	Conc. of Standard spiking found (µg/ml) Mean ± SD	% Recovery	% difference from theoretical concentration
50	50	25	75	49.89 ± 0.0416	74.84 ± 0.3927	24.94 ± 0.0208	99.76	0.24
100	50	50	100		100.2 ± 0.3579	50.28 ± 0.0585	100.56	0.56
150	50	75	125		124.6 ± 0.2510	74.66 ± 0.2212	99.55	0.45

6.8.1.5 Limit of Detection and Quantitation

The LOD and LOQ of Empagliflozin was calculated using an equation based on the ICH guideline. The LOD and LOQ for Empagliflozin was found to be 1.34 µg/ml and 4.06 µg/ml Respectively.

Table No: 6.95 LOD and LOQ data

Regression equation (y = mx+c)	y = 22299X
Slope (m)	22024
Response standard deviation (SD)	8960
LOD (µg/ml)	1.34
LOQ (µg/ml)	4.06

6.8.1.6 Robustness

Deliberate changes in various parameters such as Wavelength, flow rate, and mobile phase composition ratio resulted in a %RSD of peak area of less than 2%, indicating that the approach was robust.

Table No: 6.96 Robustness data (n=6)

Standard Mobile phase composition Acetonitrile: Water (50:50 v/v)				
Sr. No.	**Factor**	**Level**	**Peak area ± SD**	**%RSD**
1	Change in Wavelength (224 ± 2nm)	222	2234195 ± 4984	0.22
		226	2231778 ± 5919	0.26
2	Change in flow rate (0.5 ± 0.1 ml/min.)	0.4	2239896 ± 7535	0.33
		0.6	2235248 ± 5637	0.25
3	Change in Mobile Phase (± 2 ml)	48: 52	2236522 ± 11808	0.52
		52: 48	2237946 ± 12281	0.54

6.8.1.7 Specificity

Specificity was evaluated by observing the chromatograms of blank, sample and standard Empagliflozin.

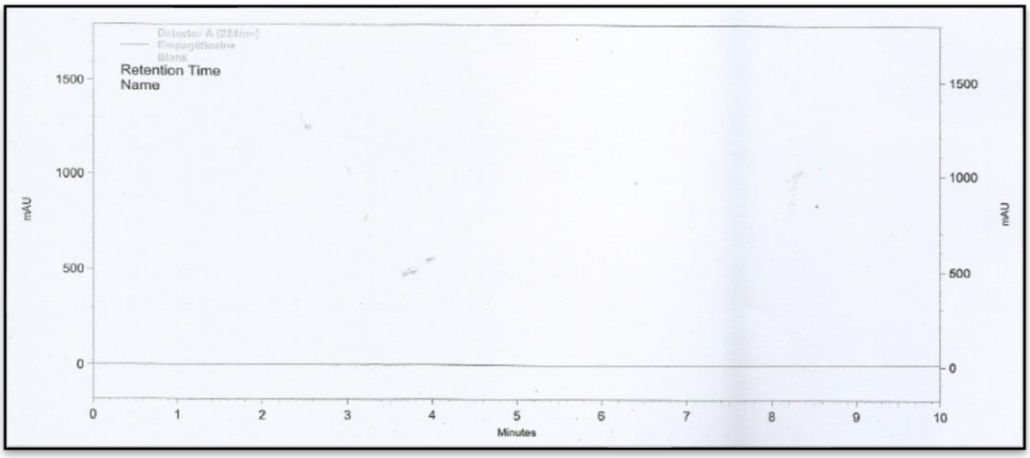


Figure No: 6.94 Chromatogram of Blank

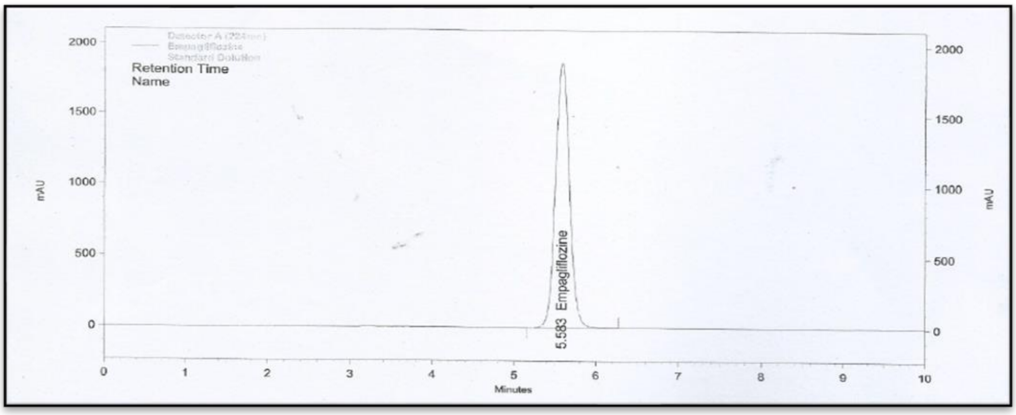


Figure No: 6.95 Chromatogram of Standard Empagliflozin API (100µg/ml)

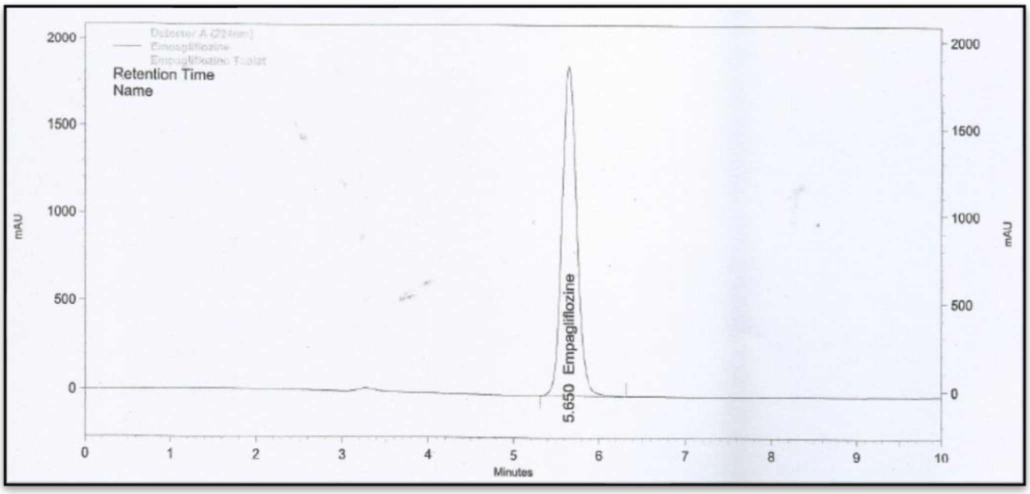


Figure No: 6.96 Chromatogram of Sample Empagliflozin (Tablet Formulation)

6.8.1.8 Application of the proposed method for assay of Empagliflozin Marketed formulation (JARDIANCE TABLETS)

The percent assay shows that there was no interference from excipients and the proposed method can successfully applied to analysis of commercial formulation containing Empagliflozin.

Label Claim: Each Tablet Contains:

Empagliflozin……25 mg
Excipients………...Q.S

Table No: 6.97 Assay data of Marketed formulation (n=3)

Sr. No	Label claim	Amount of drug found in mg	% Assay	% Difference from theoretical concentration
1.	25	24.78	99.12	0.88
2.	25	24.87	99.48	0.52
3.	25	24.9	99.6	0.40
Mean ± SD (n=3)		24.79 ± 0.06	99.40 ± 0.20	
% RSD		0.25		

6.8.2 Method Validation of Dapagliflozin

6.8.2.1 System suitability

The system suitability was evaluated by six replicates of Dapagliflozin (100 µg/ml) were injected into chromatographic system and from the chromatograms %RSD, theoretical plates and peak symmetry were calculated.

Table No: 6.98 System suitability (n=6)

Parameters	Observed Values	Specification
Retention Time (min.)	5.42 ± 0.01	-
Peak Area ± SD	356337 ± 1276	-
% RSD	0.23	Less than 2
Theoretical plates	5032 ± 9.23	More than 2000
Tailing Factor	1.15 ± 0.05	Less than 2

6.8.2.2 Linearity and Range

Linearity in the concentration range was 5-150 µg/ml for Dapagliflozin. Correlation coefficient (r^2) for calibration curve of Dapagliflozin was found to be 0.999.

Table No: 6.99 Calibration Data for Dapagliflozin (n=6)

Sr. No.	Concentration (µg/ml)	Peak area ± SD	% RSD
1	5	18555 ± 180	0.96
2	25	95127 ± 886	0.93
3	50	181605 ± 1005	0.55
4	75	266578 ± 1582	0.59
5	100	356700 ± 1236	0.34
6	125	442513 ± 2823	0.63
7	150	525612 ±3859	0.73

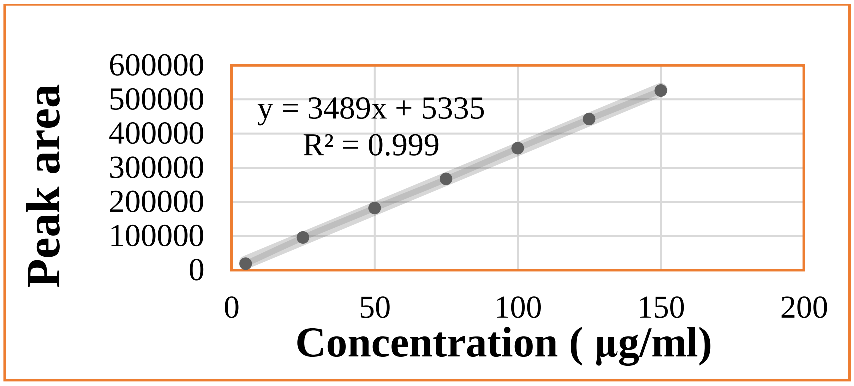


Figure No: 6.97 Calibration Curve for Dapagliflozin

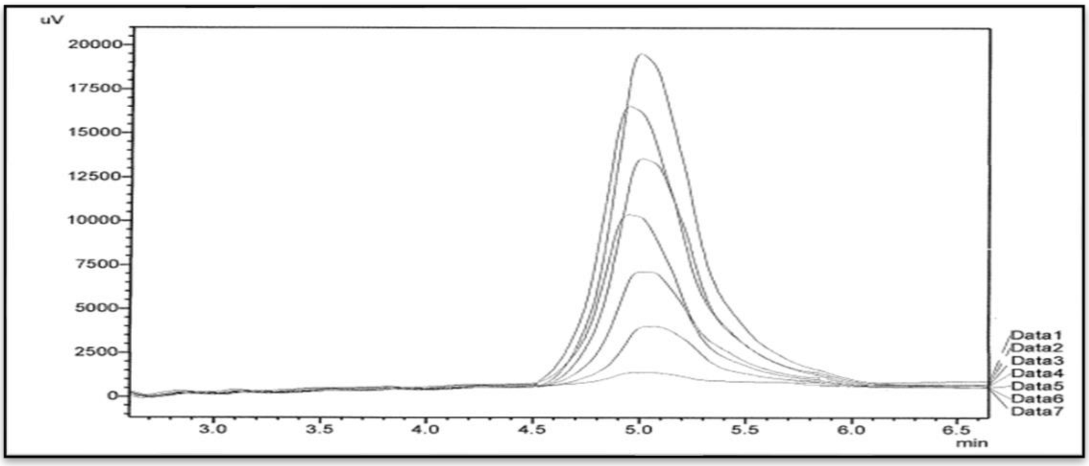


Figure No: 6.98 Chromatogram of Linearity for Dapagliflozin (5-150 µg/ml)

Table No: 6.100 Linearity data

Sr. no.	Parameter	Value
1	Wavelength	**273 nm**
2	Regression equation	y = 3489x + 5335
3	Linearity range(µg/ml)	5-150 µg/ml
4	Slope (m)	3489
5	Intercept (c)	5335
6	Correlation coefficient (r^2)	0.999

6.8.2.3 Precision

The result that the precision % RSD was less than 2 indicates that the proposed method was acceptable.

Table No: 6.101 Intraday precision data (n=3)

Concentration (µg/ml)	Peak area	Peak area ± SD	%RSD
75	265326	267837 ± 1779	0.66
	269214		
	268972		
100	353863	354576 ± 2175	0.61
	357524		
	352342		
125	441642	443565 ± 2263	0.51
	442312		
	446742		

Table No: 6.102 Interday precision data (n=3)

Concentration (µg/ml)	Peak area	Peak area ± SD	%RSD
75	262326	264837 ± 2347	0.88
	264214		
	267972		
100	351942	356443 ± 3323	0.93
	359863		
	357524		
125	441642	445899 ± 3320	0.74
	446312		
	449742		

Table No: 6.103 Repeatability data (n=6)

Conc. (µg/ml)	Sample No.	Peak area
100	**1**	352942
	2	359863
	3	357524
	4	352342
	5	353863
	6	357524
Peak area ± SD		2232586 ± 8164
% RSD		0.36

6.8.2.4 Accuracy

Accuracy of the method was determined by recovery study from synthetic mixture at three levels (50%, 100% and 150%) of standard addition. The recovery for Dapagliflozin was found to be in the range of 99.84-100.05 %

Table No: 6.104 Recovery data

Spiking Level (%)	Conc. of Formulation (µg/ml)	Conc. of Standard Spiking (µg/ml)	Total Conc. (µg/ml)	Conc. of Formulation Found (µg/ml) Mean ± SD	Total Conc. of Dapagliflozin found (µg/ml) Mean ± SD	Conc. of Standard spiking found (µg/ml) Mean ± SD	% Recovery	% difference from theoretical concentration
50	50	25	75	49.92 ± 0.0345	74.93 ± 0.2428	25.01 ± 0.0208	100.04	0.04
100	50	50	100		99.75 ± 0.4483	49.92 ± 0.0585	99.84	0.16
150	50	75	125		124.96 ± 0.5391	75.04 ± 0.2212	100.05	0.05

6.8.2.5 Limit of Detection and Quantitation

The LOD and LOQ of Dapagliflozin was calculated using an equation based on the ICH guideline. The LOD and LOQ for Empagliflozin was found to be 0.62 µg/ml and 1.87 µg/ml Respectively.

Table No: 6.105 LOD and LOQ data

Regression equation (y = mx+c)	y = 3489X
Slope (m)	3489
Response standard deviation (SD)	653
LOD (µg/ml)	0.62
LOQ (µg/ml)	1.87

6.8.2.6 Robustness

Deliberate changes in various parameters such as Wavelength, flow rate, and mobile phase composition ratio resulted in a %RSD of peak area of less than 2%, indicating that the approach was robust.

Table No: 6.106 Robustness data (n=6)

Standard Mobile phase composition Acetonitrile: Water (50:50 v/v)				
Sr. No.	**Factor**	**Level**	**Peak area ± SD**	**%RSD**
1	Change in Wavelength (273 ± 2 nm)	271	355154 ± 3332	0.93
		275	355683 ± 2574	0.72
2	Change in flow rate (0.7 ± 0.1 ml/min.)	0.6	354203 ± 2058	0.58
		0.8	355843 ± 1367	0.38
3	Change in Mobile Phase (± 2 ml)	48: 52	357163 ± 2063	0.57
		52: 48	355006 ± 2644	0.74

6.8.2.7 Specificity

Specificity was evaluated by observing the chromatograms of blank, sample and standard Dapagliflozin.

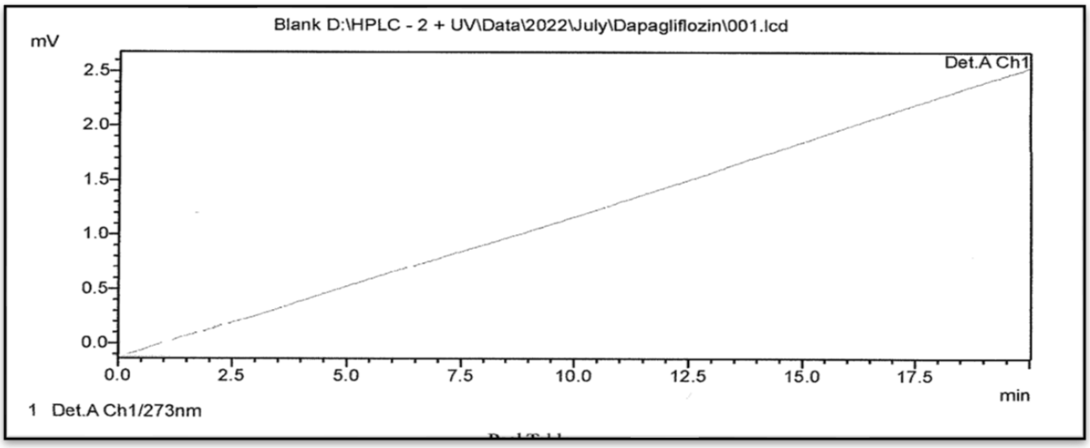


Figure No: 6.99 Chromatogram of Blank

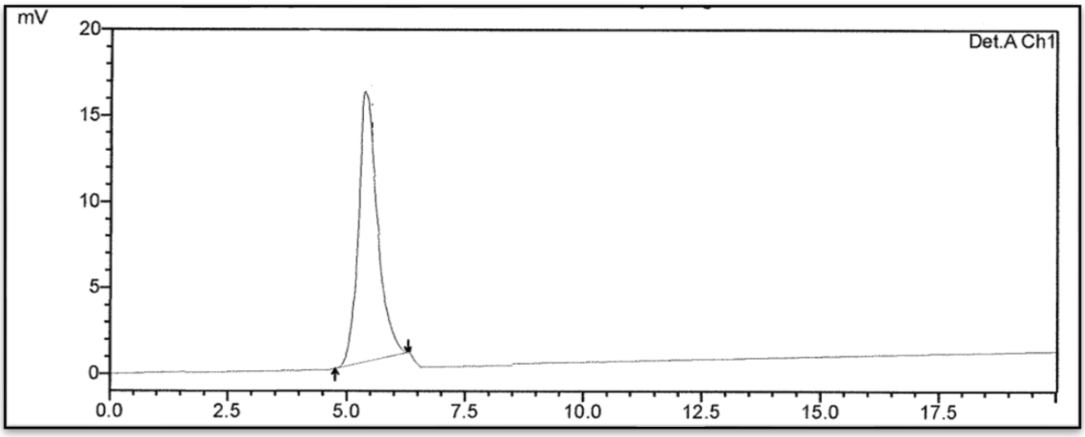


Figure No: 6.100 Chromatogram of Standard Dapagliflozin (100µg/ml)

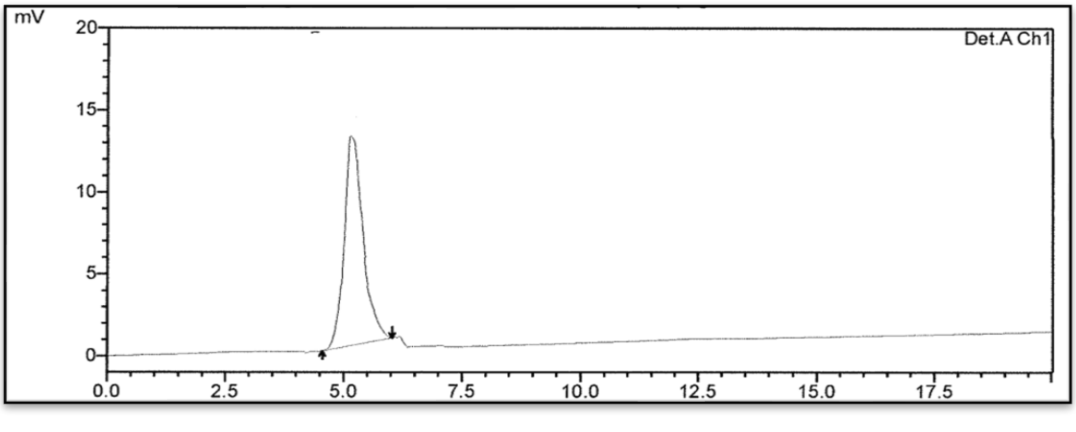


Figure No: 6.101 Chromatogram of Sample Dapagliflozin (Tablet Formulation)

6.8.2.8 Application of the proposed method for analysis of Dapagliflozin Marketed formulation (DAPARYL 10 TABLET)

The percent assay shows that there was no interference from excipients and the proposed method can successfully applied to analysis of commercial formulation containing Dapagliflozin.

Label Claim: Each Tablet Contains:

Dapagliflozin……10 mg
Excipients………...Q.S

Table No: 6.107 Assay data of Marketed Formulation (n=3)

Sr. No	Label claim	Amount of drug found in mg	%Assay	% difference from theoretical concentration
1.	10	10.04	100.4	0.40
2.	10	9.96	99.6	0.40
3.	10	9.87	98.7	1.30
Mean ± SD (n=3)		9.95 ± 0.0685	99.56 ± 0.85	
% RSD		0.68		

6.9 IMPURITY PROFILE

6.9.1 Detection, Isolation and Characterization of Empagliflozin Degradation Impurities (DP1 and DP2)

The preparative HPLC was used for isolation of degradation product of acid (DP1) and alkali (DP2) by using same developed condition. Isolated degradation product was characterized by using IR, Mass and ^{1}H NMR. The retention time for DP1 was 3.67 min and DP 2 was 3.60 min.

6.9.1.1 Proposed Structure Elucidation of Empagliflozin Acid degradation Impurity (DP1)

Chemically Empagliflozin was 2S,3R,4R,5S,6R)-2-[4-Chloro-3-[[4-[(3S)-oxolan-3-yl]oxyphenyl]methyl]phenyl]-6-(hydroxymethyl) oxane-3,4,5-triol, with empirical formula $C_{23}H_{27}CLO$ and it molecular weight was 450.91 gm/mol. When drug was degraded in acidic condition it forms DP1 with Empirical formula $C_{23}H_{29}CLO_8$ and Molecular weight was 468.92 g/mol. Standard values of different bonds in IR spectrum show in Table No: 6.104.

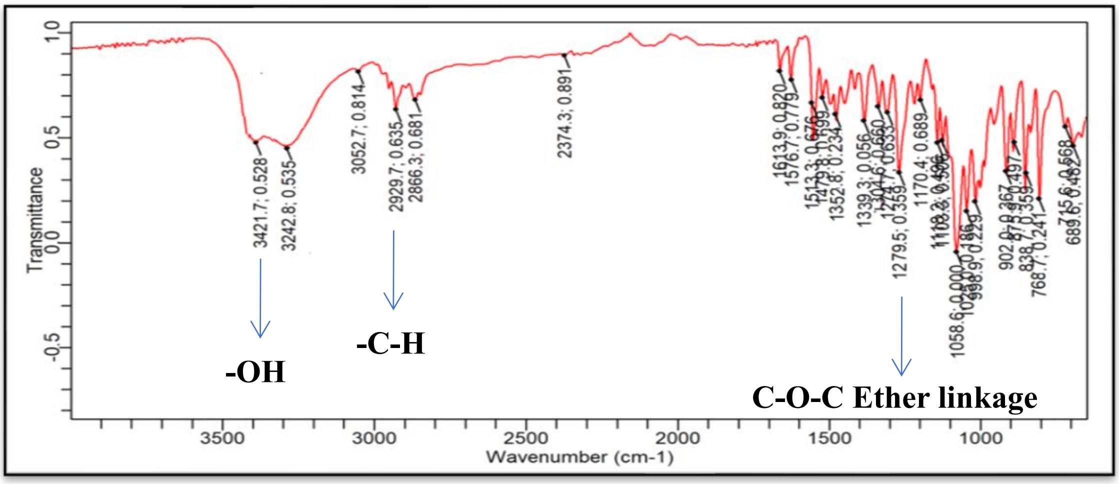


Figure No: 6.102 IR Spectra of Empagliflozin Acid degradation Impurity (DP1)

Table No: 6.108 IR Standard range of Empagliflozin Acid degradation Impurity (DP1)

Functional Group	Standard Range (cm^{-1})	Observed value (cm^{-1})
-OH	3200-3600	3421.7
Ar- C-H	2850-2970	2929.7
C-O-C (Ether linkage)	1040-1280	1279.5

MS spectra of DP1 shown *m/z* at 469.1 [M$^+$] as base peak. The Product ion peaks at *m/z* 449 (loss of water molecule), *m/z* 434 (Loss of – Cl), *m/z* 416 (Loss of – Cl from *m/z* 449), *m/z* 346 (Loss of tetrahydrofuran ring), *m/z* 313(loss of -OH group), *m/z* 327 (loss of water molecule), *m/z* 169 (loss of – $C_6H_{11}O_5$molecule).The positive mass spectrum for DP1 was shown in Fig. 7. The possible MS fragmentation of DP1 in acidic condition shown in Figure No: 6.99.

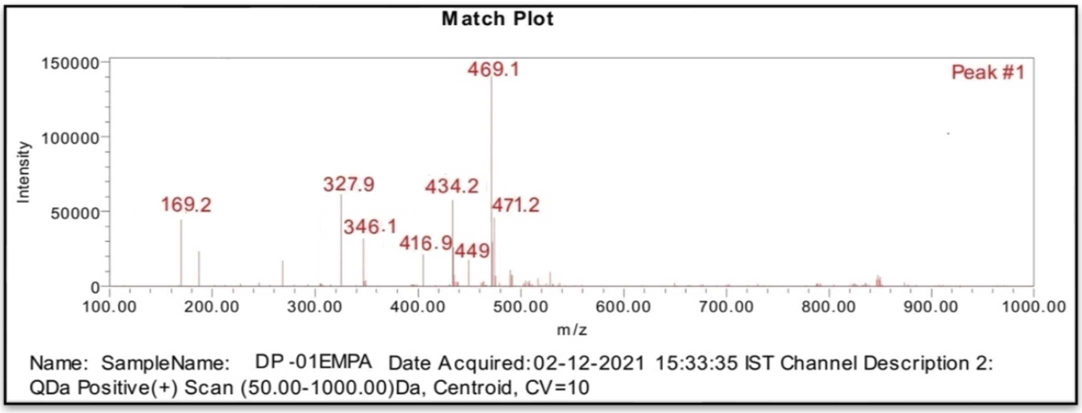


Figure No: 6.103 ESI (+ve) mass Spectrum of Empagliflozin Acid degradation Impurity (DP1)

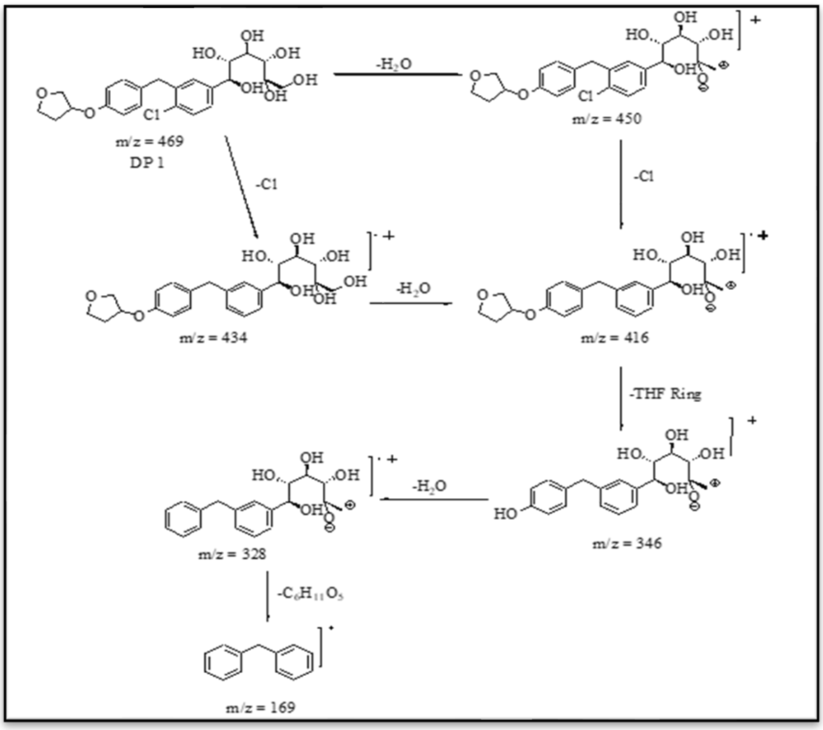
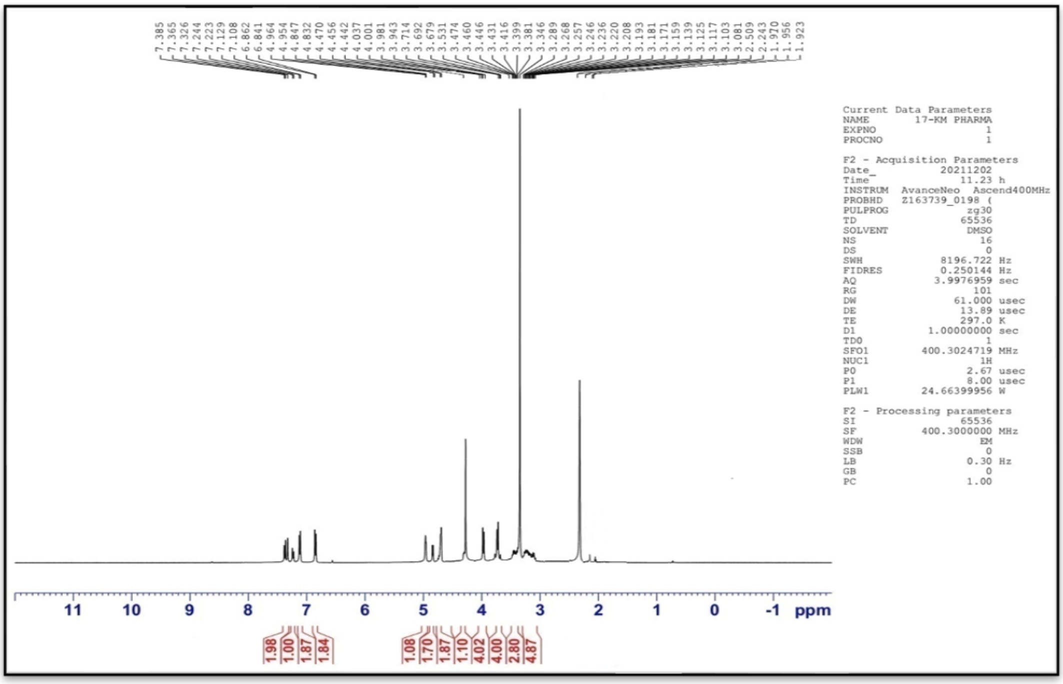


Figure No: 6.104 Possible Mass fragmentation of Empagliflozin Acid degradation Impurity (DP1)

^{1}H Analysis has been done in DMSO at 400 MHz. The chemical shift values reported on δ scale in ppm with respect to TMS (0.00 ppm). ^{1}H NMR of Empagliflozin acid impurity DP1 shown in Figure No: 6.101.^{1}H NMR values shown in Table No: 6.105 confirms structure of DP1.Based on previous data structure of DP1 was shown in Figure No: 6.102. IUPAC name of DP1 was (1S,2S,3R,4R,5S)-1-(3-((S)-4-((S)-tetrahydrofuran-3-yloxy)benzyl)-4-chlorophenyl)hexane-1,2,3,4,5,6-hexaol.

Table No: 6.109. ^{1}H NMR assignment of Empagliflozin Acid degradation Impurity (DP1)

Chemical Shift (δ ppm)	**Number of products**	**Multiplicity**	**Assignment**
3.08-3.24	5H	Multiplet	open sugarmoiety
3.41-3.71	7H	Multiplet	Tetrahydrofuran ring
3.53	4H	Multiplet	-CH_2
4.03	1H	Singlet	-OH
4.44-4.95	5H	Singlet	-OH
7.10-7.38	7H	Multiplet	Ar-H



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TE                   297.0 K
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Figure No: 6.105 ^{1}H NMR of Empagliflozin Acid degradation Impurity (DP1)

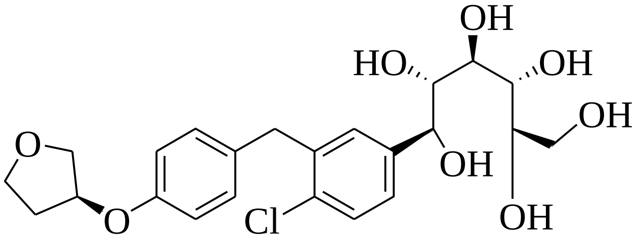

Figure No: 6.106 Possible structure of Empagliflozin Acid degradation Impurity (DP1)

On the basis of gain in molecular weight of DP1 in comparison to pure drug, in acidic hydrolysis proton attack on oxygen atom present in sugar moiety so carbocation was formed and ring opening was formed because of Hydronium ion attack on Carbocation. Further loss of proton from that position form new structure of DP1.Possible degradation pathway was shown in Figure No: 6.103**.**

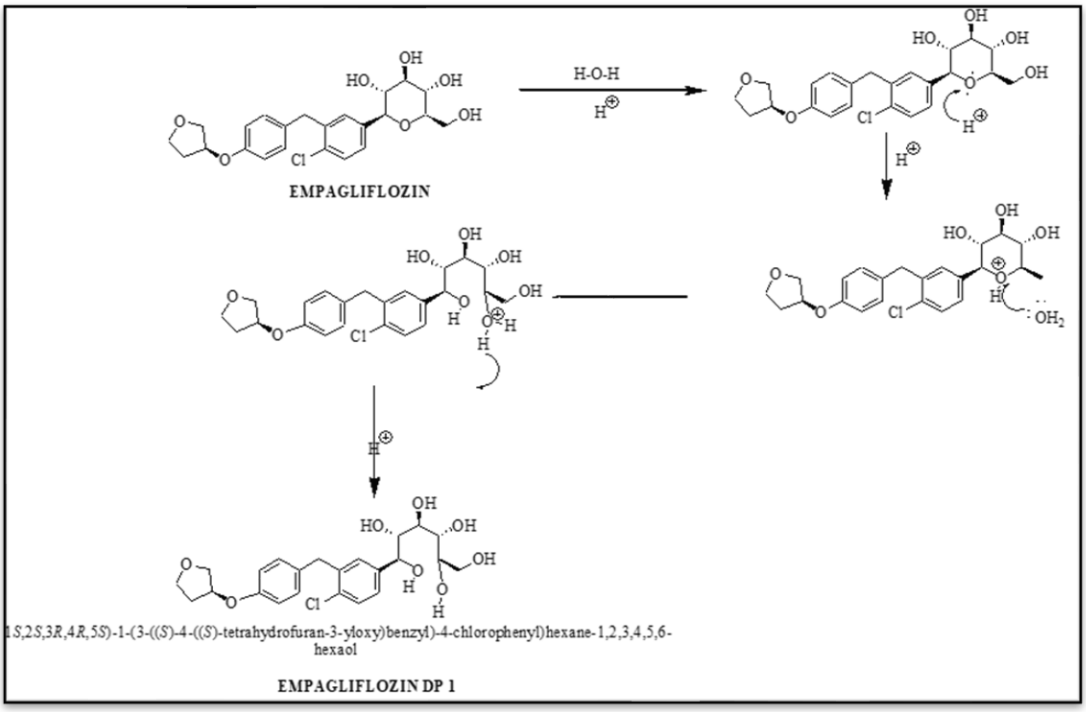

Figure No: 6.107 Possible degradation pathway of Empagliflozin Acid degradation Impurity (DP1)

6.9.1.2 Proposed Structure Elucidation of Empagliflozin Alkali degradation Impurity (DP2)

In Alkali condition it forms DP2 with Empirical formula $C_{23}H_{25}CLO_7$ and Molecular weight was 448.89 g/mol. Standard values of different bonds in IR spectrum show in Table No: 6.106.

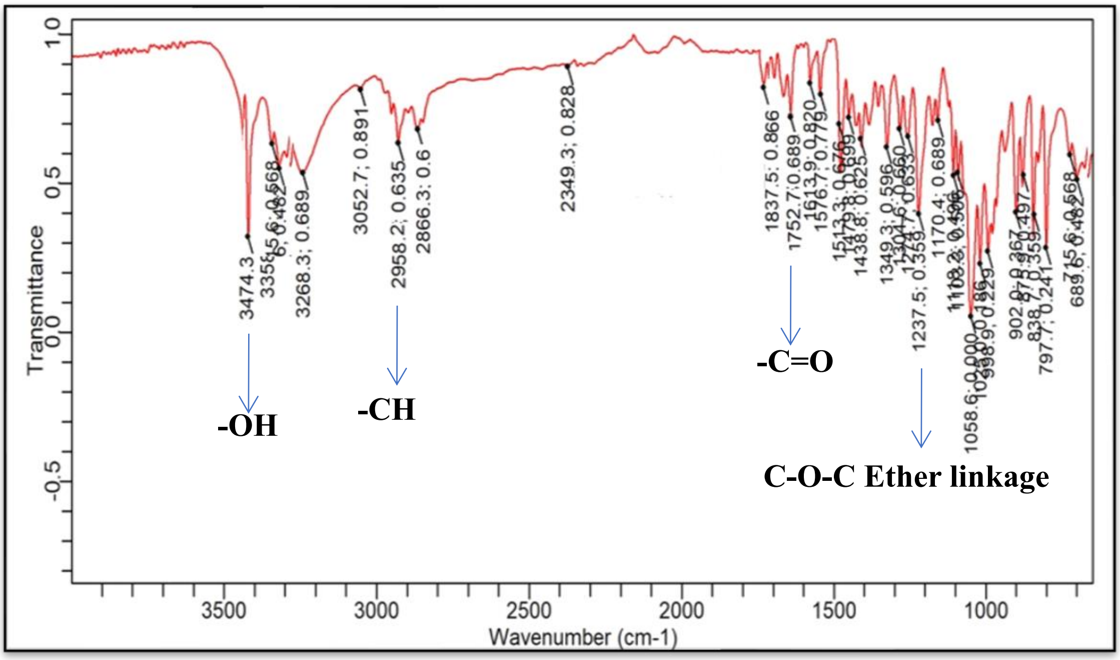

Figure No: 6.108 IR Spectra of Empagliflozin Alkali degradation Impurity (DP2)

Table No: 6.110 IR standard range of Empagliflozin Alkali degradation Impurity (DP2)

Functional Group	Standard Range (cm^{-1})	Observed value (cm^{-1})
-OH	3200-3600	3474.3
Ar- C-H	2850-2970	2958.2
C-O-C (Ether linkage)	1040-1280	1237.5
C=0	1650-1820	1752.7

MS spectra of DP2 shown *m/z* at 449.3 [M^+] as base peak. The Product ion peaks at *m/z* 443 (loss of water molecule), *m/z* 399 (Loss of – Cl), *m/z* 363 (Loss of – C_4H_7O from *m/z* 443), *m/z* 313 (Loss of – OH from *m/z* 363), *m/z* 285 (Loss of-CHO from *m/z* 313), *m/z* 169 (loss of -$C_5H_9O_3$ group from *m/z* 285), The positive mass spectrum for DP2 was shown in Figure No: 6.105.

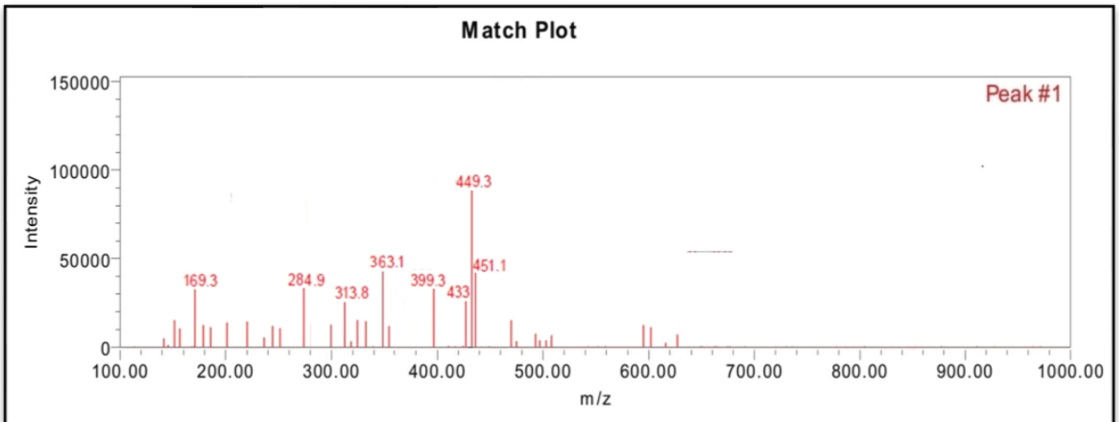

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Figure No: 6.109 ESI (+ve) mass spectrum of Empagliflozin Alkali degradation Impurity (DP2)

^{1}H NMR of Empagliflozin acid impurity DP2 shown in Figure No: 6.106.^{1}H NMR values shown in Table No: 6.107 confirms structure of DP2. Based on previous data structure of DP2 was shown in Figure No: 6.107.

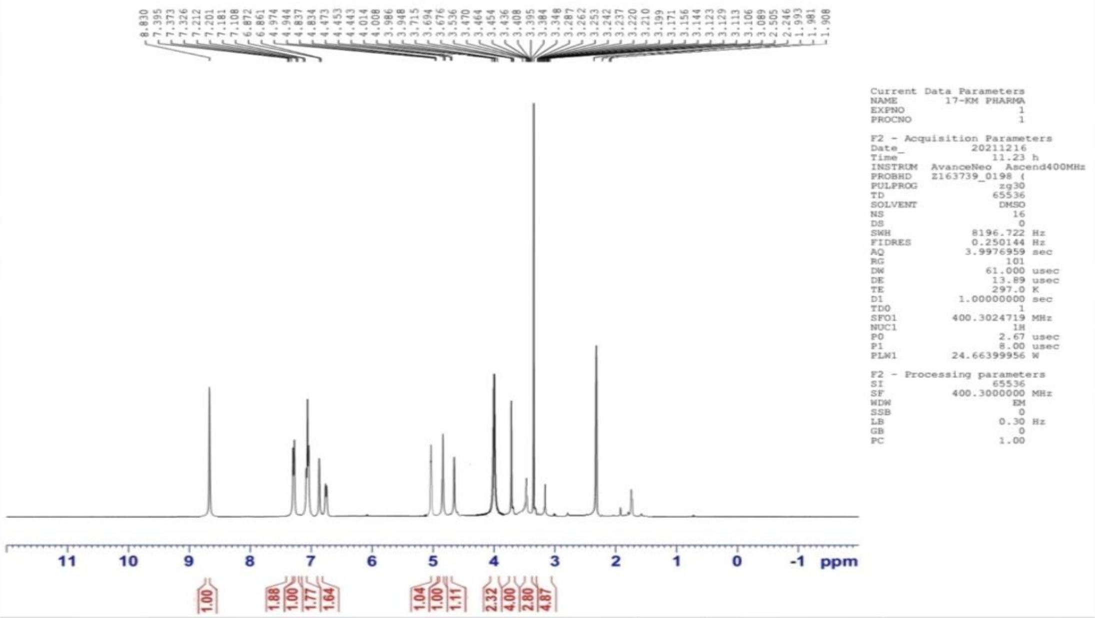

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Figure No: 6.110 ^{1}H NMR of Empagliflozin Alkali degradation Impurity (DP2)

Table No: 6.111 ^{1}H NMR Assignment of Alkali degradation Impurity (DP2)

Chemical Shift δ ppm	Number of products	Multiplicity	Assignment
3.40-3.47	5H	Multiplet	open sugarmoiety
3.53-3.98	7H	Multiplet	Tetrahydrofuran ring
4.01	2H	Singlet	$-CH_2$
4.47-4.97	3H	Singlet	-OH
7.10-7.39	7H	Multiplet	Ar-H
8.830	1H	Singlet	-CHO

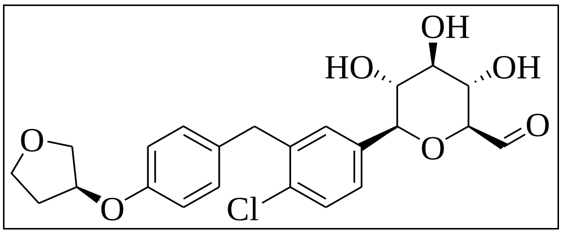


Figure No: 6.111 Possible structure of Empagliflozin Alkali degradation Impurity (DP2)

In alkali hydrolysis molecular weight was loss as compare to pure drug so in Alkali hydrolysis in presence of sodium hydroxide two hydrogen ion was removed and oxygen convert in to Aldehyde it means Alcohol convents in to Aldehyde in formed DP2. Possible degradation pathway of DP2 was shown in Figure No: 6.108. IUPAC name of DP2 was 2S,3S,4R,5R,6S)-6-(3-(4-(tetrahydrofuran-3-yloxy)benzyl)-4-chlorophenyl)-3,4,5-trihydroxy-tetrahydro-2H-pyran-2-carbaldehyde.

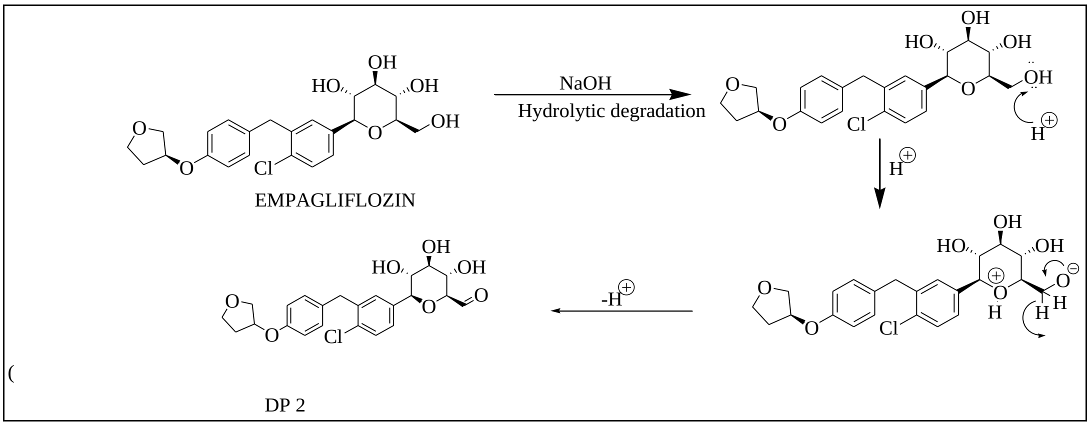


Figure No: 6.112 Possible degradation pathway of Empagliflozin Alkali degradation Impurity (DP2)

6.9.2 Detection, Isolation and Characterization of Dapagliflozin Degradation Impurities (DP1 and DP2)

The preparative HPLC was used for isolation of degradation product of acid (DP1) and alkali (DP2) by using same developed condition. Isolated degradation product was characterized by using IR, Mass and ^{1}H NMR. The retention time for DP1 was 6.57 min and DP 2 was 2.25 min.

6.9.2.1 Proposed Structure Elucidation of Dapagliflozin Acid degradation Impurity (DP1)

Chemically Dapagliflozin was (2S,3R,4R,5S,6R)-2-[4-chloro-3-[(4-ethoxyphenyl) methyl] phenyl]-6-(hydroxymethyl) oxane-3,4,5-triol, with empirical formula $C_{21}H_{25}ClO_6$ and it molecular weight was 408.9 gm/mol. When drug was degraded in acidic condition it forms DP1 with Empirical formula $C_{23}H_{27}ClO_7$ and Molecular weight was 450.14 g/mol. In Alkali condition it forms DP2 with Empirical formula $C_{21}H_{25}CLO_7$ and Molecular weight was 424.87 g/mol. Standard values of different bonds of Acidic degradation product DP1 in IR spectrum show in Table No: 6.108.

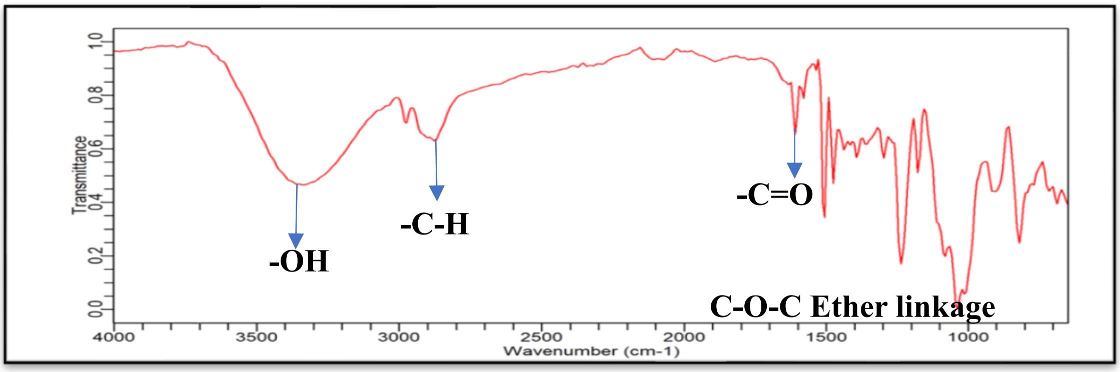


Figure No: 6.113 IR spectra of Dapagliflozin degradation product (DP1) in acidic condition

Table No: 6.112. IR Standard range of Dapagliflozin Acid degradation Impurity (DP1)

Functional Group	Standard Range (cm^{-1})	Observed value (cm^{-1})
- OH	3200-3600	3339
-C-H	2850-2970	2877
- C = O Ester Carbonyl	1650-1820	1722
C-O-C (Ether linkage)	1040-1280	1036

MS spectra of DP1 shown *m/z* at 450.3 [M^+] as base peak. The Product ion peaks at *m/z* 405 (loss of $CH_3CH_2O^-$), *m/z* 391 (loss ofCH_3COO^-), *m/z* 2 (Loss of CH_3CH_2 CH_2OH), *m/z* 202 Loss of $CH_6CH_{10}O_6$) *m/z* 168 (Loss of – Cl from *m/z* 202). The positive mass spectrum for DP1 was shown in Figure No: 6.110. The possible MS fragmentation of DP1 in acidic condition shown in Figure No: 6.111.

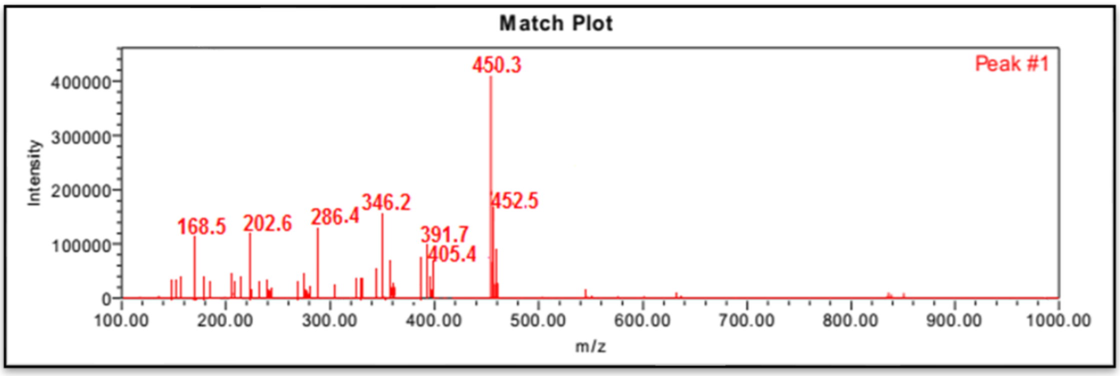


Figure No: 6.114 ESI (+ve) mass Spectrum of Dapagliflozin Acid degradation Impurity (DP1)

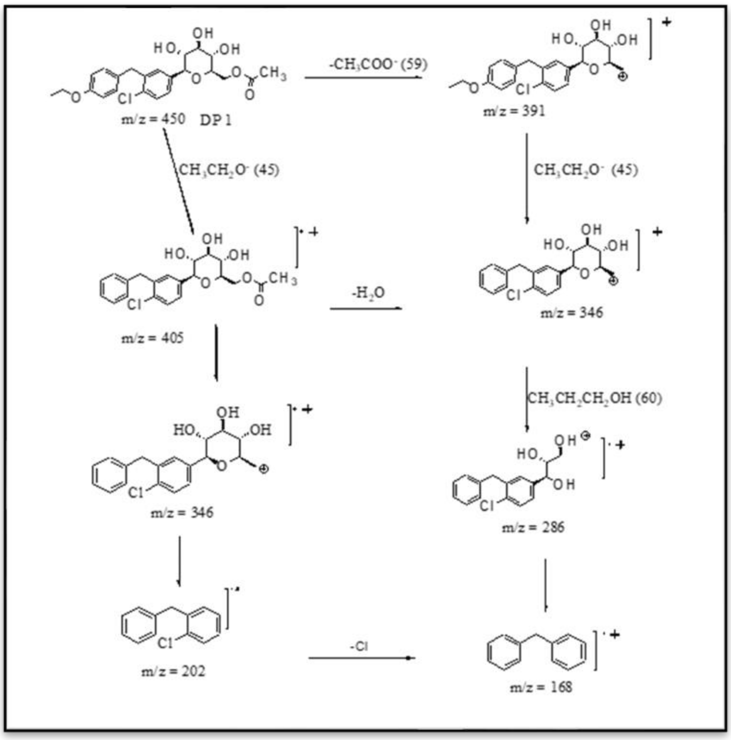
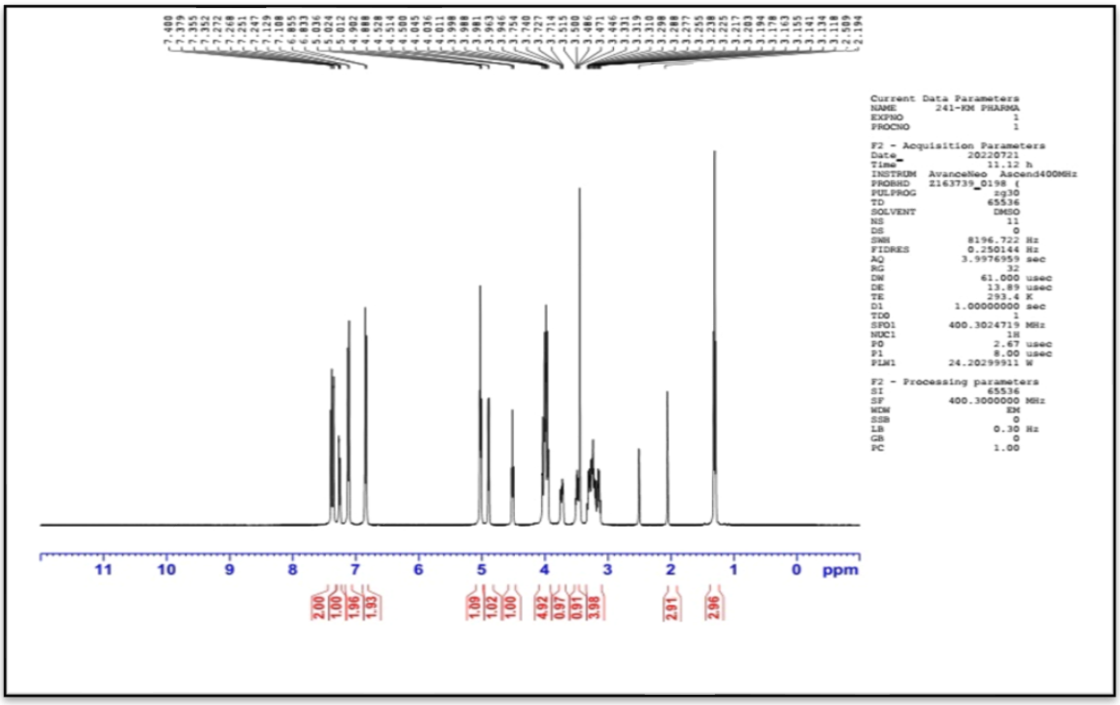


Figure No: 6.115 Possible Mass fragmentation of Dapagliflozin Acid degradation Impurity (DP1)

^{1}H Analysis has been done in DMSO at 400 MHz. The chemical shift values reported on δ scale in ppm with respect to TMS (0.00 ppm). ^{1}H NMR of Dapagliflozin acid impurity DP1 shown in Figure No: 6.112.^{1}H NMR values shown in Table No: 6.109 confirms structure of DP1.Based on previous data structure of DP1 was shown in Figure No: 6.113. IUPAC name of DP1 was (2R,3S,4R,5R,6S)-6-(3-(4-ethoxybenzyl)-4-chlorophenyl)-3,4,5-trihydroxy-tetrahydro-2H-pyran-2-yl)methyl acetate.

Table No: 6.113 ^{1}H NMR assignment of Dapagliflozin Acid degradation Impurity (DP1)

Chemical Shift δ ppm	Number of products	Multiplicity	Assignment
1.29-1.32	3H	Triplate	-CH_3
2.19	3H	Singlet	-CH_3
3.11-3.51	5H	Multiplet	Sugar Moiety
3.71- 4.08	6H	Multiplet	-CH2
4.51.5.00	3H	Doublet	-OH
6.83-7.40	7H	Multiplet	Ar-H



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RG                    32
DW                61.000 usec
DE                 13.89 usec
TE                 293.4 K
D1            1.00000000 sec
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P1                  8.00 usec
PLW1         24.20299911 W

F2 - Processing parameters
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Figure No: 6.116 ^{1}H NMR of Dapagliflozin Acid degradation Impurity (DP1)

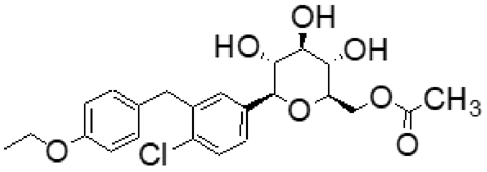


Figure No: 6.117 Possible structure of Dapagliflozin Acid degradation Impurity (DP1)

Formation of DP1, sugar moiety in the target molecule undergoes gradual decomposition to form formaldehyde /acetaldehyde and acetic acid in different proportions. The formed acetic acid involved in the esterification/Acetylation of dapagliflozin under the existing hot acidic condition. Possible degradation pathway was shown in Figure No: 6.114**.**

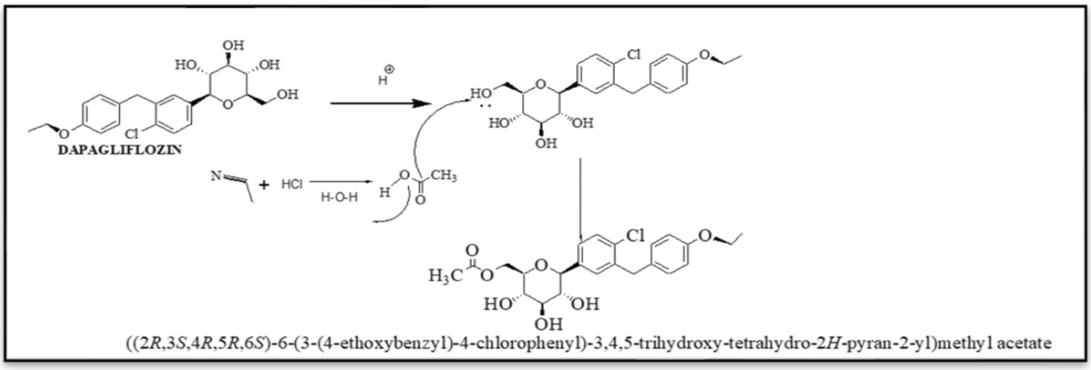


Figure No: 6.118 Possible degradation pathway of Dapagliflozin Acid degradation Impurity (DP1)

In Alkali condition it forms DP2 with Empirical formula $C_{23}H_{25}CLO_7$ and Molecular weight was 448.89 g/mol. Standard values of different bonds of Acidic degradation product DP2 in IR spectrum show in Table No: 6.110.

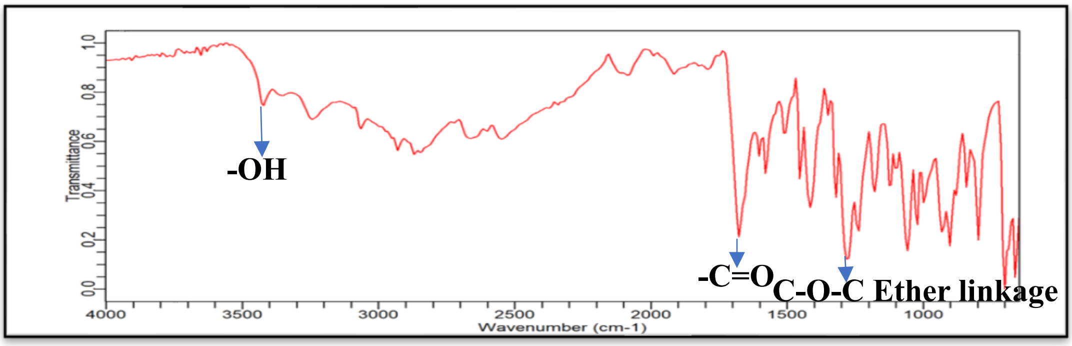


Figure No: 6.119 ESI (+ve) mass Spectrum of Dapagliflozin Alkali degradation Impurity (DP2)

Table No: 6.114: IR Standard range of Dapagliflozin Alkali degradation Impurity (DP2)

Functional Group	Standard Range (cm^{-1})	Observed value (cm^{-1})
- OH	3200-3600	3354
C-O-C **(Ether linkage)**	1040-1280	1170
C=0	1650-1820	1677

MS spectra of DP2 shown *m/z* at 424.2 [M$^+$] as base peak. The Product ion peaks at *m/z* 406 (loss of water molecule), *m/z* 389 (Loss of – Cl), *m/z* 371 (loss of water molecule from *m/z* 389) *m/z* 326 (Loss of – OCH_2CH_3 from *m/z* 371), *m/z* 308 (Loss of – OH from *m/z* 326),, *m/z* 169 (loss of $-C_5H_9O_3$ group from *m/z* 308), The positive mass spectrum for DP2 was shown in Figure No: 6.116. Possible MASS fragmentation was shown in Figure No: 6.117.

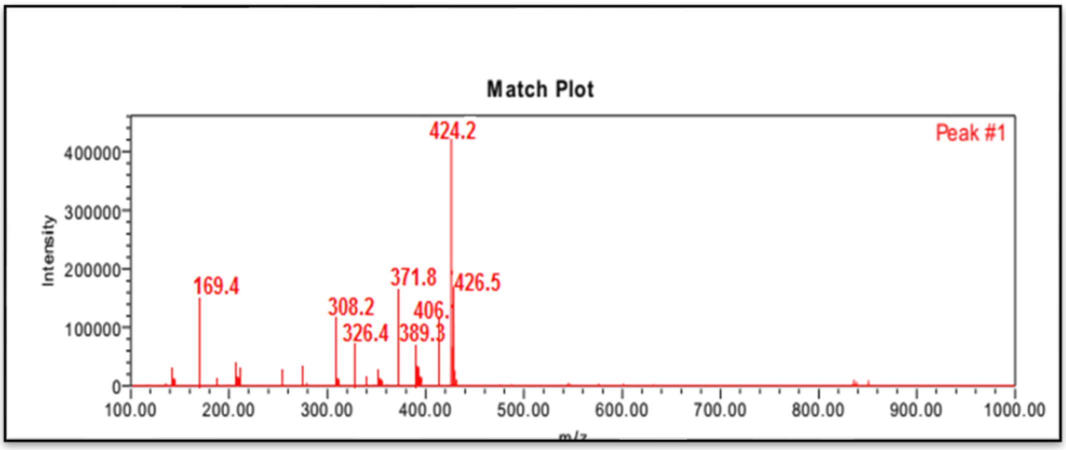


Figure No: 6.120 ESI (+ve) mass Spectrum for Dapagliflozin Alkali degradation Impurity (DP2)

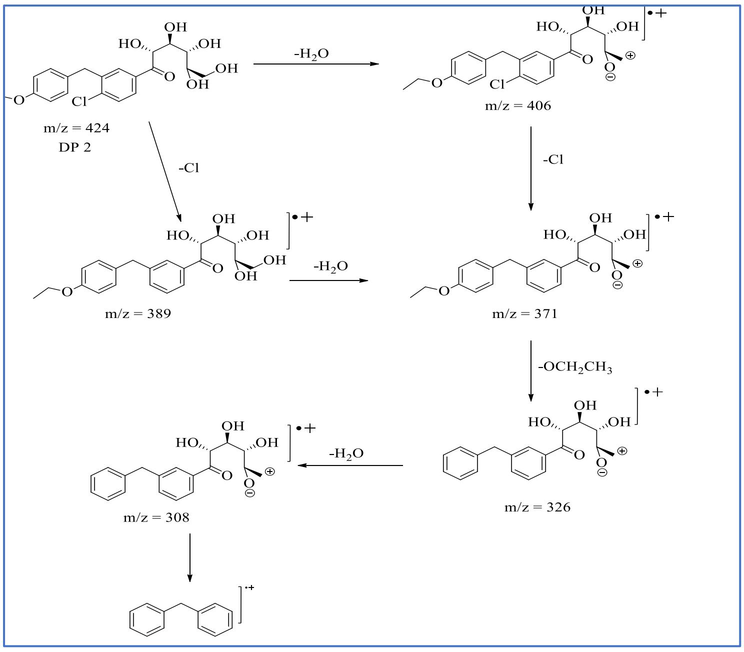
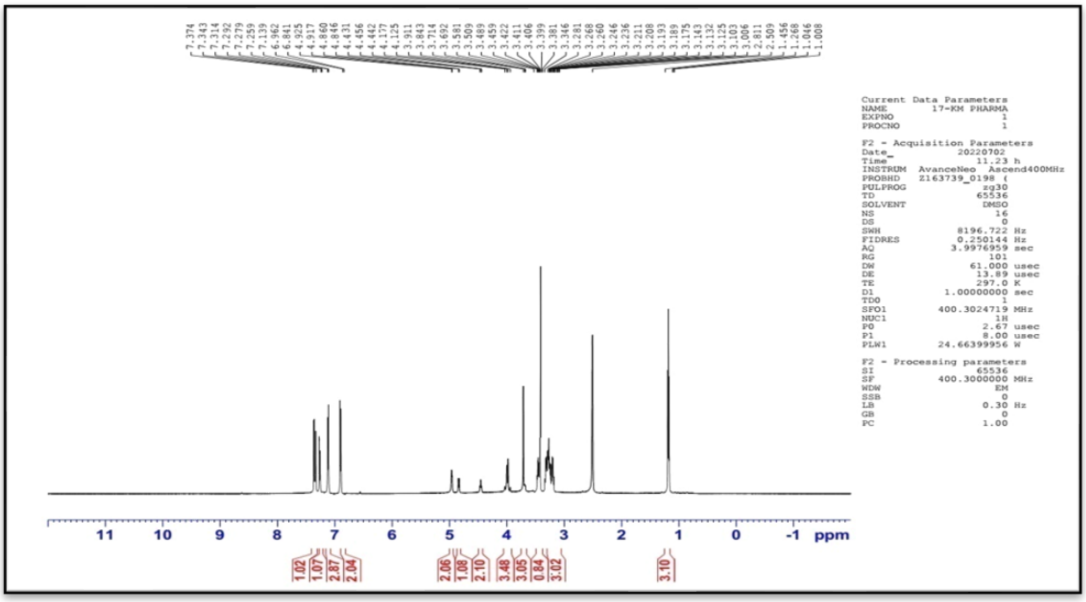


Figure No: 6.121 Possible Mass fragmentation of Dapagliflozin Alkali degradation Impurity (DP2)

^{1}H NMR of Dapagliflozin acid impurity DP2 shown in Figure No: 6.118. ^{1}H NMR values shown in Table No: 6.111 confirms structure of DP2. Based on previous data structure of DP2 was shown in Figure No: 6.119. IUPAC name of DP2 was (2R,3S,4R,5S)-1-(3-(4-ethoxybenzyl)-4-chlorophenyl)-2,3,4,5,6-pentahydroxyhexan-1-one.

Table No: 6.115: ^{1}H NMR Assignment of Dapagliflozin Alkali degradation Impurity (DP2)

Chemical Shift δ ppm	Number of products	Multiplicity	Assignment
1.00-1.45	3H	Triplate	-CH3
3.00-3.58	4H	Multiplet	open sugarmoiety
3.69-4.12	6H	Multiplet	-CH$_2$
4.44-4.92	5H	Doublet	-OH
6.84-7.37	7H	Multiplet	Ar-H



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DW                  61.000 usec
DE                   13.89 usec
TE                   297.0 K
D1              1.00000000 sec
TD0                      1
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P0                    2.67 usec
P1                    8.00 usec
PLW1           24.66399956 W

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Figure No: 6.122 ^{1}H NMR of Dapagliflozin Alkali degradation Impurity (DP2)

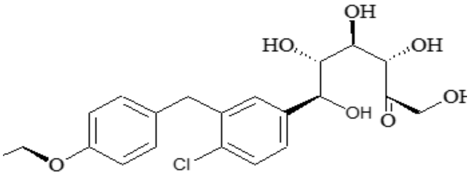


Figure No: 6.123 Possible structure of Dapagliflozin Alkali degradation Impurity (DP2)

In alkali hydrolysis molecular weight was loss as compare to pure drug so in Alkali hydrolysis in presence of sodium hydroxide two hydron ion was removed and oxygen convert in to Aldehyde it means Alcohol convents in to Aldehyde in formed DP2. Possible degradation pathway of DP2 was shown in Figure No: 6.120.

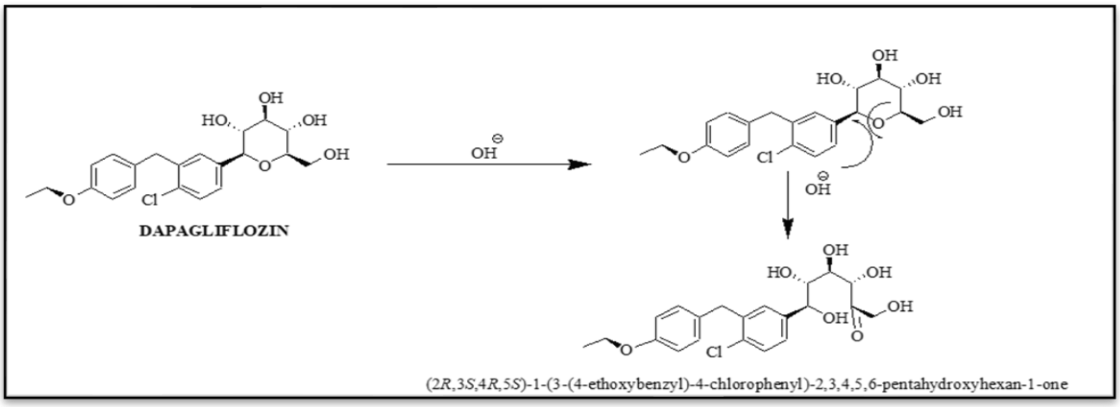


Figure No: 6.124 Possible degradation pathway of Dapagliflozin Alkali degradation Impurity (DP2)

Table No: 6.116: Summary of Degradation Impurities of Empagliflozin

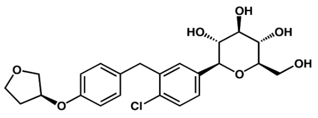
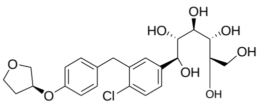
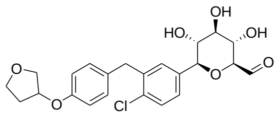
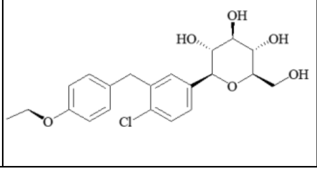
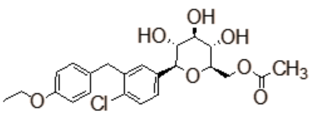
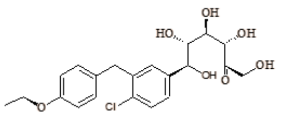
Name	**Empagliflozin**	**DP1**	**DP2**
Form	**API**	**Acidic condition (2N HCl 60°C, 24 hr)**	**Alkali Condition (2N NaOH 60°C, 24 hr)**
Molecular Formula	$C_{23}H_{27}ClO_7$	$C_{23}H_{29}ClO_8$	$C_{23}H_{25}ClO_7$
Molecular weight	**450.91 g/mol**	**468.92 g/mol**	**448.89 g/mol**
IR (Cm^{-1} KBr)	**3429.55** (-OH Str) **2939.61 (**Ar-CH str) **1239.34** (C-O-C Ether linkage)	**3421.7** (-OH Str) **2929.7** (Ar-CH str) **1279.5** (C-O-C Ether linkage)	**3474.3** (-OH Str) **2958.2** (Ar-CH str) **1237.5** (C-O-C Ether linkage) **1752.7 (C=O str)**
^{1}H NMR (DMSO-d6, δ ppm)	**3.23-3.45** (m, 5H Sugar moeity), **3.47-3.70** (q/m, 7H, THF), **4.04** (m,4H, CH_2) **4.86-4.99** (s, 4H, -OH group), **6.83-7.39** (m, 7H, Ar-H)	**3.08-3.24** (m, 5H, open sugarmoiety), **3.41-3.71** (q/m, 7H, THF), **3.53** (m, 4H, CH_2), **4.03** (s,1 H, OH) **4.44-4.95** (s,5H,OH group) **7.10-7.38** (m, 7H, Ar-H)	**3.40-3.47** (m, 5H Sugar moeity), **3.53-3.98** (q/m, 7H, THF), **4.01** (s,2H, CH_2) **4.47-4.97** (s, 3H, -OH group), **7.10-7.39** (m, 7H, Ar-H) **8.830** (s,1 H,CHO)
Mass (m/e)	**M$^+$ at 451.3** **M$^+$ 2 at 453.1**	**M$^+$ at 469.1** **M$^+$ 2 at 471.2**	**M$^+$ at 449.3** **M$^+$ 2 at 451.1**
Structure			

Table No: 6.117: Summary of Degradation Impurities of Dapagliflozin

Name	**Dapagliflozin**	**DP1**	**DP2**
Form	**API**	**Acidic condition** **(2N HCl 60ºC, 24 hr)**	**Alkali Condition** **(2N NaOH 60ºC, 24 hr)**
Molecular Formula	**$C_{21}H_{25}ClO_6$**	**$C_{23}H_{27}ClO_7$**	**$C_{21}H_{25}ClO_7$**
Molecular weight	**408.87 g/mol**	**450.14 g/mol**	**424.87 g/mol**
IR (Cm^{-1} KBr)	**3330** (-OH Str) **1237** (C-O-C Ether linkage) **2873** (Ar-CH str)	**3339** (-OH Str) **1036** (C-O-C Ether linkage) **1741** (C=O str) **2877** (Ar-CH str)	**3354** (-OH Str) 1170 (C-O-C Ether linkage) **1677** (C=O str) **2834** (Ar-CH str)
^{1}H NMR (DMSO-d6, δ ppm)	**1.08-1.243** (t,3H –CH3) **3.08-3.56** (m,5H Sugar moeity), **3.69-4.03** (m,6H, CH_2) **4.44-4.96** (d, 4H, -OH group), **6.84-7.35** (m, 7H, Ar-H)	**1.29-1.32** (t,3H –CH3) **2.19** (s,3H –CH3) **3.11-3.51** (m, 5H Sugar moeity), **3.71-4.08** (m,6H, CH_2) **4.51-5.00** (d,3H, -OH group), **6.83-7.40** (m, 7H, Ar-H)	**1.00-1.45** (t,3H –CH3) **3.00-3.58** (m, 4H Sugar moeity), **3.69-4.12** (m,6H, CH_2) **4.44-4.92** (d, 5H, -OH group), **6.84-7.37** (m, 7H, Ar-H)
Mass (m/e)	**M^+ at 409.1** **M^+ 2 at 411.3**	**M^+ at 450.3** **M^+ 2 at 452.5**	**M^+ at 424.2** **M^+ 2 at 426.5**
Structure			

CHAPTER 7

CONCLUSION

7. CONCLUSION

Impurity profiling of anti-diabetic drugs was a critical aspect of pharmaceutical research and development. The primary objectives of impurity profiling were to ensure the safety, efficacy, and quality of these drugs. Some of the key outcomes that can be achieved through effective impurity profiling include:

- **Safety Assurance:** Identification and quantification of impurities help ensure that the drug product was safe for consumption, as impurities can potentially be toxic or cause adverse effects.
- **Efficacy Enhancement:** Understanding and controlling impurities can lead to improved drug efficacy, ensuring that patients receive the intended therapeutic benefits.
- **Quality Control:** Impurity profiling was essential for maintaining consistent drug quality throughout the manufacturing process, reducing batch-to-batch variations.
- **Regulatory Compliance:** Meeting regulatory requirements for impurity limits and reporting was crucial for obtaining drug approval and maintaining compliance with various regulatory agencies.
- **Cost Reduction:** Identifying and eliminating unnecessary impurities can streamline the manufacturing process, reduce production costs, and minimize waste.
- **Product Stability:** Studying impurities helps in assessing drug stability and shelf-life, ensuring that the medication remains effective over time.

Commercial Applications:

The commercial applications of impurity profiling in the context of anti-diabetic drugs were significant and can benefit pharmaceutical companies in several ways:

- **Drug Development:** Pharmaceutical companies can use impurity profiling to develop safer and more effective anti-diabetic drugs, potentially gaining a competitive edge in the market.
- **Quality Control:** Ensuring the quality and consistency of anti-diabetic drugs through impurity profiling was crucial for maintaining a positive reputation and meeting regulatory requirements.

- **Cost Optimization:** Identifying cost-effective manufacturing processes and minimizing impurities can lead to cost savings and increased profitability.
- **Regulatory Approval:** Successful impurity profiling can expedite the regulatory approval process, allowing companies to bring their anti-diabetic drugs to market more quickly.
- **Market Differentiation:** Companies that can demonstrate superior impurity control and safety profiles may gain a competitive advantage in the diabetes treatment market.

Empagliflozin

- Method was developed for Empagliflozin by using Mobile phase Acetonitrile: water 50:50 (V/V) with flow rate 0.5 ml/min using automatic liquid chromatography Model LC-2010 (Shimadzu, Japan), Pump-single pump systems using UV-VWAS Detector at 224 nm with Software-LC Solution to acquire and process the data. Reversed-Phase YMC ODS A C-18 (150mm x 4.6 mm). The retention time was found to be 5.73 min.
- Validation was performed according to ICH Guidelines Q2(R1).
- The degradation study of Empagliflozin under stressed conditions was examined following ICH guidelines Q1(R2).
- The API was subjected to oxidative, acidic, alkaline, neutral, photolytic, and thermolytic degradation conditions.
- The drug was stable in Oxidative, thermal, and photolytic conditions, and no degradation products were observed.
- However, 2 degradation products were formed in acid (DP-1,RT:2.28 min) and Alkali stress hydrolysis conditions (DP-2, RT:2.25 min).
- DP1 was formed by attack of H+ on oxygen of oxane ring which lead to oxane ring opening in acid degradation condition.
- DP-2 was formed by attack of -OH with loss of H_2 which convert in to aldehyde.

Dapagliflozin:

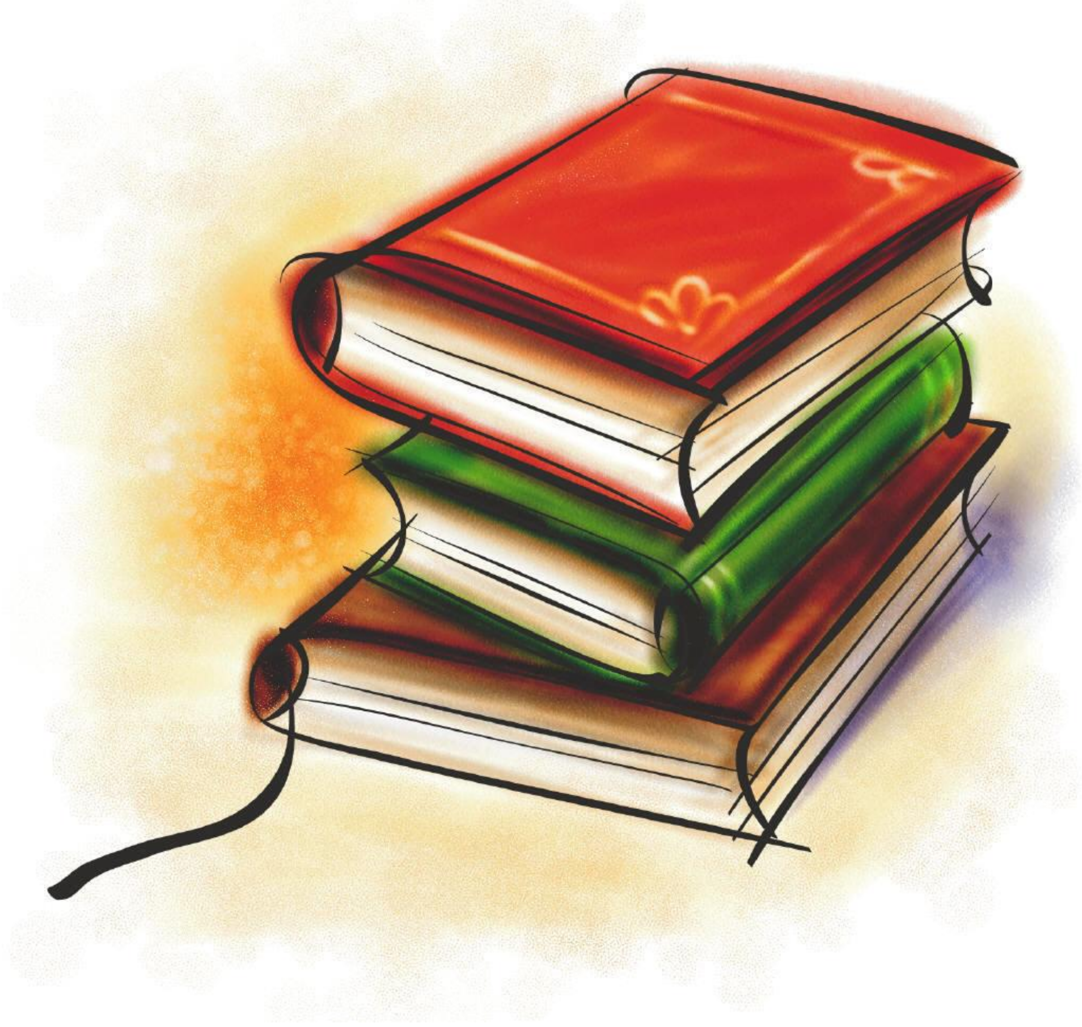
- Method was developed for Dapagliflozin by using Mobile phase Acetonitrile: water 50:50 (V/V) with flow rate 0.7 ml/min by using same at 273 nm. The retention time was found to be 5.33 min.
- Validation was performed according to ICH Guidelines Q2(R1).
- Same like Empagliflozin another drug, Dapagliflozin was also examined under stress condition according to ICH Guidelines Q1(R2) and shows degradation

under Acidic and Alkali condition but remain stable in Oxidative, thermal, and photolytic conditions, and no degradation products were observed.

- 2 degradation products formed in acid (DP-1,RT: 6.90 min) and Alkali stress hydrolysis conditions (DP-2, RT: 2.3 min).
- Formation of DP1, sugar moiety in the target molecule undergoes gradual decomposition to form formaldehyde /acetaldehyde and acetic acid in different proportions. The formed acetic acid involved in the esterification/Acetylation of dapagliflozin under the existing hot acidic condition.
- DP2 form by attack of OH- which lead to oxane ring opening.
- All degradation products were separated by Preparative HPLC using Waters semi-preparative HPLC 2489 dual UV detector, 2545 pump module, and 2707 sample manager with auto fraction collector-III. The total instrument was controlled with ChromScope-2.1 software, and in-house packed Luna C18 150 × 25 mm, 5 µm was used to isolate the degradation products. All isolated pure fractions were lyophilized using Ly freeze lyophilizer and characterized entirely using various analytical techniques like IR, ^{1}H NMR and Mass Spectroscopy. All DPs were novel products and were not reported in any literature.

CHAPTER 8

REFERENCE



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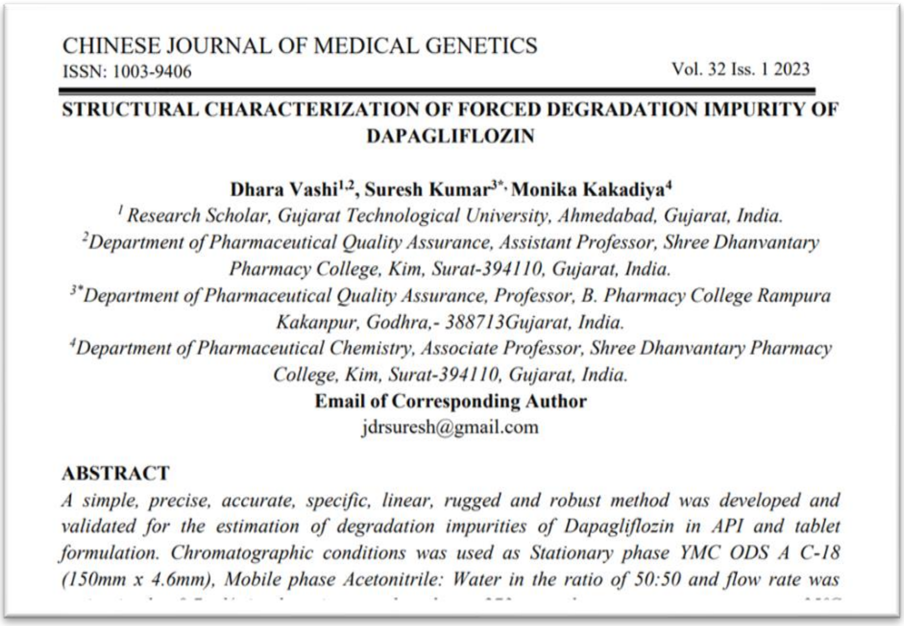
APPENDIX

APPENDIX

List of Publications

1. Research paper was published in Scopus and UGC care list group II Approved Journal Chinese Journal of Medical genetics entitled " Structural characterization of Forced degradation impurity of Dapagliflozin “. Vol. 32 Issue. 1(2023). ISSN: 1003-9406.



CHINESE JOURNAL OF MEDICAL GENETICS
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STRUCTURAL CHARACTERIZATION OF FORCED DEGRADATION IMPURITY OF DAPAGLIFLOZIN

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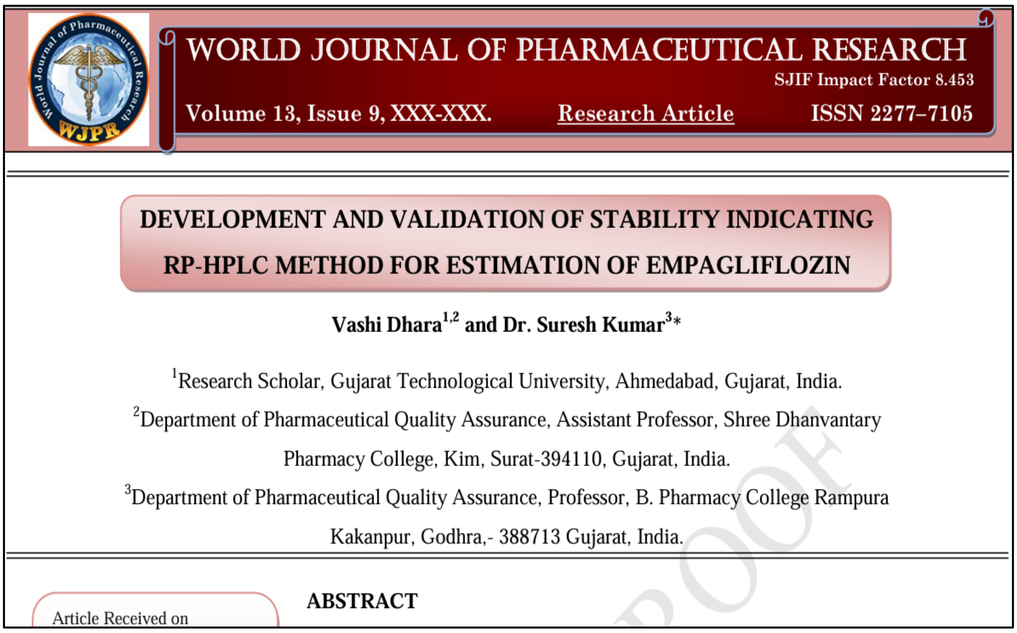
ABSTRACT

A simple, precise, accurate, specific, linear, rugged and robust method was developed and validated for the estimation of degradation impurities of Dapagliflozin in API and tablet formulation. Chromatographic conditions was used as Stationary phase YMC ODS A C-18 (150mm x 4.6mm), Mobile phase Acetonitrile: Water in the ratio of 50:50 and flow rate was

2. Research paper was published in World Journal of Pharmaceutical Research entitled “Development and Validation of Stability indicating RP-HPLC method for estimation of Empagliflozin. “Vol. 13 Issue. 9 (2024):ISSN 2277 – 7105.



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DEVELOPMENT AND VALIDATION OF STABILITY INDICATING RP-HPLC METHOD FOR ESTIMATION OF EMPAGLIFLOZIN

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Article Received on

ABSTRACT

3. Review article was published in Asian Journal of Pharmaceutical Research entitled “Impurity identification and Characterisation some Anti-Diabetic drugs using of various analytical methods”. Vol. 9 Iss. 4 (2019). ISSN: 2231-5691

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Impurity Identification and Characterization of some Anti-Diabetic Drugs using various Analytical Methods

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