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Stability-Indicating RP-HPLC Method Development and Validation for Simultaneous Estimation of Dapagliflozin Propanediol Monohydrate and Linagliptin in Pharmaceutical Dosage Form

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ABSTRACT

A new, repaid, reliable, simple, and reproducible stability-indicating RP-HPLC method has been developed for simultaneous estimation of linagliptin and dapagliflozin propanediol monohydrate in tablets. Separation of dapagliflozin and linagliptin was achieved using gradient program setting with mobile phase A phosphate buffer (pH 3.5) and mobile phase B Acetonitrile. Inertsil ODS-3V, 150 x 4.6 mm, 5µ column, set at 25 °C, was used as a stationary phase. Flow rate was kept 1.0 mL/minute and detection was carried out at 233 nm. Retention times were found to be 2.86 min and 7.45 min for linagliptin and dapagliflozin respectively. The method was found to be stability indicating with all the degradants well-separated from linagliptin and dapagliflozin peaks. This developed method has been validated according to ICH guideline with respect to system suitability, specificity, precision, linearity, accuracy and robustness. This method is specific and showed excellent linear response with correlation coefficient (R2) values of 0.998 and 0.999 for linagliptin and dapagliflozin respectively in the concentration ranges of 2.5-7.5 µg/ml and 5-15 µg for linagliptin and dapagliflozin, respectively. The method has future potential to be used for routine quality control analysis as well as accelerated stability testing.

Keywords: Dapagliflozin, linagliptin, RP-HPLC, validation, stability-indicating method

CHAPTER 1: INTRODUCTION [1-16]

1.1 Diabetes Mellitus[1-3]

Diabetes, documented as early as ancient Egypt, was named "diabetes mellitus" by the Greek physician Aertaeus, referring to the passing of sweet-tasting urine. Diabetes mellitus (DM) is a metabolic disorder resulting from a defect in insulin secretion, insulin action, or both which in turn results into hyperglycemia. Chronic hyperglycemia brought on by an insulin shortage also causes problems with protein, lipid, and carbohydrate metabolism. It is the most prevalent endocrine condition, and by 2010.

Global industrialization and obesity have caused a diabetes epidemic. Prevalence varies, but it's predicted to rise from 4% in 1995 to 6.4% by 2025. Undiagnosed cases are common.

The rate of diabetes has been rising, and factors like changes in life expectancy and the quality of healthcare have played a part. Diabetes patients are prone to both short- and long-term complications, as well as premature death, which has an impact on urban areas more than other regions of the world and increases the load on healthcare systems globally.

Type 1 and Type 2 diabetes are the two most common forms of diabetes mellitus

Diabetes Type 1:

Diabetes type 1 is brought on by an autoimmune disorder. The pancreatic beta cells that make insulin are wrongly attacked and destroyed by the immune system. Little or no insulin is produced as a result.

Insulin Dependence: People with Type 1 diabetes need to take insulin for the rest of their lives to stay alive.

Prevalence: About 5–10% of all diabetes cases are type 1 diabetes.

Diabetes Type 2:

Cause: Insulin resistance, or the body's cells not responding well to insulin, and a gradual relative insulin deficit are the main features of type 2 diabetes. It's also linked to decreased pancreatic insulin secretion.

Risk factors: Genetics, obesity, a sedentary lifestyle, a bad diet, and advancing age are risk factors. A big part is played by family history.

Prevalence: Type 2 diabetes makes up the bulk of cases (about 90–95%) of diabetes.

Gestational Diabetes: It is a condition that is characterized by an elevated level of blood gluose during pregnancy that typically resolves after delivery.

Treatment options include lifestyle changes (diet, exercise). Depending on the patient's needs and the course of the condition, the treatment is customized with inclusion of oral anti-diabetic drugs (oral hypoglycemic agents) monotherapy or combination.

Oral hypoglycemic agents can be classified into several categories based on their mechanisms of action and therapeutic effects. Figure 1 represents classification of modern oral hypoglycemic agents.

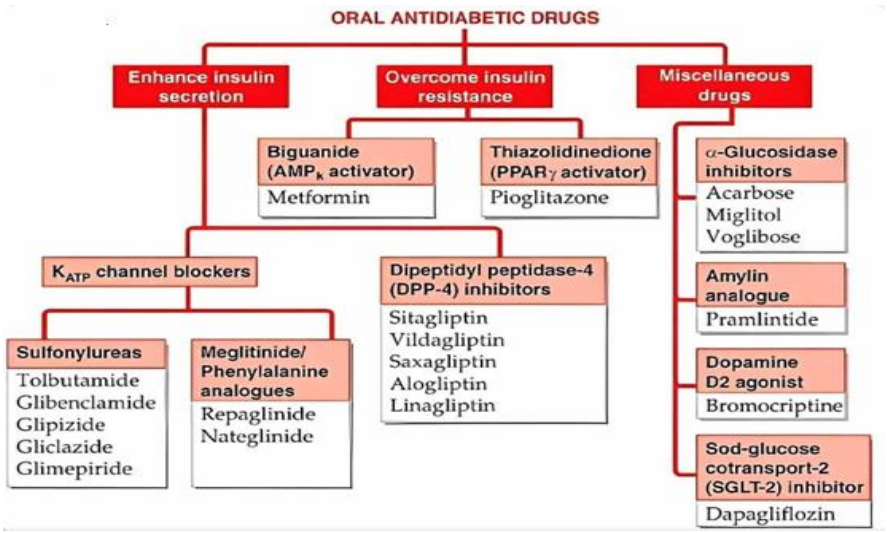

Figure 1: Schematic diagram of classification of oral antidiabetics

The invention of new medications and therapeutic agents with the goal of enhancing patient outcomes and quality of life drives ongoing growth in the field of pharmaceutical sciences. In this perspective, it is impossible to overrate the importance of analytical chemistry in pharmaceutical research and development. Characterization, quantification, and quality evaluation of pharmaceutical substances all depend on accurate and exact analytical techniques.

1.2 Analytical Chemistry in Pharmaceutical R&D [4]

The foundation of pharmaceutical research and development (R&D) is analytical chemistry. It includes a broad range of tools and procedures that let researchers delve deeply into the molecular and chemical makeup of medicinal molecules. From the early discovery of prospective therapeutic agents to their formulation into safe and effective pharmaceutical products, these procedures are essential at various phases of drug development.

Analytical chemistry fundamentally enables researchers to:

* Identify and measure impurities and active pharmaceutical ingredients (APIs) in medicine formulations.

* Analyze the stability and purity of medicinal substances throughout time.

* Ensure that pharmaceuticals are of a high standard and consistency.

* Find out more about the pharmacokinetics and bioavailability of medications in the human body.

* Obey strict legal requirements and quality control guidelines.

Below are some commonly used analytical techniques in pharmaceutical research and development (R&D) and their main applications:

* High-Performance Liquid Chromatography (HPLC): Quantification of pharmaceutical compounds, stability testing, impurity analysis, and assay determination.

* Gas Chromatography (GC): Analysis of volatile compounds, such as residual solvents, volatile impurities, and the characterization of volatile components in drug products.

* Liquid Chromatography-Mass Spectrometry (LC-MS): Identification, quantification, and structural elucidation of pharmaceutical compounds, including the detection of metabolites and degradation products.

* Fourier-Transform Infrared Spectroscopy (FT-IR): Identification of functional groups, structural analysis, and characterization of solid-state forms of pharmaceuticals.

* Ultraviolet-Visible Spectroscopy (UV-Vis): Quantification of compounds with chromophores, such as drug assays and determination of drug concentrations in formulations.

* Nuclear Magnetic Resonance Spectroscopy (NMR): Structural elucidation, confirmation of chemical structure, and investigation of molecular interactions in drug development.

* Mass Spectrometry (MS): Molecular weight determination, identification of impurities, and quantitative analysis of drug compounds.

1.3 Chromatography in Pharmaceutical Analysis [5]

Chromatography stands out as a versatile and very essential tool in pharmaceutical analysis. According to their affinities for stationary phases and interactions with mobile phases, the components of a complex mixture can be identified and quantified using a variety of separation techniques collectively referred to as "chromatography".

There are several types of chromatography methods, each designed for specific applications. Here are some of the most common types of chromatography:

Certainly, here's a list of various types of chromatography without definitions:

- Liquid Chromatography (LC)
- High-Performance Liquid Chromatography (HPLC)
- Gas Chromatography (GC)
- Gas-Liquid Chromatography (GLC)
- Ion Exchange Chromatography (IEC)
  - Cation Exchange Chromatography
  - Anion Exchange Chromatography

- Size-Exclusion Chromatography (SEC) or Gel Filtration Chromatography
- Affinity Chromatography
- Reverse-Phase Chromatography (RPC)
- Normal Phase Chromatography
- Thin-Layer Chromatography (TLC)
- Paper Chromatography
- Column Chromatography
- Supercritical Fluid Chromatography (SFC)

1.4 High-Performance Liquid Chromatography (HPLC)[6-11]

High-Performance Liquid Chromatography (HPLC) is a powerful and widely used chromatographic technique in pharmaceutical research and various other fields of chemistry, biotechnology, forensic science, environmental sciences, etc.

Here, we will discuss HPLC in depth because our research work is based on the HPLC instrument.

1.4.1 Principles of HPLC

With the help of a mobile phase (solvent or buffer) and a stationary phase (column packed with packing material), sample components are separated under high-pressure. The following are the main terms used in HPLC analysis:

* Stationary Phase: The stationary phase often consists of a solid substances that has been compressed into a column. Depending on the kind of analytes to be separated, it can be formed of a variety of materials, such as silica, polymer, or other specialty phases.

* Mobile Phase: The mobile phase, a liquid solvent or buffer, transports the sample over the column.

* Sample Injection: The sample is injected into the column of the HPLC system, frequently using an autosampler to precisely measure and inject the sample.

* Separation Mechanisms: HPLC can employ different separation mechanisms, including partition, adsorption, ion exchange, size exclusion, and affinity interactions, depending on the type of column and stationary phase used.

* Detection: The separated compounds pass through a detector, commonly a UV-Visible spectrophotometer, fluorescence detector, or mass spectrometer, which quantifies and identifies the analytes based on their unique properties.

1.4.2 Components of HPLC instrumentation:

1. **Reservoir:** Glass bottles used for holding the mobile phase solvents.
2. **Column:** Columns are a critical component of the HPLC system and play a key role in the separation and analysis of samples.

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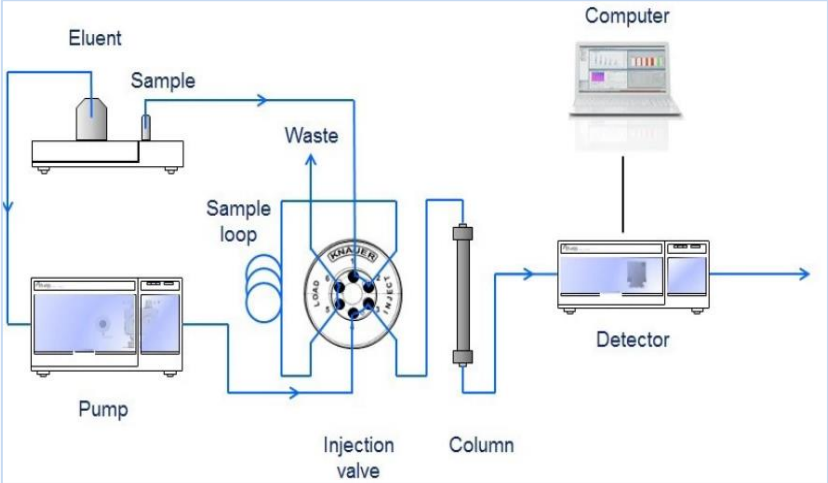

Figure 2: Schematic layout of HPLC instrumentation

Different types of HPLC columns and their characteristics:

1. Normal Phase HPLC Columns:

Stationary Phase: Typically made of polar materials such as silica.

Mobile Phase: Nonpolar solvents.

Applications: Separation of compounds based on differences in polarity, used for nonpolar and moderately polar compounds.

2. Reverse-Phase HPLC Columns:

Stationary Phase: Hydrophobic materials such as C18 (octadecylsilane) or C8.

Mobile Phase: Polar solvents, often with an organic component.

Applications: Separation of polar and moderately polar compounds, including pharmaceuticals, peptides, and proteins.

3. Ion-Exchange HPLC Columns:

Stationary Phase: Contains ion-exchange sites with either positively charged (cation-exchange) or negatively charged (anion-exchange) functional groups.

Mobile Phase: Aqueous buffer solutions with varying ionic strength and pH.

Applications: Separation of charged compounds, such as amino acids, proteins, and nucleic acids.

4. Size-Exclusion (SEC) or Gel Filtration Columns:

Stationary Phase: Porous beads with different pore sizes.

Mobile Phase: Aqueous buffer solutions.

Applications: Separation of molecules based on size, commonly used for proteins, polymers, and biomolecules.

5. Affinity Chromatography Columns:

Stationary Phase: Functionalized with ligands that specifically bind to target molecules (e.g., antibodies or enzymes).

Mobile Phase: Buffer solutions.

Applications: Highly selective separation and purification of specific biomolecules.

6. Chiral Chromatography Columns:

Stationary Phase: Contains chiral selectors, allowing separation of enantiomers (mirror-image isomers).

Mobile Phase: Various solvent systems.

Applications: Separation of chiral compounds, essential in pharmaceuticals, flavors, and fragrances.

3. Column Oven:

Controls the temperature of the column to optimize separation.

4. Pump:

The HPLC pump is a vital component of the High-Performance Liquid Chromatography (HPLC) system, used for delivery of the mobile phase at a precisely controlled and constant flow rate.

To effectively push the mobile phase through the chromatographic column, these pump runs under high pressure, typically between 2,000 and 15,000 psi.

Modern pumps can operate in both isocratic and gradient modes. In order to ensure flow rate stability and the repeatability of chromatographic analyses, HPLC pumps frequently include solvent degassing, reduced pulsation, pressure monitoring, and automated operation.

5. Injector:

Injector introduces the sample into the system where it enters the flow of the mobile phase and travels through the column for separation.

HPLC injectors employ various sample injection methods, including manual injection, partial-loop injection, and full-loop injection, depending on the system's design and requirements.

Injectors are designed to minimize injection variability, reducing the risk of bias in quantitative analyses; hence autosamplers are often fitted with the injector, allowing for multiple sample injections in a sequence without manual intervention.

For temperature-sensitive samples, some injectors have temperature control capabilities to maintain sample integrity during injection.

6. Detector:

Responsible for detecting and quantifying analytes as they elute from the chromatographic column.

It plays a central role in obtaining meaningful chromatograms and analytical data.
HPLC detectors are available in various types, each designed to detect specific types of compounds or provide unique detection capabilities. Common HPLC detectors include UV-Visible detectors, fluorescence detectors, refractive index detectors (RID), conductivity detectors, electrochemical detectors, and mass spectrometers (LC-MS).

7. Data System:

Gathers and processes data, equipped with chromatography software for analysis.

1.4.3 Advantages of HPLC

* Sensitivity: HPLC can detect compounds at low concentrations, making it suitable for trace-level analysis.
* High Resolution: HPLC offers excellent resolution, allowing the separation of closely related compounds.
* Quantitative: HPLC provides precise measurements of compound concentrations during quantitation analysis.
* Wide Applicability: It can analyze a broad range of compounds, from small molecules to large biomolecules.
* Reproducibility: Standardized methods offer reproducibility across different laboratories.
* Versatility: Various HPLC column types and detection techniques allow customization of methods for specific applications.

1.4.4 Applications of HPLC

Various applications of HPLC are as follows:

* Pharmaceutical Analysis: Quantification of APIs, dissolution testing, impurity profiling, finished drug product analysis.
* Food Analysis: Determination of pesticides, food additives, and food quality assessment.
* Environmental Monitoring: Detection of pollutants and contaminants in water, air, and soil.
* Biotechnology: Characterization and purification of biomolecules like nucleic acids, proteins, and peptides.
* Clinical Chemistry: Measurement of analytes in biological samples for diagnostics purposes.
* Forensic Science: Detection of narcotics, toxins, and poisons in forensic investigations.
* Chemical Research: General chemical analysis, identification of unknown compounds, and reaction monitoring.

1.4.5 Disadvantages

- Cost: Initial investment in chromatographic equipment can be substantial.
- Complexity: Routine operation and maintenance needs skillful people.
- Time-Consuming: Sometimes can be time-intensive depending on the method and sample preparation techniques.

1.4.6 Method development by HPLC

* Define the Objective: Begin by clearly defining the purpose of the analysis. Understand the type of compounds you need to separate and quantify, as well as the specific analytical requirements (e.g., sensitivity, speed, selectivity).
* Select the HPLC Columns: Choose an appropriate HPLC column based on the characteristics of the analytes.
* Choose the Mobile Phase: Select the mobile phase solvent or solvent mixture based on the analytes' polarity and chemical properties.
* Optimize the Mobile Phase Composition: Systematically vary the composition of the mobile phase (e.g., solvent ratios, pH) to achieve desirable chromatographic separation. Optimize conditions for selectivity and peak resolution.
* Set Column Temperature: Determine the appropriate column temperature to optimize separation. Temperature can affect analyte retention and peak shape.

* Adjust Flow Rate: Optimize the flow rate of the mobile phase for the best compromise between separation efficiency and analysis time.

* Choose Detection Wavelength (for UV Detectors): If using a UV detector, select an appropriate detection wavelength based on the analytes' UV absorbance properties.

* Determine Injection Volume: Optimize the injection volume to ensure that it falls within the linear range of the detector and provides sufficient sensitivity.

* Optimize Gradient Conditions: If isocratic separation is not feasible, gradient setting needs to be employed. If using gradient elution, develop a gradient profile that ensures efficient separation.

* Test Sensitivity and Specificity: Evaluate the method's sensitivity and specificity by analyzing samples and standards. Ensure that analyte peaks are well-resolved from interferences.

* Method Validation: Validate the developed HPLC method in accordance with ICH guidelines by assessing its linearity, accuracy, precision, limits of detection (LOD), quantification (LOQ), and robustness.

* Documentation: Document all method development and validation steps, including chromatograms, calibration data, and validation results, for future reference and regulatory compliance.

* Routine Maintenance: Implement regular maintenance of the HPLC system, including column conditioning, detector checks, and system suitability tests to ensure method reproducibility.

1.5 Stability-indicating analytical methods [12-14]

All High-Performance Liquid Chromatography (HPLC) methods need to be stability indicating. These methods serve as essential tools to ascertain the safety and efficacy of drug products during their entire shelf life.

Forced degradation studies constitute an integral component of the development of stability-indicating HPLC methods.

Before new medicines can be approved, they need to undergo stability studies to make sure they remain safe and effective over time. These studies last for 12 months (long term) and 6 months (accelerated), but the tests to check for harmful changes take time. Instead of waiting for months, scientists can speed up the process by forced degradation and make the pharmaceutical drug product degrade in just a few weeks. This helps them create tests to check for harmful changes in the medicine much faster than actual stability testing.

1.5.1 Forced Degradation Studies

The goal of forced degradation study is to induce degradation and identify potential degradation products and pathways.

During forced degradation, APIs or drug products are exposed to extreme stressors like heat, humidity, UV, acid/alkali, oxidizing agents, etc. to generate significant amount of degradants. To develop the analytical method, we use degraded samples and known impurities, creating a worst-case scenario which may happen during actual stability testing. It's essential that all peaks (main peak, degraded peaks, and known impurities) are well-separated. If not, the method must be redeveloped for proper separation. When an analytical method can separate all peaks from degraded samples, it becomes suitable for analyzing stability samples, making it stability-indicating.

Forced degradation data aids in identifying root causes during out-of-specification (OOS) or lab investigations. If unexpected peaks appear during sample analysis but not during forced degradation, contamination during manufacturing or analysis is a likely cause.

The suggested degradation conditions as accepted by the FDA during DMF/ANDA/NDA and IND submissions for regulatory approval are listed below in table 1. These parameters are based on existing industrial practices.

Table 1 Suggested Experimental Conditions for Forced Degradation Studies

Degradation Type	Concentration of Reagent	Conditions to be applied	Time	Remarks
Acid	5N HCl	80° C	1 hour	Concentration, condition, and time can be changed to optimize degradation.
Alkali	5N NaOH	80° C	1 hour	
Peroxide	10% H2O2	80° C	1 hour	
Heat/Thermal	80°C	80° C	1 hour	
UV	Expose under UV light at 254 nm wavelength.	Ambient temperature.	24 hours	Time can be changed to optimize degradation.
Control	NA	NA	NA	NA

Numerous additional degradant peaks can be produced from the analyte peak during the forced degradation studies. After the study is complete, the percent degradation of the analyte peak will be determined.

The FDA accepts the following formula as a standard method for calculating the percent deterioration in the pharmaceutical industry.

$$\%Degradation = 100 - \frac{\text{Acid Sample Main Peak Area}}{\text{Control Sample Main Peak Area}} \times \frac{\text{Control Spl.Wt}}{\text{Acid Spl.Wt}} \times 100$$

Mass balance for each degradant peak should be assessed using the % degradation. Taking into account the analysis's margin of error, the mass balance should be close to 100%.

1.5.2 Benefits of Stability-Indicating HPLC Methods and Forced Degradation Studies:

- Identifies and quantifies degradation products and ensures the stability and safety of the finished drug products.
- Gives details on probable impurities and degradation mechanisms.
- Shows product stability and supports regulatory submissions.
- Aids in establishing recommended shelf lives and acceptable storage conditions.
- Aids drug manufacturers in making well-informed formulation and packaging selections.

1.6 Analytical Method Validation [15-16]

In a number of scientific fields, including pharmaceuticals, environmental analysis, food safety, and others, method validation is an essential procedure. It includes a number of methodical steps and requirements meant to make sure that an analytical method is appropriate for the test and yields accurate, precise and reliable findings.

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1.6.1 Parameters to be checked for method validation

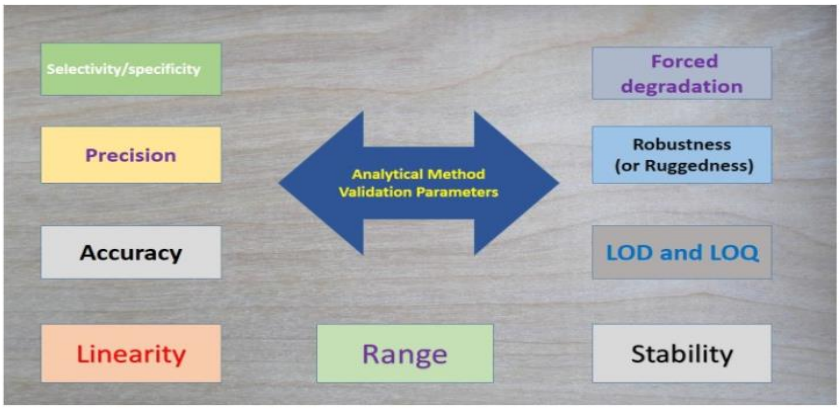


Figure 3: Parameters to be checked for validation

1. Specificity:

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc.

2. Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found.

3. Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility.

4. Detection Limit (LOD) and Quantitation Limit (LOQ)

The detection limit of an individual analytical procedure is the lowest amount of analyte in as ample which can be detected but not necessarily quantitated as an exact value.

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in sample which can be quantitatively determined with suitable precision and accuracy.

5. Linearity and Range:

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample.

6. Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

7. System Suitability:

System suitability is an essential aspect of analytical method validation and quality control in pharmaceutical analysis. As a quality assurance tool, system suitability tests are used to confirm that the chromatographic system is appropriate for the desired analysis. They aid in recognizing and averting problems that can compromise the precision and accuracy of analytical results.

Common System Suitability Parameters:

● Resolution (Rs): Determines the distance between neighboring peaks. It ensures that closely related analytes may be distinguished by the chromatographic technique.

● Retention Time: Ensures that the column and system are operating consistently by determining whether the retention periods of the target analytes are within a specified range.

● Tailings Factor (T): Evaluates the chromatographic peaks' symmetry. By doing this, the peak shape is made sure to be appropriate and not distorted.

● Theoretical Plates (N or N/m): Analyzes column performance. It gauges how effectively a column can separate different chemicals.

● Relative Standard Deviation (%RSD): Calculates how accurately repeat injections are performed. It ensures that the approach yields reliable outcomes.

Regularly monitoring the adequacy of the system allows QC laboratories to identify instrument- or method-related issues early and take corrective action, eliminating the need for reanalysis and cutting expenses.

1.6.2 Regulatory guidelines for validation

Some of the key standard bodies and guidelines related to analytical method validation for Pharmaceuticals and Drug Manufacturing:

International Council for Harmonization (ICH):

* ICH Q2(R1) - Validation of Analytical Procedures: Text and Methodology

* ICH Q2(R2) - Validation of Analytical Procedures: Methodology

* U.S. Pharmacopeia (USP):

* USP General Chapter <1225> - Validation of Compendial Procedures

* USP General Chapter <1226> - Verification of Compendial Procedures

* European Medicines Agency (EMA):

* EMA Guideline on Bioanalytical Method Validation

* EMA Guideline on Validation of Analytical Procedures

1.6.3 Methods to be validated

* Identification tests.

* Quantitative tests for impurities' content.

* Limit tests for the control of impurities.

* Quantitative tests of the active moiety in samples of drug substance or drug product or other selected component(s) in the drug product.

Table 2: Parameters to be checked for method validation

Type of analytical procedure characteristics	IDENTIFICATION	TESTING FOR IMPURITIES quantitat.	limit	ASSAY - dissolution (measurement only) - content/potency
Accuracy	-	+	-	+
Precision				
Repeatability	-	+	-	+
Interm.Precision	-	+ (1)	-	+ (1)
Specificity (2)	+	+	+	+
Detection Limit	-	- (3)	+	-
Quantitation Limit	-	+	-	-
Linearity	-	+	-	+
Range	-	+	-	+

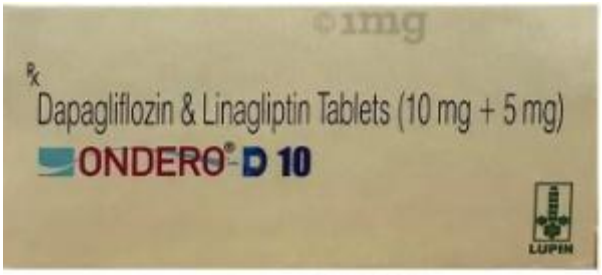
- \- signifies that this characteristic is not normally evaluated
- \+ signifies that this characteristic is normally evaluated
- (1) in cases where reproducibility (see glossary) has been performed, intermediate precision is not needed
- (2) lack of specificity of one analytical procedure could be compensated by other supporting analytical procedure(s)
- (3) may be needed in some cases

1.7 COMBINATION DOSAGE FORMS:

CDSCO approval: Approval granted to conduct phase III trials to Exemed Pharmaceuticals for Fixed dose combination of Linagliptin 5 mg + Dapagliflozin propanediol monohydrate 10 mg on November 4, 2022.

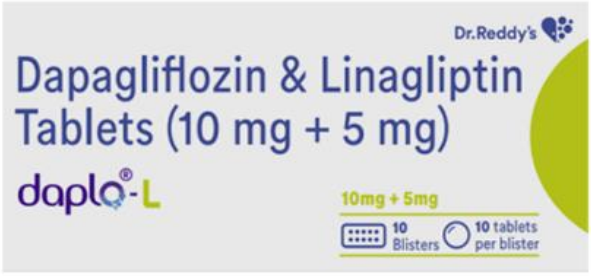
Table 3: Combination dosage form details

Brand Names	Dosage Form	Label Claims	Manufacturer
Ondero-D	Tablet	Linagliptin 5 mg Dapagliflozin 10 mg	Lupin Ltd
Daplo-L	Tablet	Linagliptin 5 mg Dapagliflozin 10 mg	Dr Reddy's Laboratories Ltd
Dapanorm-L10	Tablet	Linagliptin 5 mg Dapagliflozin 10 mg	Alkem Laboratories Ltd


Dapagliflozin & Linagliptin Tablets (10 mg + 5 mg)
ONDERO D 10
LUPIN


Dapagliflozin & Linagliptin Tablets (10 mg + 5 mg)
ONDERO D 10
Composition :
Each film coated tablet contains:
Linagliptin ..... 5 mg
Dapagliflozin Propanediol Monohydrate
eq. to Dapagliflozin ..... 10 mg
Excipients ..... q.s.
Colours: Ferric oxide Red NF &
Titanium dioxide IP
Dosage : As directed by the Physician.
Store below 30°C.
Keep the medicine out of reach of children.


Dapagliflozin 10 mg
and Linagliptin 5mg Tablets
DAPANORM-L10


Dr.Reddy's
Dapagliflozin & Linagliptin
Tablets (10 mg + 5 mg)
daplo-L
10mg + 5mg
10 Blisters
10 tablets per blister

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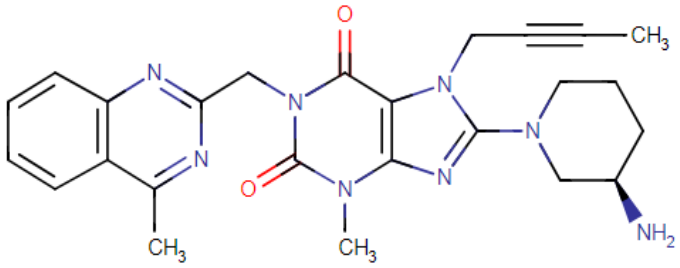
CHAPTER 2: DRUG PROFILE [17-18]

2.1 DRUG PROFILE OF LINAGLIPTIN [17]

Table 4: Drug profile of linagliptin

Name	**Linagliptin**
Chemical Structure	
Molecular Formula	$C_{25}H_{28}N_8O_2$
Molecular Weight	472.54 g/mol
IUPAC Names	8-[(3R)-3-Aminopiperidin-1-yl]-7-(but-2-yn-1-yl)-3-methyl-1-[(4-methylquinazolin-2-yl)methyl]-3,7-dihydro-1H-purine-2,6-dione
CAS No.	668270-12-0
Appearance	White to Orange Solid
Solubility	Chloroform (Slightly), DMSO (Slightly), Methanol (Slightly)
pKa (Strongest Basic)	9.86
Log P	2.8
Melting Point	190-196°C
Pharmacodynamics	
Mechanism of Action	Linagliptin is a competitive, reversible DPP-4 inhibitor. Inhibition of this enzyme slows the breakdown of GLP-1 and glucose-dependant insulinotropic polypeptide (GIP) 3, 5. GLP-1 and GIP stimulate the release of insulin from beta cells in the pancreas while inhibiting release of glucagon from pancreatic beta cells5. These effects together reduce the breakdown of glycogen in the liver and increase insulin release in response to glucose
Therapeutic indication	Type II diabetes
Adverse effects	Hypoglycemia very rarely, skin redness
Pharmacokinetics	
Bioavailability	Oral, bioavailability of linagliptin is 30%
Protein Binding	Linagliptin is 99% protein bound at a concentration of 1nmol/L
Elimination Half-life	155 hours
Excretion	Via feces

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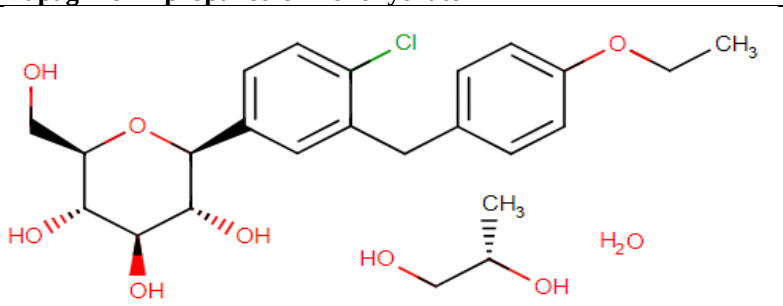
2.2 DRUG PROFILE OF DAPAGLIFLOZIN PROPANEDIOL MONOHYDRATE [18]

Table 5: Drug profile of dapagliflozin propanediol monohydrate

Name	**Dapagliflozin propanediol monohydrate**
Chemical Structure	
Molecular Formula	$C_{24}H_{35}ClO_9$
Molecular Weight	502.99 g/mol
IUPAC Names	(2S,3R,4R,5S,6R)-2-[4-chloro-3-[(4-ethoxyphenyl)methyl]phenyl]-6-(hydroxymethyl)oxane-3,4,5-triol;(2S)-propane-1,2-diol;hydrate
CAS No.	668270-12-0
Appearance	White to off white powder
Solubility	Chloroform (Slightly), DMSO (Slightly), Methanol (Slightly)
pKa (Strongest Acidic)	9.86
pKa (Strongest Basic)	-3
Log P	2.52
Melting Point	74 - 78°°C
Pharmacodynamics	
Mechanism of Action	Dapagliflozin inhibits the sodium-glucose contransporter 2(SGLT2) which is primarily located in the proximal tubule of the nephron1. SGLT2 facilitates 90% of glucose reabsorption in the kidneys and so its inhibition allows for glucose to be excreted in the urine. This excretion allows for better glycemic control and potentially weight loss in patients with type 2 diabetes mellitus
Therapeutic indication	Type II diabetes
Adverse effects	Feeling or being sick, feeing very thirsty, feeling confused or unusually tired, stomach pain.
Pharmacokinetics	
Bioavailability	Oral dapagliflozin is 78% bioavailable
Protein Binding	91%
Elimination Half-life	Mean plasma terminal half-life is approximately 12.9 hours
Excretion	75.2% of dapagliflozin is recovered in the urine with 1.6% of the dose unchanged by metabolism. 21% of the dose is excreted in the feces with 15% of the dose unchanged by metabolism

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CHAPTER 3: LITERATURE REVIEW [19-43]

Official Methods

Neither Dapagliflozin nor Linagliptin monograph is given in IP, BP, USP, JP, or EP.

Published Methods

Table 6: Published methods for Linagliptin

Sr. No.	Title	Author name, publication	Chromatographic Parameters	References
1.	A Validated RP-HPLC Method for the Determination of Linagliptin	Badugu, L. R. American Journal of Pharmaceutical Technology Research, 2012	Mobile Phase: Methanol: Water (83:17, v/v) Column: Gemini C-18 (250 x 4.6mm, 5µm) Flow Rate: 1.0 ml/min Wavelength: 241 nm	19
2.	A novel RP - HPLC method for the quantification of linagliptin in formulations	Lakshmi, B., & Reddy, T. V Journal of Atoms and Molecules; 2012,	Mobile Phase: Acetonitrile: Water: Methanol (25:50:25, v/v/v). Column: Symmetry Chromosil C18 (250x4.6mm, 5µm in particle size). Flow Rate: 1.0 ml/min. Wavelength: 238 nm.	20
3.	Development and Validation of a RP-HPLC Method for Quantitative Analysis of Linagliptin in Bulk and Dosage Forms	Rajbangshi, J. C., Alam, M. M., Dhaka University Journal of Pharmaceutical Sciences, 2018,	Mobile Phase: A mobile phase consisting of a 70:30 v/v mixture of phosphate buffer (pH 6.8±0.2) and acetonitrile was used. Column: A C18 column with dimensions of 150 x 4.6 mm i.d. and a particle size of 5µm was employed. Flow Rate: The flow rate was maintained at 1 ml/min. Wavelength: Detection was performed at 239 nm	21
4.	A Validated HPLC Method for the Determination of Linagliptin in Rat Plasma. Application to a Pharmacokinetic Study	Hanafy, A., & Mahgoub, H. Journal of Chromatographic Science, 2016	Mobile Phase: 75% methanol: 25% formic acid 0.1%, pH 4.1. Column: Zorbax Eclipse XDB C18. Flow Rate: 1.0 mL/min. Wavelength: UV detection at 254 nm.	22
5.	Stability-Indicating HPLC-DAD Method for the Determination of Linagliptin in Tablet Dosage Form: Application to Degradation Kinetics	Mourad, S. S., El-Kimary, E. I., Hamdy, D. A., & Barary, M. A Journal of Chromatographic Science, 2016	Mobile Phase: Methanol: water containing 0.3% TEA (40:60, pH 4.5). Column: zorbax Eclipse XDB-C18 (4.6 × 150 mm, 5 µm) column Flow Rate: 1 mL/min. Wavelength: Detection at 225 nm.	23

Table 7: Published methods for Linagliptin in combination with other drugs

Sr. No.	Title	Author name, publication	Chromatographic Parameters	References
1.	Development and Validation of RP-HPLC Method for the Simultaneous Estimation of Linagliptin, Empagliflozin and Metformin in Solid Dosage Forms	T. Naga Ravi Kiran, P. Parvathi, J.N. Suresh Kumar. Asian J. Pharm. Ana. 2020	Mobile Phase: Buffer: Acetonitrile (45:55, v/v). Column: Kromosil, 250 x 4.6 mm, 5µm. Flow Rate: The flow rate is not explicitly mentioned in the provided paragraph. Wavelength: Detection at 233 nm using a PDA detector.	24
2.	Quality-by-Design Approach for Chromatographic Analysis of Metformin, Empagliflozin and Linagliptin	Gurrala, S., Raj, S., Cvs, S., & Anumolu, P. D. Journal of Chromatographic Science, 2022	Mobile Phase: Acetonitrile: phosphate buffer pH 5 (38:62% v/v). Column: Std. Discovery C18 column at 30°C. Flow Rate: 1 mL/min by isocratic elution. Wavelength: UV detection at 222 nm.	25
3.	Development and Validation of Stability Indicating HPTLC Method for Simultaneous Determination of Linagliptin and Metformin	Srivani, J., Umamahesh, B., & Veeresham, C. International Journal of Pharmacy and Pharmaceutical Sciences, 2016	HPTLC Plates: Silica gel 60 F254 HPTLC plates. Mobile Phase: Acetone: methanol: chloroform: formic acid (3:1:5:1, v/v). Detection Wavelength: Densitometric quantification at 230 nm.	26
4.	QbD Stressed Development and Validation of Stability-Indicating RP-HPLC Method for the Simultaneous Estimation of Linagliptin and Metformin HCl in Pharmaceutical Dosage Form	Khushbu, P., Ujashkumar, A. S., Hirak, V. J., Jayvadan, K. P., Research Journal of Pharmacy and Technology, 2022	Mobile Phase: Acetonitrile and methanol (75:25 % v/v). Column: Water C18 column (250mm × 4.5mm × 5µm). Flow Rate: 1.0 ml/min. Wavelength: Detection at 245 nm.	27
5.	Optimization of a single HPLC-PDA method for quantifying Metformin, Gliclazide, Pioglitazone, Dapagliflozin, Empagliflozin, Saxagliptin, Linagliptin and Teneligliptin using central composite design	Kant, R., Bodla, R. B., Kapoor, G., Bhutani, R. " Bioorganic Chemistry, 2019	Mobile Phase: Not mentioned in the provided information. Column: Waters Reliant™ HPLC Columns (250 mm × 4.6 mm i.d., 5 µm particle size). Flow Rate: Not mentioned in the provided information. Wavelength: Detection at 230 nm.	28
6	Development of RP-HPLC, Stability Indicating Method for Degradation Products of Linagliptin in Presence of Metformin HCl by Applying 2 Level Factorial Design; and Identification of Impurity-VII, VIII and IX and Synthesis of Impurity-VII	Jadhav, S. B., Reddy, P. S., Narayanan, K. L., Sci. Pharm., 2017	Mobile Phase: KH2PO4 buffer solution (0.02M) pH 3.0 adjusted with OPA used as mobile phase-A, and mobile phase-B, ACN:MeOH (90:10, v/v) Column: Zorbax SB-Aq, 250 × 4.6 mm, 5 µm. Flow Rate: 1.0 ml/min Wavelength: Detector wavelength of 225 nm.	29

7	A Validated Method for The Simultaneous Estimation of Linagliptin and Metformin in Tablet Dosage Forms by RP-HPLC	Sivagami, B., Purushotham, A., Sikdar, P., Chandrasekar, R., Research Journal of Pharmacy and Technology, 2020	Mobile Phase: Methanol, Acetonitrile, and Water in the ratio of 40:40:20. Column: Inertsil ODS C18, 250 x 4.6 mm, 5 µm. Flow Rate: 1 ml/min. Wavelength: Optimized wavelength for Metformin and Linagliptin was 258 nm.	30
8	Validated HPLC-UV method for simultaneous estimation of linagliptin and empagliflozin in human plasma	Donepudi, S., & Achanta, S. International Journal of Applied Pharmaceutics, 2018	Column: Discovery C18 (250×4.6×5) Mobile Phase: Buffer: Acetonitrile (68:32) Flow Rate: 1 ml/min Wavelength for Detection: 218 nm	31

Table 8: Published methods of Dapagliflozin

Sr. No.	Title	Author name, publication	Chromatographic Parameters	References
1.	A New RP-HPLC Method Development and Validation of Dapagliflozin in Bulk and Tablet Dosage Form	Debata, J., Kumar, S., Jha, S. K., International Journal of Drug Development and Research, 2017	Mobile Phase: Phosphate buffer and acetonitrile in the ratio of 60:40 v/v. Column: Waters C18, 5 µm particle size, 25 cm × 4.6 mm i.d. Flow Rate: 1.0 ml/min. Wavelength for UV Detection: 237 nm.	32
2.	Development and Validation of stability-Indicating RP-HPLC method for determination of Dapagliflozin	Sanagapati M., Dhanalakshmi K., Reddy N. Sreenivasa S, Journal of Advanced Pharmacy Education & Research, 2014	Column: BDS column Mobile Phase: Mixture of acetonitrile and ortho phosphoric acid (55:45) Flow Rate: 1 ml/min Detection Wavelength: 245 nm	33
3	RP-HPLC Method for Estimation of Dapagliflozin from its Tablet	Mante, G. V., Hemke, A. T., & Umekar, M. J International Journal of ChemTech Research, 2018	Mobile Phase: A mixture of Acetonitrile: 0.1% Triethylamine (pH-5.0) in the ratio of 50:50v/v. Column: Princeton C18 column. Flow Rate: 1 mL/min. Detection Wavelength: 224 nm.	34
4	Stability-Indicating RP-HPLC Method Development for Simultaneous Determination and Estimation of Dapagliflozin in Raw and Tablet Formulation	Manoharan, G., Ismaiel, A. M., & Ahmed, Z. M. Chemistry Research Journal, 2018,	Mobile Phase: Methanol: Water (75:25 v/v) Column: C(18) column reversed-phase Flow Rate: 1 mL/minute Wavelength: Absorption maxima observed at 230 nm	35

Table 9: Published methods of Dapagliflozin with other drug combinations

Sr. No.	Title	Author name, publication	Chromatographic Parameters	References
1	Development and validation of RP-HPLC method for simultaneous estimation of dapagliflozin and metformin in bulk and in synthetic mixture	Urooj, A., Sundar, P. S., Vasanthi, R., World Journal of Pharmacy and Pharmaceutical Sciences 2015	Mobile Phase: Acetonitrile: Water (75:25% v/v) Column: Phenomenex Luna C18 (4.6mm I.D. × 250mm, 5µm) Flow Rate: 1 ml/min Wavelength: 285nm	36
2	Stability indicating HPLC method for the simultaneous determination of dapagliflozin and saxagliptin in bulk and tablet dosage form	Deepan, T., & Dhanaraju, M. D. Current Issues in Pharmacy and Medical Sciences, 2018	Mobile Phase: Acetonitrile: Water (60:40) Column: Xterra RP18 (4.6×150 mm, 5 µm particle size) Flow Rate: 1 mL/min Wavelength: 248 nm	37
3	Development and validation of a stability-indicating RP-HPLC method for simultaneous determination of dapagliflozin and saxagliptin in fixed-dose combination	Singh, N., Bansal, P., Maithani, M., & Chauhan, Y. New Journal of Chemistry, 2018	Mobile Phase: Buffer and acetonitrile (53 : 47 v/v) Column: Xterra C-18 analytical column (150 mm × 4.6 mm i.d., particle size 3.5 µ) Flow Rate: 1.2 mL min−1 Wavelength: 230 nm	38
4	Validated RP-HPLC method for simultaneous determination of canagliflozin, dapagliflozin, empagliflozin and metformin.	Khalil, G. A., Salama, I., Gomaa, M. S., & Helal, M. A. International Journal of Pharmaceutical, Chemical & Biological Sciences, 2018,	Mobile Phase: Acetonitrile and 0.05 M potassium dihydrogen phosphate buffer (pH 4) in a ratio [65:35, v/v] Column: C18 column (250×4.6 mm, 5µm particle size) Inertsil® ODS Flow Rate: 1 mL/min Wavelength: 212 nm	39
5	Development of a new stability indicating RP-HPLC method for simultaneous estimation of saxagliptine and dapagliflozin and its validation as per ICH guidelines	Kommineni, V., Chowdary, K. P. R., & Prasad, S. V. U. M. IAJPS, 2017,	Mobile Phase: Phosphate buffer (pH 4) and Acetonitrile (50:50 v/v) Column: XTerra C18 column (150mm x 4.6mm x 5µm particle size) Flow Rate: 1 ml/min Wavelength: UV detection at 225nm	40
6	RP- HPLC Method for Simultaneous Estimation of Dapagliflozin and Saxagliptin in Bulk Samples	Rao, B. Rama, Rao, V. Venkata, & Venkateswarlu, B. S. Journal of Pharmaceutical Sciences and Research, 2019	Mobile Phase: 10mM phosphate buffer (pH 6.8): Acetonitrile; 40:60 (v/v) Column: Phenomenex Luna C18 (25 cm x 4.60 mm, particle size 5 µm) Flow Rate: Not specified Wavelength: UV detection at 260 nm	41
7	Application of quality by design approach in RP-HPLC method development for simultaneous estimation of saxagliptin and dapagliflozin in tablet dosage form	Braz. J. Pharm. Sci. Braz. J. Pharm. Sci., 2019	Mobile Phase Composition: Acetonitrile and ortho phosphoric acid (0.1%) in the ratio of 50:50 Flow Rate: 0.98 ml/min Column Temperature: 31.4 °C Wavelength: 210 nm	42

8	Development of Validated Stability Indicating Assay Method for Simultaneous Estimation of Metformin and Dapagliflozin by RP- HPLC	Deepan, T., Rao, M. V. B., & Dhanaraju, M. D European Journal of Applied Sciences, 2017	Mobile phase: Acetonitrile and 0.1M orthophosphoric acid buffer (70:30, v/v) Column: Inspire (4.6 x 150mm, 5µm) micro column Flow rate: 1.0 mL/min Detection wavelength: 260 nm	43

CHAPTER 4: PATENT SEARCH AND ANALYSIS REPORT (PSAR) [44-49]

Table 10: Patent Search and Analysis Report

Sr. No.	Patent Application No.	Title of Patent	Reference No.
1.	EP2853257A1	Pharmaceutical formulations of linagliptin	44
2.	WO2013098775A1	Improved process for preparation of pure linagliptin	45
3.	US20120107398A1	Pharmaceutical compositions	46
4.	WO2015128853A1	Dapagliflozin compositions	47
5.	US9845303B2	Process for the preparation of dapagliflozin	48
6.	201013321103	Methods for treating extreme insulin resistance in patients resistant to previous treatment with other anti-diabetic drugs employing an SGLT2 inhibitor and compositions thereof	49

CHAPTER 5: RATIONALE OF THE DRUG

Recently, Central Drugs Standard Control Organization (CDSCO) has granted an approval to fixed dose combination of Linagliptin 5 mg + Dapagliflozin propanediol monohydrate 10 mg on November 4, 2022.

Our research focus is to develop analytical methods for drugs that are currently in demand or under investigation for specific therapeutic purposes. Linagliptin and dapagliflozin has been the subject of research and development due to their clinical relevance.

Linagliptin, a DPP-4 inhibitor, and dapagliflozin, an SGLT2 inhibitor, can be used together to lower blood sugar levels because they each have different mechanisms of action.

This combination medication may improve glucose management, lower the risk of hypoglycemia, and give further cardiovascular advantages. [49]

Combination of two or more active pharmaceutical compound in a single dosage form is known as fixed dose combinations (FDCs), have gained more attention in pharmaceutical research and development due to several advantages over single compounds taken individually, including improved therapeutic impact, enhanced pharmacokinetics, safety, and patient compliance. In the Indian pharmaceutical industry, FDCs have witnessed substantial growth, and their popularity continues to rise.

A comprehensive literature review revealed there are numerous analytical methods, including spectroscopic, HPLC, fluoroscopic, LC-MS, for the determination of linagliptin and dapagliflozin either individually or in combination. However, to the best of our knowledge and critical observation, it was found that there is a gap in the available analytical methods because there is no stability-indicating method available for the simultaneous determination of both Linagliptin and Dapagliflozin in a combined dosage form.

Hence, it is imperative to develop a robust and comprehensive RP-HPLC method that can be used for simultaneous assay of linagliptin and dapagliflozin in a combined dosage form.

CHAPTER 6: AIM AND OBJECTIVES

AIM:

- To establish an RP-HPLC method capable of separating and quantifying linagliptin and dapagliflozin simultaneously in the tablet formulation.
- To assess the stability-indicating properties of the method by performing forced degradation studies.
- To validate the developed method to demonstrate its accuracy, precision, specificity, linearity, and robustness.

RESEARCH OBJECTIVE:

- The primary objective of this research project is to develop a simple, reliable, sensitive, rapid, economic, and stability-indicating reversed-phase high-performance liquid chromatography (RP-HPLC) method for the simultaneous estimation of linagliptin and dapagliflozin in a tablet dosage form.
- This method will serve as a crucial tool for quality control, ensuring the safety and efficacy of the FDC, and supporting regulatory compliance.

CHAPTER 7: EXPERIMENTAL WORK

7.1 Instrumentation

Table 11: List of Instrumentation

Name	Brand	Software
HPLC	Shimadzu LC 2010 CHT	LC Solution
FT-IR	Agilent Cary 630	Agilent MicroLab
UV-Visible Spectrophotometer	Shimadzu 1800	UV Probe
Sonicator	Athena Technology	NA
Analytical Balance	Athena Technology	NA
Micropipette	Eppendorf	NA

7.2 Standard/Sample Procurement

- Linagliptin and dapagliflozin propanediol monohydrate APIs were received as a gift sample from Torrent Pharmaceuticals.
- Dapalo-L was purchased from local medical store.

7.3 Chemicals and Reagents

Table 12: List of reagents and chemicals

Sr. No.	Name of Chemicals	Make	Grade
1	Water	MiliQ	HPLC Grade
2	Methanol	Rankem	HPLC Grade
3	Acetonitrile	Rankem	HPLC Grade
4	Potassium Dihydrogen Phosphate	Rankem	AR Grade
5	Orthophosphoric acid	Rankem	AR Grade

7.4 Identification Tests for API

7.4.1 Melting point determination

To determine the melting point of APIs, a small quantity of the API was placed in a clean and dry capillary tube using a spatula. The capillary tube was subsequently immersed in a heated paraffin bath. The temperature of the paraffin bath was gradually elevated while monitored with a thermometer. Once the compound within the capillary tube began to liquefy till the conversion into complete liquid, the temperature range was recorded. The results were compared with reference range.

7.4.2 FT-IR

For FT-IR spectroscopy analysis, APIs were positioned on the sample holder of the FT-IR instrument. After switching on the FT-IR spectrometer, a warming-up period of at least 20 minutes was allowed. Subsequently, the API samples were subjected to scanning within the spectral range of 4000-400 cm^{-1}. The resulting spectrum was then compared to a reference spectrum to identify any disparities. Specific peaks and their corresponding wavenumbers for linagliptin as well as dapagliflozin propanediol monohydrate were documented.

7.5 Assay method development by RP-HPLC

7.5.1 Solubility study for solvent selection:

To identify the suitable solvent, the solubility of 25 mg of linagliptin and 25 mg of dapagliflozin propanediol monohydrate APIs were assessed. Individual API was placed in a small test tube, and the solvent was gradually added in 1 mL increments. The test tube was vigorously shaken until saturation was reached. Solubility was determined based on the amount of solute dissolved per unit volume of the solvent.

7.5.2 UV-spectrophotometry for wavelength selection:

Solution preparation (5 µg/ml linagliptin and 10 µg/ml dapagliflozin): Precisely measuring 5 mg of linagliptin and 10 mg of dapagliflozin propanediol monohydrate, it was transferred to a 100 ml volumetric flask and dissolved in 20 ml of methanol through sonication. The volume was then adjusted up to the mark with methanol. Subsequently, 1 ml of this solution was taken into a 10 ml volumetric flask and diluted to the mark with methanol.

Procedure: The above-mentioned solutions were subjected to UV scanning in the range of 200–400 nm individually. Methanol was used as a blank. Linagliptin and dapagliflozin spectrum were overlain on each other (Figure 4) for determination of absorbance maxima and isosbestic point.

7.5.3 Solution preparation

- **Phosphate Buffer**: A 20 mM potassium dihydrogen phosphate buffer was made by dissolving 2.72 g of potassium dihydrogen phosphate in 1000 ml of MilliQ water. The pH was adjusted to 3.5 using orthophosphoric acid.

- **Mobile Phase**: Mobile phase involved gradient program of buffer as mobile phase A and acetonitrile as mobile phase B, filtered through a 0.45-micron filter under vacuum.

- **Diluent**: Buffer : Acetonitrile in 50:50% ratio.

- **Linagliptin Stock Solution** (50 µg/ml Linagliptin): 5 mg of Linagliptin API was accurately weighed and transferred into a 100 ml volumetric flask containing approximately 50 ml of diluent. After thorough mixing and sonication for dissolution, the volume was adjusted to the mark with diluent. The stock solution was refrigerated for a week, and subsequent dilutions were made from this stock solution.

- **Dapagliflozin Stock Solution** (100 µg/ml Linagliptin): 12.4 mg of dapagliflozin propanediol monohydrate API (equivalent to 10 mg of dapagliflozin, correction factor 0.81) was accurately weighed and transferred into a 100 ml volumetric flask containing approximately 50 ml of diluent. After thorough mixing and sonication for dissolution, the volume was adjusted to the mark with diluent. The stock solution was refrigerated for a week, and subsequent dilutions were made from this stock solution.

- **Standard Solution** (10 µg/ml dapagliflozin and 5 µg/ml linagliptin): 2 mm of linagliptin stock solution and 2 mm of dapagliflozin stock solution were transferred into a 20 ml volumetric flask, and the volume was adjusted to the mark with diluent.

- **Sample Preparation for Quantitation** (10 µg/ml dapagliflozin and 5 µg/ml linagliptin): The weight of five tablets of Daplo-L was measured to determine the average weight per tablet. The weight equivalent to one tablet was then transferred into a 100 mL volumetric flask, to which 20 mL of diluent was added. The mixture underwent sonication for 20 minutes.

Subsequently, the volume was adjusted with diluent and filtered through 0.45 micron syringe filter. From the filtered solution, 2 ml was withdrawn using a pipette and placed into a 20 ml volumetric flask. The volume was then adjusted with diluent.

7.5.4 Method development and optimization

An HPLC method was developed to quantify dapagliflozin and linagliptin in pharmaceutical dosage form. The optimization steps and parameters employed to achieve distinct separation between the two drug substances, which are outlined below:

- **Stationary Phase Selection:**

Considering the polar nature of linagliptin and less polar nature of the dapagliflozin, the C18 stationary phase with column length of 150 mm, internal diameter of 4.6 mm and particle size of 5 µ exhibited adequate separation and peak shapes.

- **Effect of Buffer Concentration and pH:**

Various phosphate buffers and concentrations (10 – 50 mM) and pH ranges (3.5 – 5.0) were explored. Ultimately, 20 mM potassium dihydrogen phosphate (KH_2PO_4) solution adjusted to a pH of 3.5 ± 0.05 with diluted orthophosphoric acid was selected. Higher pH levels affected the peak shape of dapagliflozin.

- **Effect of Organic Modifier:**

Initial use of methanol as the organic modifier resulted in poor peak shape of dapagliflozin. Acetonitrile, when directly used as mobile phase B led to improved peak shape. Therefore acetonitrile was used as a organic modifier in mobile phase.

- **Elution mode selection**

There is a significant difference in polarities of linagliptin and dapagliflozin. Therefore it was difficult to achieve separation by isocratic elution.

Various attempts were made by using different buffer and organic modifier combination with various flow rates and temperature, but failed to resolve both the peaks in a shorter time. Therefore, to make the assay method short, gradient elution system was employed.

7.6 Forced Degradation Studies:

The optimized method needs to be stability-indicating, hence forced degradation studies were conducted.

The objective was to demonstrate the method's capability to accurately measure the APIs even in the presence of expected degradants resulting from various degradation conditions applied to the sample.

In the forced degradation analysis, aliquots of the stock solutions were individually subjected to different conditions: 1 ml of 0.1 N HCl for acid stability, 1 ml of 0.1 N NaOH for alkaline stability, 1 ml of 0.3% H_2O_2 for oxidative degradation, and exposure of the sample drug solution in a hot air oven at 70°C for 24 hours for thermal degradation. The stability of these treated samples was compared with a fresh sample analyzed on the same day.

The comprehensive degradation conditions for each analysis are outlined as follows:

- **Oxidation:**

2 ml stock solutions of linagliptin and dapagliflozin + 1 ml of 0.3% hydrogen peroxide (H_2O_2) were taken into 20 ml volumetric flasks. The solution was kept for 4 hours at room temperature and finally made up to volume with diluent. For HPLC study, the resultant solution was diluted and injected into the system and the chromatograms were recorded to assess the stability of sample.

- **Acid Degradation Studies:**

2 ml stock solutions of linagliptin and dapagliflozin + 1 ml of 0.1 N hydrochloric acid were taken into 20 ml volumetric flasks. The solution was kept for 4 hours at room temperature and then neutralized with equal amount of 0.1 N sodium hydroxide. Finally made up to volume with diluent. For HPLC study, the resultant solution was diluted and injected into the system and the chromatograms were recorded to assess the stability of sample.

- **Alkali Degradation Studies:**

2 ml stock solutions of linagliptin and dapagliflozin + 1 ml of 0.1 N sodium hydroxide were taken into 20 ml volumetric flasks. The solution was kept for 4 hours at room temperature and then neutralized with equal amount of 0.1 N hydrochloric acid. Finally made up to volume with diluent. For HPLC study, the resultant solution was diluted and injected into the system and the chromatograms were recorded to assess the stability of sample.

- **Thermal-induced degradation:**

Linagliptin and dapagliflozin samples was kept in Hot air oven at 70 °C for 24 hours. Then the sample solutions were prepared as per the proposed method and injected into HPLC and analyzed.

- **Photolytic degradation**

The samples were kept in photo chamber at 350 nm, for 24 hours. Solutions were made as per the method parameters and then injected into the system and analyzed.

7.7 Analytical Method Validation:

We conduced comprehensive validation of the method in accordance with the guidelines set forth by the International Conference on Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH). The validation process covered various critical aspects, including Linearity, Precision, Specificity, Accuracy, Limit of Detection, Limit of Quantification, and Robustness as outlined in guidelines Q2R1.

System suitability:

Before starting with the validation, system suitability study was undertaken to ensure that the HPLC system or proposed analytical method are suitable for the intended analysis. System suitability was assessed by conducting five consecutive injections from freshly prepared standard solutions.

Specificity:

The method's specificity was assessed by injecting a blank solution (absent of any sample) followed by a 30 µl injection of drug solution, 30 µl injection of sample solution, 30 µl individual linagliptin and dapagliflozin standard solutions. This procedure aimed to demonstrate the effective separation of the two analyte peaks, linagliptin and dapagliflozin, from any other peaks or baseline noise.

Linearity

Aliquots of 1, 1.5, 2, 2.5, and 3 mL were withdrawn from a stock solution containing 50 µg/ml of linagliptin and 100 µg/ml of dapagliflozin.

These aliquots were transferred into 20 ml volumetric flasks and then diluted up to the mark with the diluent. Consequently, the final concentrations fell within the range of 2.5 to 7.5 µg/ml of linagliptin and 5 to 15 µg/ml of dapagliflozin. Subsequently, 30µl volumes of each prepared solutions were injected and a calibration curve was prepared in Microsoft Excel by plotting the peak area against the drug concentration.

Table 13: Linearity solution preparation

Concentration level	Stock taken in mL	Dilution performed	Concentration of Linagliptin	Concentration of Dapagliflozin
50%	0.50	10.0	2.50	5.00
75%	0.75	10.0	3.75	7.50
100%	1.00	10.0	5.00	10.00
125%	1.25	10.0	6.25	12.50
150%	1.50	10.0	7.50	15.00

Precision

Precision refers to the degree of closeness of data values in repeated measurements under the same analytical conditions. Precision was tested for intraday and interday levels.

Intraday precision:

Six different sample solutions containing linagliptin (5 µg/ml) and dapagliflozin (10 µg/ml) were prepared from Daplo-L tablet of the same batch as per the test method sample preparation. The resultant solutions were then injected and responses were noted. Assay were calculated against standard solutions.

Inter-day Precision:

Interday precision for linagliptin and dapagliflozin was assessed by determining corresponding responses of six sample preparation from Daplo-L tablet on consecutive day, by another analyst and on other instrument. The percent relative standard deviation (%RSD) was calculated from the assay.

Accuracy (Recovery studies):

To assess the accuracy of the proposed developed method, a standard additions approach was used to measure the recovery of the drugs. A consistent quantity of the sample was taken, and standard drug additions were made to make concentrations of 50%, 100%, and 150% levels and three replicates at each level.

Table 14: Recovery solution preparation

%Level	Sample wt. in mg	Wt. of Linagliptin in mg	Wt. of Dapagliflozin in mg	Volume made upto with diluent
50%	100	0.10	0.05	20
	100	0.10	0.05	20
	100	0.10	0.05	20
100%	100	0.20	0.10	20
	100	0.20	0.10	20
	100	0.20	0.10	20
150%	100	0.30	0.15	20
	100	0.30	0.15	20
	100	0.30	0.15	20

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Limit of Detection and Limit of Quantification:

LOD and LOQ were calculated by regression data from calibration curve using standard deviation (σ) of the response and slope of the calibration curve with the help of the following formulas:

$$\text{Limit of detection} = -\frac{\sigma \times 3.3}{S}$$

$$\text{Limit of quantification} = \frac{\sigma \times 10}{S}$$

Where, σ = standard deviation of the response.

S = slope of the calibration curve (of the analyte).

Robustness:

Robustness testing involved systematically varying proposed chromatographic method parameters. Minor adjustments were made to the flow rate (±0.1 ml/min), the wavelength (±2 nm), and column temperature (±5 °C).

CHAPTER 8: RESULTS AND DISCUSSION

8.1 Identification tests for API

8.1.1 Melting point determination

Melting point results are summarized in table – 15.

Table 15: Melting point determination

Compound	Reference Range	Results
Linagliptin	190-196 °C	191-195 °C
Dapagliflozin	74-78 °C	75-76 °C

Results: Melting points were determined and compared with literature reference ranges and were found to be well within the range.

8.1.2 FT-IR

FT-IR spectra were obtained and compared with reference standard spectra. Structural elucidation is also done.

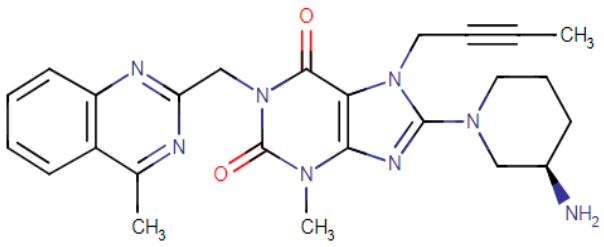

Figure 4: Structure of linagliptin

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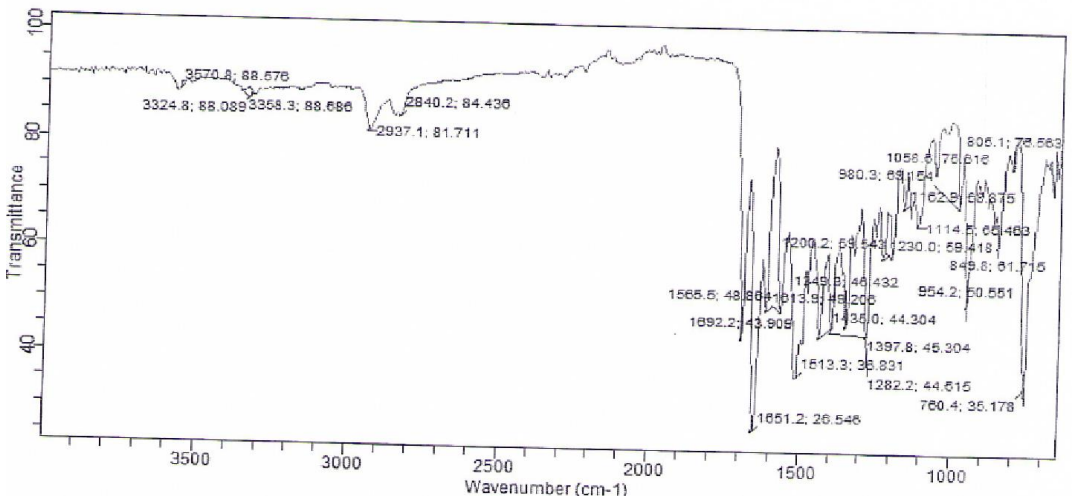

Figure 5: FT-IR spectrum of linagliptin

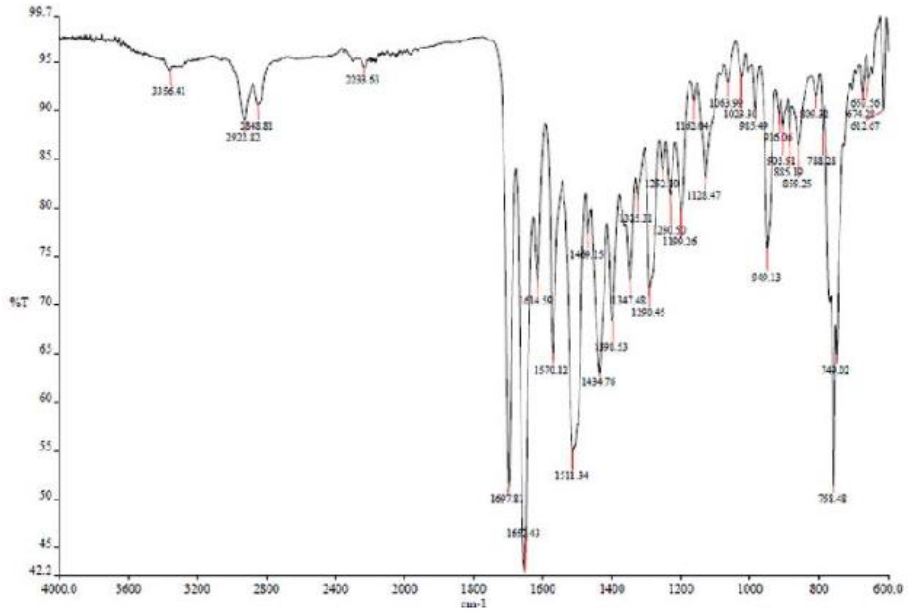

Figure 6: Reference FT-IR spectrum of linagliptin[50]

Table 16: FT-IR interpretation of Linagliptin

Group	Observed Absorption (cm-1)	Reference Absorption (cm-1)
N-H stretching	3324.8	3400-3300
C-H stretching	2840.2	3000-2840
C=O stretching	1692.2	1710-1685
C-N stretching (aromatic amine)	1282.2	1342-1266
C-H bending1,2-disubstituted	760.4	755 ± 20

Dapagliflozin FT-IR

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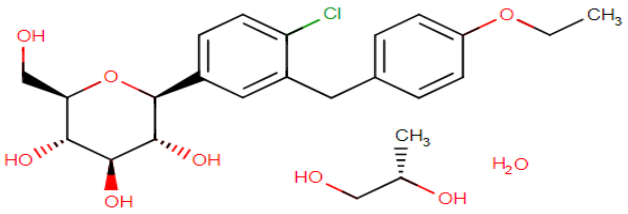


Figure 7: Dapagliflozin Structure

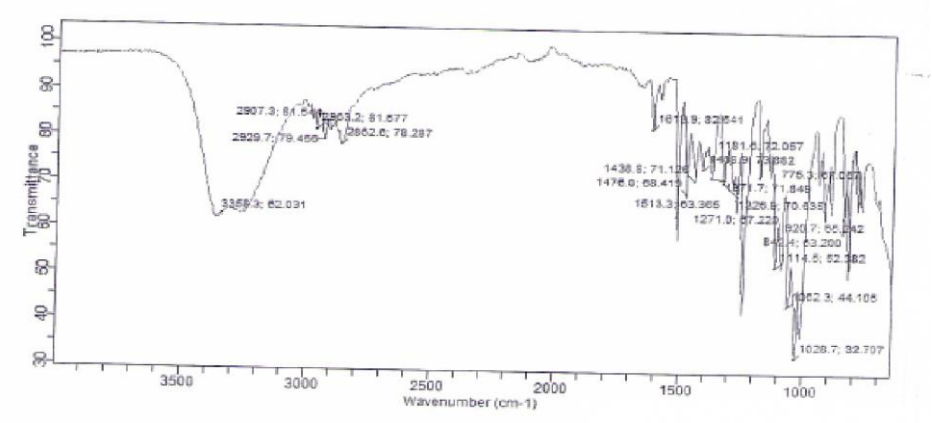


Figure 8: FT-IR spectrum of dapagliflozin

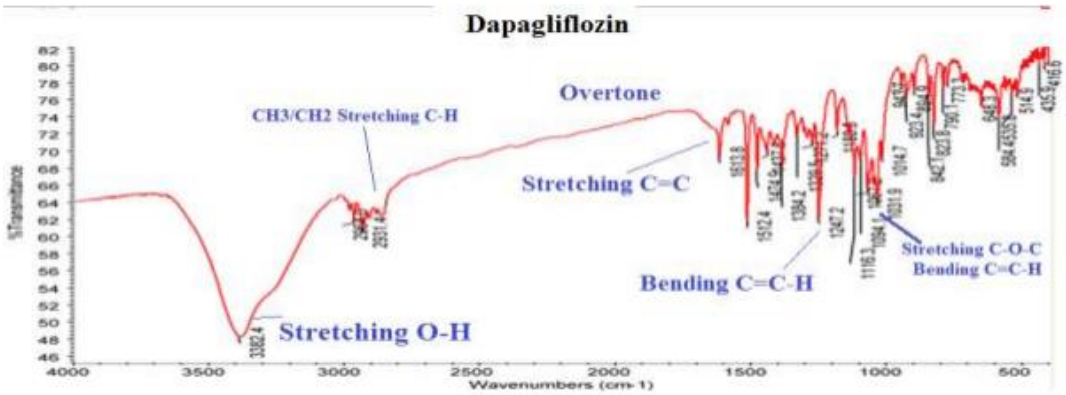


Figure 9: Reference FT-IR spectrum of dapagliflozin[51]

Table 17: FT-IR interpretation of Dapagliflozin

Group	Observed Absorption (cm-1)	Reference Absorption (cm-1)
O-H stretching	3369.3	3550-3200
C-H stretching	2802.2	2830-2695
C-O stretching	1271.02	1310-1250
C-Cl stretching	820.7	850-550

Results: Received API were correct as concluded from above interpretation.

8.2 Assay method development of by RP-HPLC

8.2.1 Solubility study for solvent selection

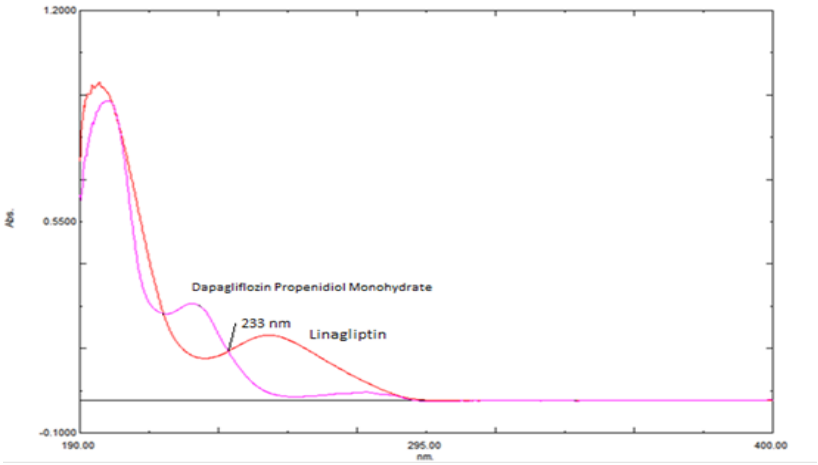
Solubility study results are summarized in table 18.

Table 18: Solubility results

Solvent	Linagliptin	Dapagliflozin
Water	Insoluble	Insoluble
Methanol	Slightly	Slightly
Acetonitrile	Freely soluble	Freely soluble
Phosphate Buffer:Acetonitrile (50:50%)	Freely soluble	Freely soluble

Results: Both linagliptin and dapagliflozin are freely soluble in aqueous buffer and ACN mixture, therefore it can be chosen as a solvent as well as mobile phase also.

8.2.3 UV-spectrophotometry for wavelength selection



Results: The isosbestic point was found when UV spectrum of linagliptin and dapagliflozin were overlain, 210 nm, which can be used for HPLC method development experiment.

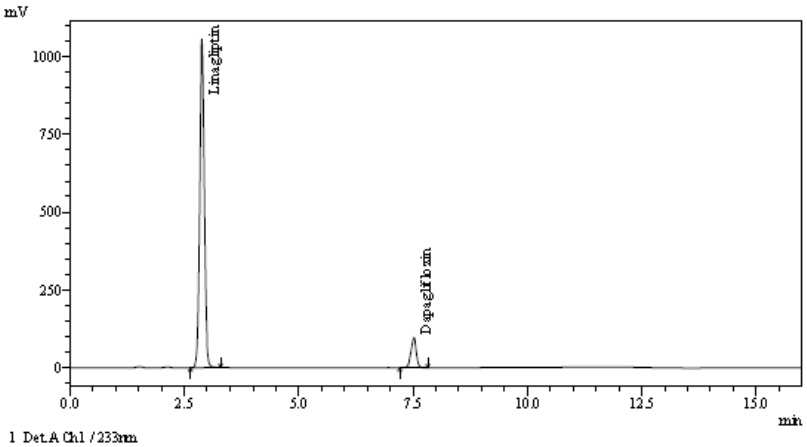
8.2.4 Method development and optimization

Method development trials are demonstrated in table 19

Table 19: Method optimization summary

Sr. No.	Mobile phase	Retention time	Remark
1.	Column: Inertsil ODS-3V, 150 x 4.6 mm, 5µ Mobile Phase: Water:ACN 50:50%v/v	Linagliptin : 8.06 min	Poor peak shape
2.	Column: Inertsil ODS-3V, 150 x 4.6 mm, 5µ Mobile Phase: 20 mM KH_2PO_4, pH 3.5:ACN 50:50%v/v	Linagliptin : 7.74 min	Poor peak shape
3.	Column: Inertsil ODS-3V, 150 x 4.6 mm, 5µ Mobile Phase: 20 mM KH_2PO_4, pH 3.5:ACN 30:70%v/v	Linagliptin : 2.281 min Dapagliflozin; 10.88 min	Linagliptin elutes in dead volume with poor peak shape
4.	Column: Inertsil ODS-3V, 150 x 4.6 mm, 5µ 20 mM KH_2PO_4, pH 3.5:ACN 40:60%v/v	Linagliptin : 3.46 min	Poor peak shape of linagliptin

<table>
<tr><td></td><td></td><td>Dapagliflozin: 13.98 min</td><td></td></tr>
<tr><td>5.</td><td>Column: Inertsil ODS-3V, 150 x 4.6 mm, 5µ
20 mM KH_2PO_4, pH 3.5 : ACN 45:55%v/v</td><td>Linagliptin : 3.09 min
Dapagliflozin: 21.7 min</td><td>Dapagliflozin peak is very broad, unidentifiable from baseline</td></tr>
<tr><td>6.</td><td>Column: Inertsil ODS-3V, 150 x 4.6 mm, 5µ
MPA: Buffer, MPB: ACN
<table>
<tr><th>Time (min)</th><th>% MP A</th><th>% MP B</th></tr>
<tr><td>0</td><td>50</td><td>50</td></tr>
<tr><td>15</td><td>70</td><td>30</td></tr>
<tr><td>16</td><td>50</td><td>50</td></tr>
<tr><td>20</td><td>50</td><td>50</td></tr>
</table></td><td>Linagliptin : 5.45 min
Dapagliflozin: 13.22 min</td><td>Linagliptin peak shape is still poor with less theoretical plates</td></tr>
<tr><td>7.</td><td>Column: Inertsil ODS-3V, 150 x 4.6 mm, 5µ
MPA: Buffer, MPB: ACN
<table>
<tr><th>Time (min)</th><th>% MP A</th><th>% MP B</th></tr>
<tr><td>0</td><td>35</td><td>65</td></tr>
<tr><td>11</td><td>63</td><td>37</td></tr>
<tr><td>12</td><td>35</td><td>65</td></tr>
<tr><td>15</td><td>35</td><td>65</td></tr>
</table></td><td>Linagliptin : 2.87 min
Dapagliflozin: 7.51 min</td><td>Appropriate peak shapes of linagliptin and dapagliflozin. Satisfactory peak resolution, theoretical plates, tailing factor</td></tr>
</table>



PeakTable

Detector A Ch1 233nm

Peak#	Ret. Time	Name	Area	Area %	T. Factor	T.Plate	Resolution
1	2.878	Linagliptin	7451133	90.59	1.09	3450	0.0
2	7.514	Dapagliflozin	773735	9.41	0.94	18184	22.1
Total			8224868	100.00			

Figure 10: Chromatographic presentation of optimized method

Proposed HPLC method for Linagliptin and Dapagliflozin:

Mode of separation: Gradient mode

Flow rate: 1.0 ml/min

Column: Inertsil ODS-3V, 150 x 4.6 mm, 5µ

Mobile Phase: Mobile phase A: pH 3.5, 20 mM potassium dihydrogen phosphate buffer. Mobile phase B: Acetonitrile in gradient program.

Time (min)	% MP A	% MP B
0	35	65
11	63	37
12	35	65
15	35	65

Detector wave length: 233 nm

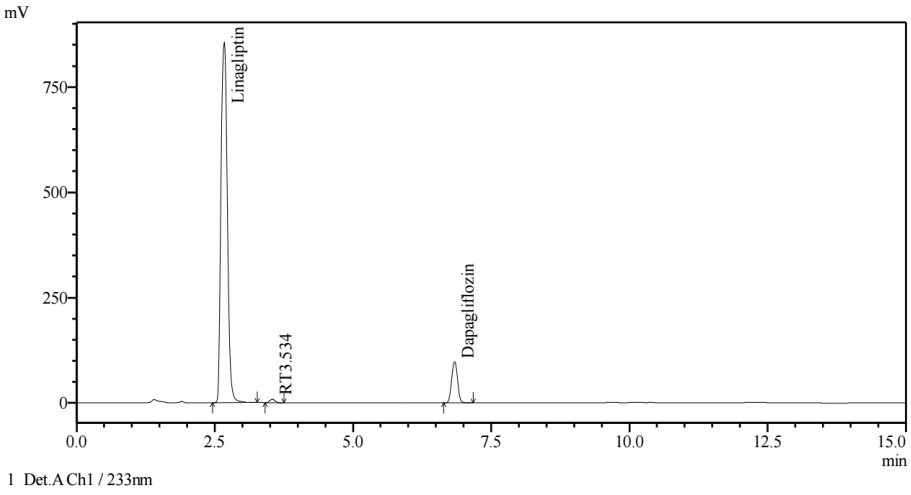
Column temperature: Ambient

Injection volume: 30 µl

Run time: 15 min

8.3 Forced degradation studies:

The HPLC chromatograms of the degraded products exhibited no interference at the respective analyte peaks. Chromatograms are provided in Figures 11 to 21, and detailed results are presented in Table-21.



PeakTable

Detector A Ch1 233nm

Peak#	Ret. Time	Name	Area	Area %	T. Factor	T.Plate	Resolution
1	2.667	Linagliptin	6543321	89.85	1.16	2541	0.0
2	3.534	RT3.534	52593	0.69	1.14	5819	4.4
3	6.836	Dapagliflozin	700720	9.46	1.06	17735	16.9
Total			7296634	100.00			

Figure 11: Chromatogram of acidic degradation of Daplo-L

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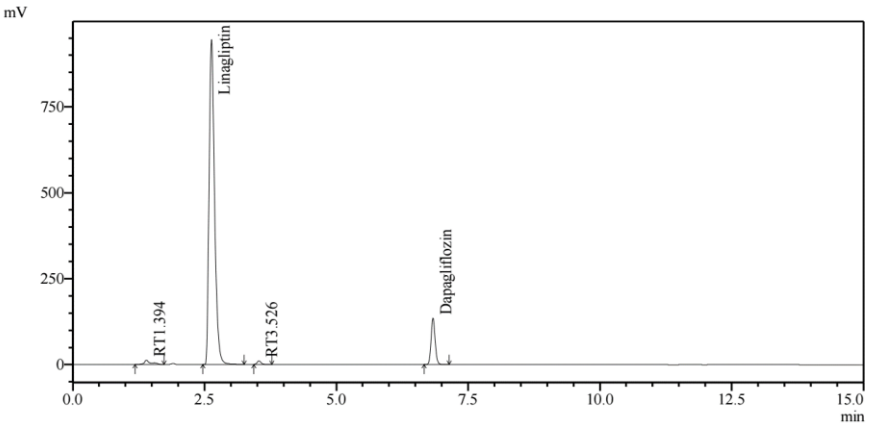
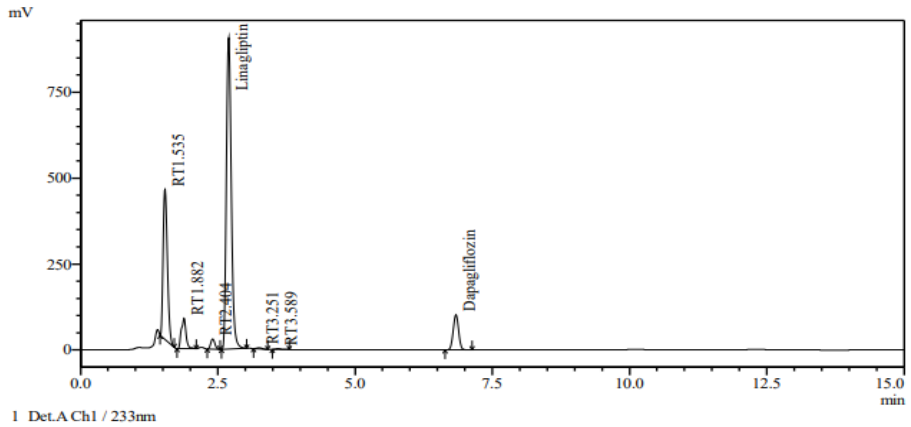


PeakTable

Detector A Ch1 233nm

Peak#	Ret. Time	Name	Area	Area %	T. Factor	T.Plate	Resolution
1	1.394	RT1.394	111555	1.45	1.59	1131	0.0
2	2.628	Linagliptin	6414054	88.58	1.39	2810	6.8
3	3.526	RT3.526	49539	0.64	1.22	9285	5.2
4	6.831	Dapagliflozin	687210	9.32	1.16	31891	22.1
Total			7262358	100.00			

Figure 12: Chromatogram of acidic degradation of API



PeakTable

Detector A Ch1 233nm

Peak#	Ret. Time	Name	Area	Area %	T. Factor	T.Plate	Resolution
1	1.535	RT1.535	2353539	25.01	1.38	1541	0.0
2	1.882	RT1.882	521195	5.54	1.07	1943	2.1
3	2.404	RT2.404	158713	1.69	1.15	3887	3.2
4	2.693	Linagliptin	5649127	60.02	1.28	3828	1.8
5	3.251	RT3.251	19427	0.21	1.03	5385	3.2
6	3.589	RT3.589	13656	0.15	1.01	6908	1.9
7	6.835	Dapagliflozin	696099	7.40	1.05	20314	17.8
Total			9411756	100.00			

Figure 13: Chromatogram of alkali degradation of Daplo-L

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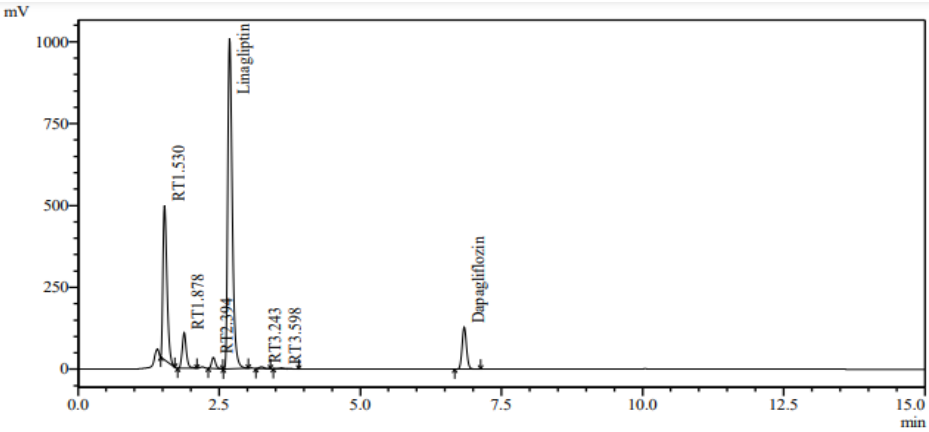
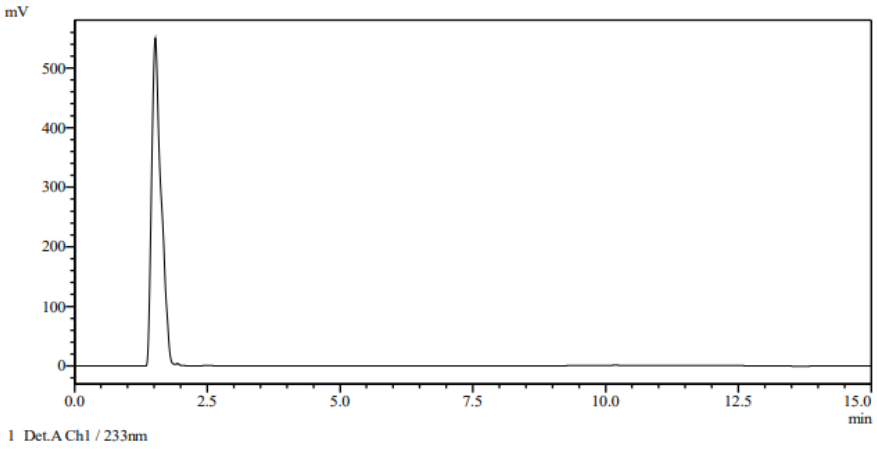


PeakTable

Detector A Ch1 233nm

Peak#	Ret. Time	Name	Area	Area %	T. Factor	T.Plate	Resolution
1	1.530	RT1.530	2322264	24.82	1.60	1792	0.0
2	1.878	RT1.878	544903	5.82	1.20	2759	2.4
3	2.394	RT2.394	156994	1.68	1.26	4844	3.7
4	2.681	Linagliptin	5582709	59.68	1.53	4737	2.0
5	3.243	RT3.243	29919	0.32	1.17	6086	3.5
6	3.598	RT3.598	23294	0.25	1.53	6401	2.0
7	6.839	Dapagliflozin	694897	7.43	1.15	31068	19.3
Total			9354981	100.00			

Figure 14: Chromatogram of alkali degradation of API



PeakTable

Detector A Ch1 233nm

Figure 15: Chromatogram of peroxide blank

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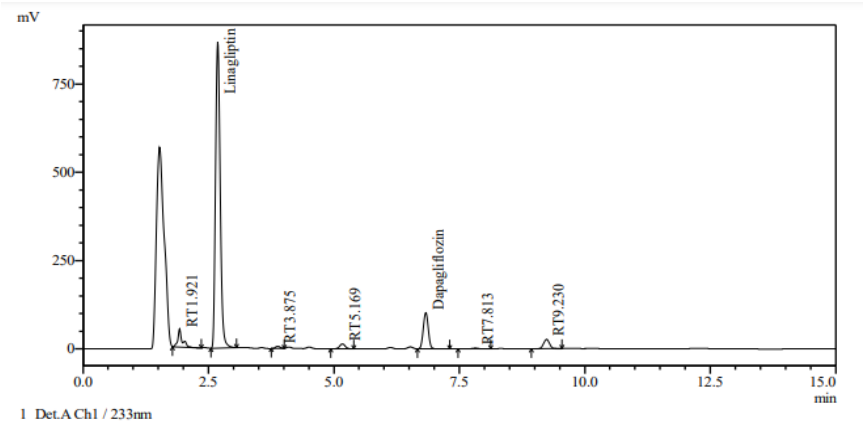
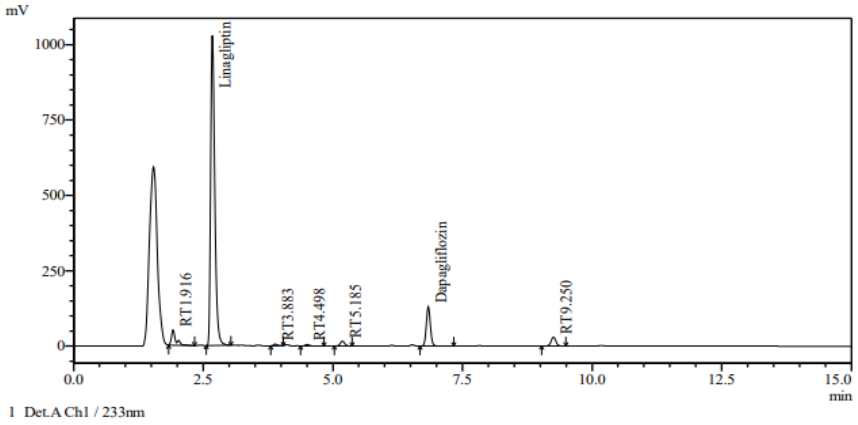


PeakTable

Detector A Ch1 233nm

Peak#	Ret. Time	Name	Area	Area %	T. Factor	T.Plate	Resolution
1	1.921	RT1.921	305841	4.39	1.40	3816	0.0
2	2.681	Linagliptin	5588872	80.16	1.24	3547	5.0
3	3.875	RT3.875	38023	0.55	1.04	7772	6.7
4	5.169	RT5.169	103701	1.49	1.05	9776	6.7
5	6.829	Dapagliflozin	708197	10.16	1.06	19797	8.2
6	7.813	RT7.813	15938	0.23	1.11	20739	4.8
7	9.230	RT9.230	211732	3.04	1.06	27091	6.4
Total			6972302	100.00			

Figure 16: Chromatogram of oxidation degradation of Daplo-L



PeakTable

Detector A Ch1 233nm

Peak#	Ret. Time	Name	Area	Area %	T. Factor	T.Plate	Resolution
1	1.916	RT1.916	293417	4.19	1.81	3234	0.0
2	2.672	Linagliptin	5664047	80.92	1.49	4717	5.2
3	3.883	RT3.883	24901	0.36	1.10	11842	8.1
4	4.498	RT4.498	25605	0.37	1.21	12244	4.0
5	5.185	RT5.185	94678	1.35	1.14	15309	4.2
6	6.838	Dapagliflozin	707552	10.11	1.13	30731	10.2
7	9.250	RT9.250	189656	2.71	1.05	42461	14.4
Total			6999857	100.00			

Figure 17: Chromatogram of oxidation degradation of API

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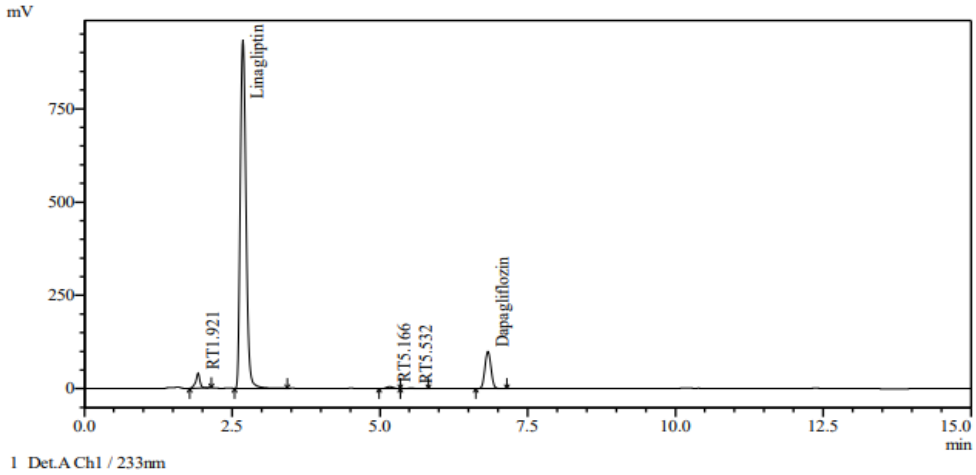
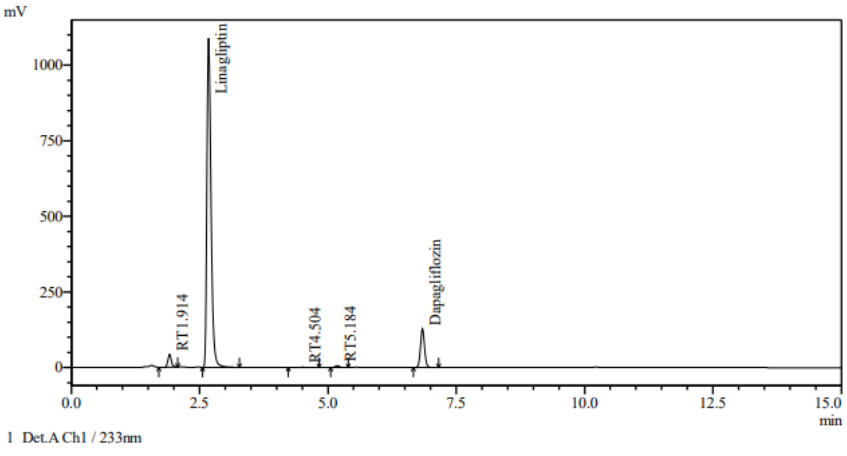


PeakTable

Detector A Ch1 233nm

Peak#	Ret. Time	Name	Area	Area %	T. Factor	T.Plate	Resolution
1	1.921	RT1.921	188202	2.61	0.90	3599	0.0
2	2.679	Linagliptin	6275948	87.00	1.27	3339	4.8
3	5.166	RT5.166	33840	0.47	1.13	10139	12.7
4	5.532	RT5.532	7517	0.10	1.09	11208	1.8
5	6.825	Dapagliflozin	708166	9.82	1.09	18599	6.3
Total			7213674	100.00			

Figure 18: Chromatogram of photolytic degradation of Daplo-L



PeakTable

Detector A Ch1 233nm

Peak#	Ret. Time	Name	Area	Area %	T. Factor	T.Plate	Resolution
1	1.914	RT1.914	199018	2.84	1.19	3014	0.0
2	2.672	Linagliptin	6078307	86.60	1.49	4635	5.1
3	4.504	RT4.504	6418	0.09	1.31	10805	11.1
4	5.184	RT5.184	33769	0.48	1.12	16117	4.0
5	6.839	Dapagliflozin	701060	9.99	1.11	30293	10.3
Total			7018573	100.00			

Figure 19: Chromatogram of photolytic degradation of API

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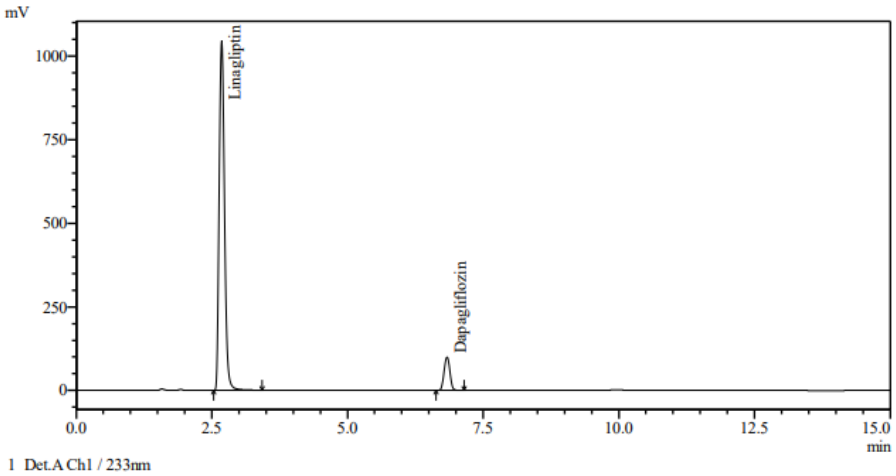
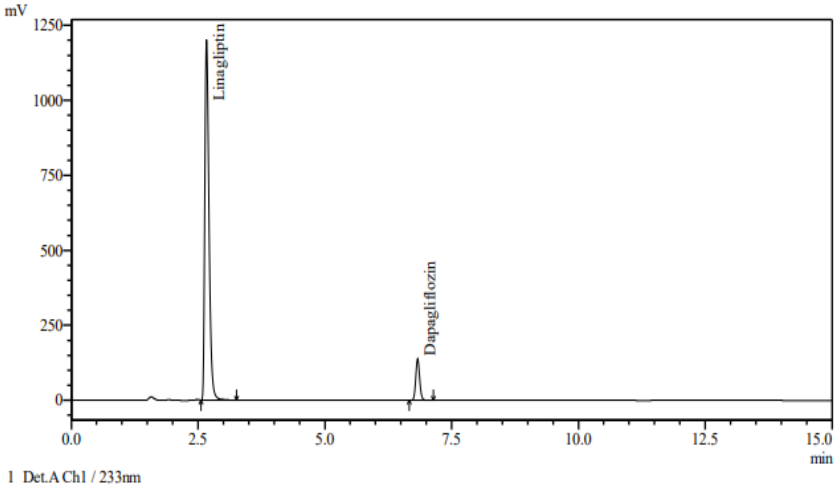


PeakTable

Detector A Ch1 233nm

Peak#	Ret. Time	Name	Area	Area %	T. Factor	T.Plate	Resolution
1	2.677	Linagliptin	7132088	90.59	1.26	3232	0.0
2	6.831	Dapagliflozin	741061	9.41	1.06	17498	21.0
Total			7873149	100.00			

Figure 20: Chromatogram of thermal degradation of Daplo-L



PeakTable

Detector A Ch1 233nm

Peak#	Ret. Time	Name	Area	Area %	T. Factor	T.Plate	Resolution
1	2.663	Linagliptin	6889575	90.35	1.54	4340	0.0
2	6.827	Dapagliflozin	735513	9.65	1.16	31812	26.5
Total			7625088	100.00			

Figure 21: Chromatogram of thermal degradation of API

Table 20: Degradation summary

Condition	% Degradation of Dapagliflozin		% Degradation of Linagliptin	
	Sample	Standard	Sample	Standard
Acid	8.3	6.9	5.4	6.6
Base	20.8	19.0	6.1	5.5
Peroxide	21.6	17.8	4.4	3.8
Thermal	5.4	4.6	5.8	5.2
Photo	12.0	11.8	4.4	4.7

Results: All the degradants are well separated from the analyte peaks. So it was concluded that the method was deemed specific and stability-indicating.

8.4 Analytical method validation

System suitability:

Results of the system suitability indicated that the observed Relative Standard Deviation (%RSD) values were well within the generally accepted limits (≤2%). Other parameters such as theoretical plates, tailing factor, and resolution for linagliptin and dapagliflozin were also determined and were found to be well within the acceptable range. The summarized results are presented in Table-18.

Table 21: System Suitability parameter results

Drug Name	Linagliptin	Dapagliflozin	Acceptance criteria
R_t(min)	2.86	7.45	Consistent
Average Area	7692594	775914	-
% Relative Standard Deviation	0.0	0.1	NMT 2
USP Resolution	-	20.7	NLT 2
USP tailing	1.06	0.92	NMT 2
USP plate count	3061	15829	NLT 2000

*NMT = Note more than, NLT = Not less than

Results: All system suitability parameters were met and system was found to be suitable to perform further studies.

Specificity

The absence of any interference and consistent retention time confirmed the specificity of the method. Chromatograms depicting the blank, standard, sample and individual standard solutions are illustrated in Figures 22 to 24.

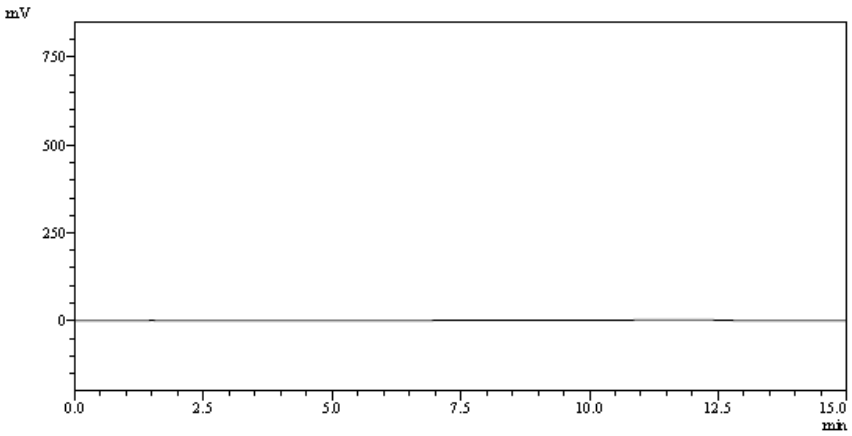


Figure 22: Chromatogram of diluent

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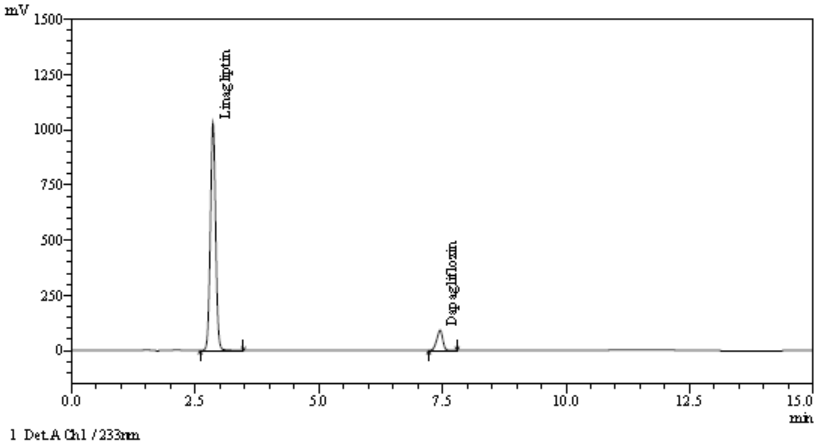
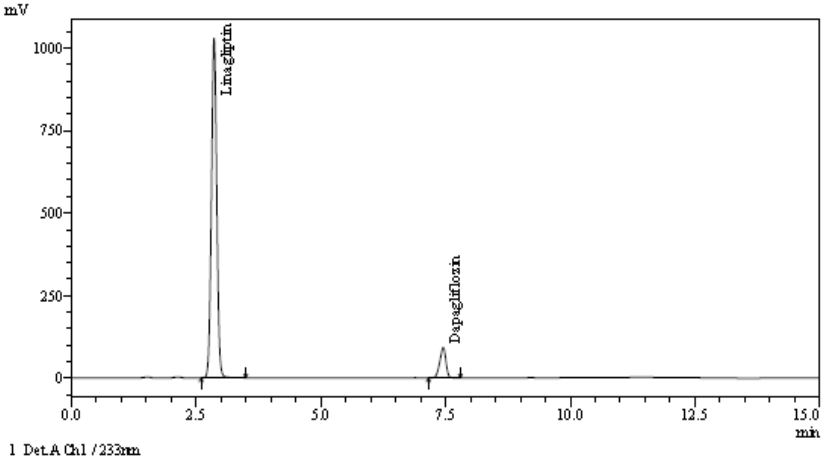


PeakTable

Detector A Ch1 233nm

Peak#	Ret. Time	Name	Area	Area %	T. Factor	T.Plate	Resolution
1	2.855	Linagliptin	7645198	90.83	1.06	3061	0.0
2	7.452	Dapagliflozin	771988	9.17	0.92	15829	20.7
Total			8417186	100.00			

Figure 23: Chromatogram of sample



PeakTable

Detector A Ch1 233nm

Peak#	Ret. Time	Name	Area	Area %	T. Factor	T.Plate	Resolution
1	2.855	Linagliptin	7645835	90.78	1.06	3061	0.0
2	7.452	Dapagliflozin	776396	9.22	0.91	15780	20.7
Total			8422230	100.00			

Figure 24: Chromatogram of standard

Results: No interference found at retention times of linagliptin and dapagliflozin. So method is specific.

Linearity

The results of linearity tests and the calibration curve are presented in Table 22 and Figures 25-26.

Table 22: Linearity test results of Linagliptin

Sr. No.	Concentration (µg/mL)	Peak Area
1	2.50	3547951
2	3.75	5196136
3	5.00	6869125
4	6.25	8314245
5	7.50	9896883
Correlation (R)	**0.9996**	
Correlation Coefficient (R^2)	**0.9993**	
Y-Intercept	**438478.80**	
Slope	**1267813.46693**	

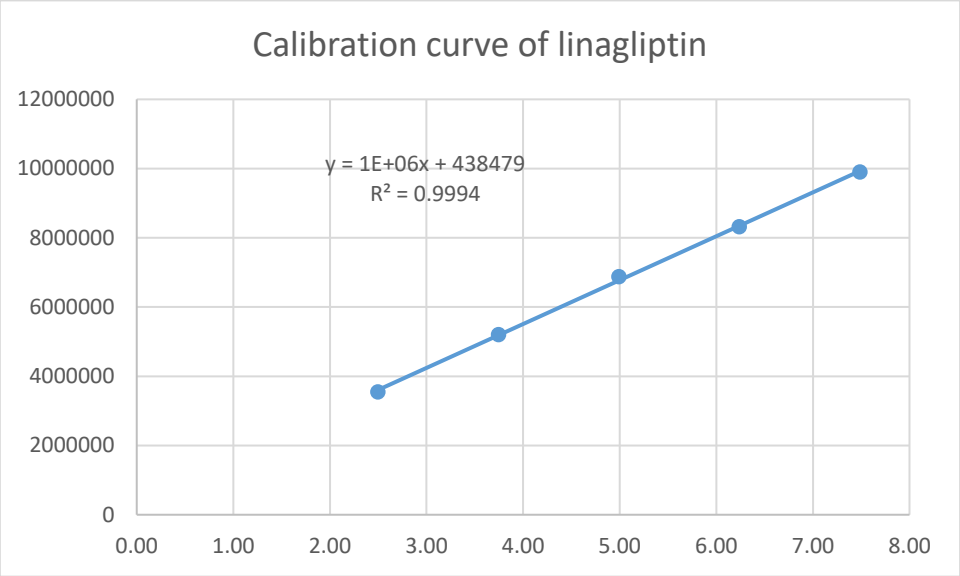


Figure 25: Calibration curve of linagliptin

Table 23 Linearity test results of dapagliflozin

Sr. No.	Concentration (µg/mL)	Peak Area
1	5.0	355733
2	7.5	538438
3	10.0	734804
4	12.5	921893
5	15.0	1073138
Correlation (R)	**0.9990**	
Correlation Coefficient (R^2)	**0.9981**	
Y-Intercept	**-2504.80**	
Slope	**73018.58922**	

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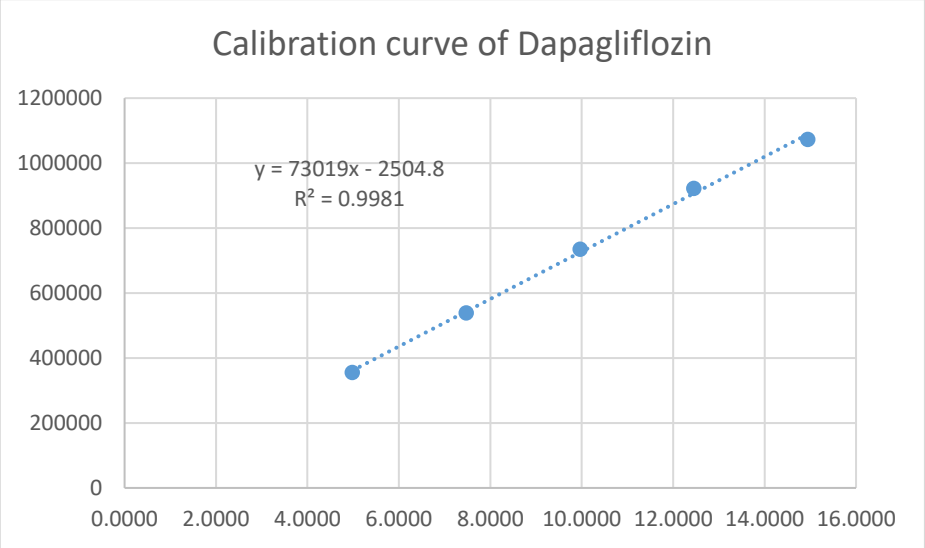

Figure 26: Calibration curve of Dapagliflozin

Results: Correlation coefficient R^2 for linagliptin was 0.999 and dapagliflozin was found to be 0.998. Above results revealed a linear relationship between the peak areas and concentration within the specified range of 50% to 150% of the targeted concentration.

Precision

Intraday precision

Results of the intraday precision are detailed in Table-24.

Table 24: Intraday precision

Sr. No.	Linagliptin assay	Dapagliflozin assay
1.	101.8	99.1
2.	101.5	99.7
3.	98.6	98.4
4.	100.8	98.1
5.	100.4	99.3
6.	101.0	97.7
%RSD	1.1	0.8

Interday precision

The detailed results of interday precision are presented in Table-25.

Table 25: Interday precision

Sr. No.	Linagliptin assay	Dapagliflozin assay
1.	98.1	97.6
2.	101.0	100.6
3.	100.5	100.7
4.	98.8	98.6
5.	98.2	99.0
6.	99.9	99.9
%RSD	1.2	1.2

Results: Both intraday and interday precision data shows relative standard deviation of less than 2, which indicates the method is highly precise.

Accuracy (Recovery studies):

The added amounts and the amounts found for both linagliptin and dapagliflozin were determined, and individual recovery values as well as the mean recovery values were reported. %RSD were also evaluated at each level. The outcomes were summarized, and it was confirmed that the results fell within acceptable limits. The detailed accuracy results are provided in Table-26.

Table 26: Recovery Results of Linagliptin

Table 27

Levels	Area	Mg added	Mg found	%Recovery	Mean	RSD
50%	3545194	0.046	0.047	101.1	101.0	0.1
50%	3545871	0.046	0.047	101.1		
50%	3533573	0.046	0.047	100.9		
100%	6868106	0.093	0.092	99.1	99.3	0.3
100%	6858306	0.093	0.093	99.7		
100%	6827626	0.093	0.092	99.2		
150%	10570666	0.139	0.140	100.5	99.9	0.8
150%	10416931	0.139	0.138	99.0		
150%	10532392	0.139	0.139	100.1		

Table 28: Recovery results of dapagliflozin

Table 29

Levels	Area	Mg added	Mg found	%Recovery	Mean	RSD
50%	357575	0.115	0.113	98.6	98.9	0.4
50%	358341	0.115	0.114	98.8		
50%	360527	0.115	0.114	99.4		
100%	731529	0.230	0.232	100.8	100.7	0.5
100%	734020	0.230	0.233	101.2		
100%	726719	0.230	0.230	100.2		
150%	1080831	0.345	0.343	99.3	99.7	0.8
150%	1081042	0.345	0.343	99.3		
150%	1095118	0.345	0.347	100.6		

Results: Recovery of linagliptin was found between 99.3 to 101% and recovery of dapagliflozin was found to be between 99.7 to 100.7%. and RSD at each concentration level was found to be less than 2. Therefore, the method was found to be accurate.

Limit of Detection and Limit of Quantification

LOD was found to be 0.059 and 0.19 µg/mL for linagliptin and dapagliflozin respectively. LOQ was found to be 0.18 and 0.59 µg/mL for linagliptin and dapagliflozin respectively.

Results: Low levels of LOD and LOQ showed sensitivity of the method

Robustness

Notably during robustness testing, slight modifications in method parameters did not cause significant alterations in the retention time of the peak of interest.

Table 30: Robustness results of linagliptin

	Temp. - 5°C	Temp. +5°C	Flow -0.1 ml/min	Flow +0.1 ml/min	Wavel-ength - 2 nm	Wavel-ength +2 nm
Mean Area	6961290	7715172	6338876	6298240	8499011	5186082
% RSD	0.1	0.1	0.2	0.1	0.2	0.1
Tailing factor	1.34	1.32	1.37	1.45	1.34	1.37
Theoretical plates	3922	4136	4010	4146	4199	4404
Retention time	2.65	2.96	2.44	2.44	2.68	2.68

Table 31: Robustness results of Dapagliflozin

	Temp. - 5°C	Temp. +5°C	Flow -0.1 ml/min	Flow +0.1 ml/min	Wave 2 nm	Wave +2 nm
Mean Area	6961290	7715172	6338876	6298240	8499011	5186082
% RSD	0.1	0.1	0.2	0.1	0.2	0.1
Tailing factor	1.34	1.32	1.37	1.45	1.34	1.37
Theoretical plates	3922	4136	4010	4146	4199	4404
Retention time	2.65	2.96	2.44	2.44	2.68	2.68

Results: Key performance indicators, including plate count (not less than 2000), tailing factor (not more than 2.0), resolution (not less than 2.0), and the %RSD for five replicate injections (not more than 2.0), remained well within the specified acceptance criteria. The results, detailed in Table-28-29, demonstrated robustness of the method.

8.5 Validation summary

Table 32: Validation summary

Parameter		Value (Linagliptin)	Value (Dapagliflozin)
Linearity (µg/mL)		2.5-7.5	4.5-15.5
Regression coefficient (R^2)		0.999	0.998
Specificity		No interference	No interference
% Recovery (Mean±Std Deviation)	50%	101.0±0.18	98.9±0.39
	100%	99.3±0.48	100.7±0.49
	150%	99.9±0.76	99.7±0.68
Intra-day precision (%RSD)		1.1	0.8
Inter-day precision (%RSD)		1.2	1.2
LOD (µg/mL)		0.059	0.19
LOQ (µg/mL)		0.18	0.59
Robustness		System suitability criteria met	System suitability criteria met

8.6 Conclusion

The analysis of Linagliptin and Dapagliflozin was successfully carried out using newly developed RP-HPLC method. The mobile phase consisted of a mixture of 20 mM potassium dihydrogen phosphate pH 3.5 and acetonitrile in gradient program setting. Inertsil ODS-3V, 150 x 4.6 mm, 5µ column was taken as a stationary phase, with detection performed using a UV detector set at 233 nm. The chromatographic conditions maintained a constant flow rate of 1.0 ml/min. Key system suitability parameters such as efficiency, resolution, and tailing factor were calculated to ensure the reliability of the method.

The method demonstrated specificity during forced degradation studies. The method's robustness and suitability were evident from recovery studies, and there was no interference observed due to excipients. Precision was evaluated through repeated injections, providing consistent values. The method underwent comprehensive validation for linearity, accuracy, precision, and robustness.

In summary, the RP-HPLC method developed is deemed suitable for routine analysis of tablet formulations containing linagliptin and dapagliflozin. It has demonstrated linearity, accuracy, precision, and robustness, making it a reliable choice for pharmaceutical quality assessment.

LIST OF ABBREVIATIONS

FDC: Fixed dose combination

DAPA: Dapagliflozin

LINA: Linagliptin

HPLC - High-Performance Liquid Chromatography

UV - Ultraviolet (detector)

LOD - Limit of Detection

LOQ - Limit of Quantitation

RSD - Relative Standard Deviation

RT - Retention Time

API - Active Pharmaceutical Ingredient

ICH - International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use

USP - United States Pharmacopeia

EP - European Pharmacopoeia

BP - British Pharmacopoeia

IP - Indian Pharmacopoeia

PDA - Photodiode Array

DAD - Diode Array Detector

SOP - Standard Operating Procedure

PPM - Parts Per Million

QC – Quality control

IUPAC - International Union of Pure and Applied Chemistry

µg - Microgram

mg - Milligram

mL - Milliliter

µL – Microliter

mm - Millimeter

µ - Micron

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