

**Development and Validation of a Robust HPLC-UV/PDA
Method for Simultaneous Analysis of Six Antidiabetic Drugs
in Pharmaceuticals, Ensuring Quality and Efficacy**

2023-2025

Muhammad Asif Malik

SU92-MSCHW-S23-027

FOS



SUPERIOR UNIVERSITY

Thesis Submitted to

The Superior University Lahore

**In Partial Fulfillment of the
Requirement for the Degree of
M. Phil Chemistry**

By

Muhammad Asif Malik

Roll No. SU92-MSCHW-S23-027

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Supervisor Name: Dr. Muhammad Mudassir Iqbal

Designation: Assistant Professor

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Designation: Lecturer

Signature: _____

CERTIFICATE OF APPROVAL

This is to certify that the research work presented in this thesis, titled **“Development and Validation of a Robust HPLC-UV/PDA Method for Simultaneous Analysis of Six Antidiabetic Drugs in Pharmaceuticals, Ensuring Quality and Efficacy”** was conducted by **Muhammad Asif Malik** under the supervision of **Dr. Muhammad Mudassir Iqbal**.

No part of this thesis has been submitted anywhere else for any other degree. This thesis is submitted to the Faculty of Sciences, The Superior University, Lahore in partial fulfillment of the requirements for the degree of Master of Science/Master of Philosophy in the field of **“Chemistry”** in Faculty of Sciences at The Superior University, Lahore.

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DEDICATION

This is dedicated to Almighty Allah, the Lord of Lords; to my Parents, my Family, and my friends; and to all those who love, support, and encourage me throughout my studies.

ACKNOWLEDGMENTS

All praise and gratitude are due to Almighty Allah, the source of all knowledge and wisdom, who granted me the strength, guidance, and perseverance to complete this research. May peace and blessings be upon the Holy Prophet Muhammad (PBUH), who illuminated the path of righteousness and humanity.

I dedicate this research to my beloved parents and family, whose unwavering support, sacrifices, and encouragement have been the foundation of my academic journey. Their patience, hard work, and prayers have been a constant source of motivation throughout my studies.

The studies and findings presented in this thesis are the result of dedicated efforts, extensive research, and the valuable contributions of many individuals. I want to express my sincere gratitude to **Prof. Dr. Muhammad Naveed Babur**, Dean Faculty of Allied health Sciences, **Prof. Dr. Uqba Mehmmod**, Head of Department, **Dr. M. Mudassir Iqbal**, Assistant Professor and Head of the Department of Chemistry at the Superior University, Lahore, for his continuous supervision, insightful guidance, constructive criticism, and valuable suggestions throughout the experimental work and preparation of this thesis.

I am also thankful to the authors and researchers whose published articles and journals provided valuable information and support for this study.

Special thanks are extended to **Mr. M. Ishaque (Manager QC)**, **Mr. Hassan Javed (Lead Quality Control)**, **Mr. Ali Haider Magsi**, **Mr. Suleman Anees**, **Mr. Usman Abbas**, and **Mr. Attiq Ahmad Butt (Executive Officer Quality Control Department)** at Highnoon Laboratories Limited, Multan Road, Lahore, for their cooperation, technical support, and assistance during the completion of this research work.

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ABSTRACT

Fixed dose combinations have been widely used in recent times because of their improved effectiveness and patient compliance owing to the increasing prevalence of diabetes mellitus. This current investigation aims to develop and validate a novel, accurate and reliable HPLC methodology with UV/PDA detector for estimating the six common antidiabetic drugs, Metformin, Linagliptin, Empagliflozin, Dapagliflozin, Ertugliflozin and Sotagliflozin in different pharmaceutical formulations. By applying reversed phase C18 column, mobile phase of buffer (pH 4.0) and acetonitrile and detecting at wavelength 225 nm, the optimal gradient technique was obtained within 16 minutes, thereby ensuring full resolution of each analyte. The parameters such as specificity, linearity, accuracy, precision, LOD, LOQ and robustness were validated as per ICH Q2(R1) guideline. The precision of the developed method was evaluated, and the %RSD values were found to be below 2% demonstrating excellent repeatability and precision (Results: Metformin 0.81%, Linagliptin 0.96%, Empagliflozin 1.17%, Dapagliflozin 0.27%, Ertugliflozin 0.91% & Sotagliflozin 0.50%) with a correlation coefficient (R^2) of more than 0.99, study showed a strong correlation between concentration and response with correlation coefficient (R^2) value of (Metformin 0.9998, Linagliptin 1.000, Empagliflozin 1.0000, Dapagliflozin 1.000, Ertugliflozin 0.9998 & Sotagliflozin 0.9943). The recovery studies found the values of accuracy within an acceptable range of 98–102%, the observed recovery values were within the required range (Metformin **(60%)** 101.25%, **(100%)** 99.00% & **(140%)** 98.43%, Linagliptin **(60%)** 99.53%, **(100%)** 100.97%, **(140%)** 101.61%, Empagliflozin **(60%)** 99.69%, **(100%)** 100.15%, **(140%)** 99.09%, Dapagliflozin **(60%)** 101.32%, **(100%)** 98.67%, **(140%)** 98.80%, Ertugliflozin **(60%)** 100.60%, **(100%)** 99.93%, **(140%)** 99.56% & Sotagliflozin **(60%)** 100.60%, **(100%)** 99.67%, **(140%)** 99.52%), Despite the very low concentrations, the LOD and LOQ values were sufficiently low to detect the drugs and estimate their content. LOD and LOQ were found to be (Metformin 0.51 µg./mL, Linagliptin 5.52 µg./mL, Empagliflozin 2.93 µg./mL, Dapagliflozin 0.90 µg./mL, Ertugliflozin 1.09 µg./mL & Sotagliflozin 0.46 µg./mL) and (Metformin 1.55 µg./mL, Linagliptin 16.73 µg./mL, Empagliflozin 8.89 µg./mL, Dapagliflozin 2.72 µg./mL, Ertugliflozin 3.32 µg./mL & Sotagliflozin 1.38 µg./mL) respectively. Specificity study showed that excipients and diluent did not interfere with the chromatographic peaks of the target compounds, ensuring accurate determination of the active pharmaceutical ingredients. With robustness studies, it became clear that even minor changes in the chromatography parameters had no effect on the developed technique. Thus, the analysis of pharmaceutical commercial formulations containing the selected medicines was successfully conducted through the application of the tested technique. Consequently, the method proved its reliability in terms of being used for routine control and stability tests in the pharmaceutical industry. Thus, the developed technique is helpful in ensuring medicine quality and patient safety due to being a reliable analytical technique for simultaneous quantitation of antidiabetic drugs.

CHAPTER NO.1

INTRODUCTION

1.1 Method Development

In order to manufacture pharmaceutical dosage forms of the best possible quality, a proper HPLC technique is necessary. The manufacturing of medicines should be in accordance with certain regulations that will help ensure that the quantity of the active pharmaceutical ingredient (API) corresponds to the information on the medicine label. Analytical methods provide guarantees that the pharmaceutical substances used in the formulation of medicines will be precisely and reliably detected and quantified. HPLC is an advanced analytical technique that has been extensively used in the pharmaceutical industry to analyze medicinal substances due to its high accuracy and capability to distinguish various compound mixtures [1].

An understanding of the physical and chemical characteristics of the compounds under study is needed in order to create the HPLC method to study a variety of medications in combination. The antidiabetics consist of metformin, linagliptin, empagliflozin, dapagliflozin, ertugliflozin and sotagliflozin. It is essential that there should be no overlapping or interaction between the medications; therefore, it is necessary to optimize the approach [2]. Various chromatographic conditions are altered during the process of optimization. These include the composition of the mobile phase, nature of the column used, flow rates, and the wavelength used for detection [3]. All these conditions need to be varied so that maximum resolution and separation can be ensured without having any interference from the excipients and degradation products of the pharmaceutical dosage form. In order to confirm the stability-indicating capacity of the developed HPLC method, forced degradation studies should be considered. This involves exposing the pharmaceutical formulation under different stressed conditions such as heat, light, and oxidation, in order to determine any kind of degradation product produced.

The forced degradation testing should form part of the development of the method in order to guarantee the stability indicating nature of the HPLC technique. This is necessary since the potential degradants have to be identified by subjecting the drug formulation under extreme conditions such as heat, light, and oxidation in the forced degradation tests. One technique that can help in recognizing and analyzing each element, even the degradant elements, necessary for producing the final

pharmaceutical product to make it stable and consistent [4]. In terms of method development in the pharmaceutical industry, another critical aspect that is needed to consider includes proving that the method developed will comply with any regulatory requirements. Method validation is very important in making sure that the HPLC technique used complies with various regulatory guidelines, including those pertaining to specificity, sensitivity, linearity, precision, accuracy, and robustness [5]. The method should also have the capability to analyze the active compounds in the sample to be analyzed within acceptable detection and quantification levels. This can be decided by the conduction of examinations like precision testing, accuracy testing, robustness test etc [6].

The ultimate goal of a chromatographic method development is to develop a highly sensitive, precise, and repeatable HPLC method to analyze multiple types of AAD drug in one single run. For this purpose, the technique will be developed to be able to process pharmaceutical preparations containing the various excipients and active components [7]. This type of system ensures efficiency, effectiveness and low costs in the development of quality control methods as it eliminates the need for different analysis. A reliable method, however, ensures the quality of pharmaceuticals, which is a key to patients' safety and health.

1.1.2 Simultaneous Estimation of Antidiabetic Drugs

More than one drug can be estimated at the same time from the same specimen of sample by using a single method, which is called the simultaneous estimation. It is often used in the pharmaceutical industry to save in terms of cost and time. It allows for the analysis of multiple APIs in combination medications, without having to conduct multiple tests [8]. This is crucial to ensure that each component of the antidiuretics combination (cymbalta) drug is measured accurately.

When it comes to estimation of medicines such as metformin, linagliptin, empagliflozin, dapagliflozin, ertugliflozin and sotagliflozin, HPLC-UV/PDA methods provide a very effective solution to quality control of these drugs. It satisfies all conditions regarding the safety of patients and efficiency of drugs by making sure that the active ingredients are contained in sufficient quantities [9]. Monitoring the content of drugs and ensuring their consistency is highly important in clinical applications and quality control in general.

The use of simultaneous estimation allows reducing risks connected with

possible errors resulting from several different measurements. Using a single estimation technique helps pharmaceutical companies to fulfill regulatory requirements and operate more efficiently [10]. Thus, simultaneous estimation serves as a very important instrument for creating and evaluating drug combinations.

1.1.3 Key Information for Method Development in Simultaneous Antidiabetic Drug Analysis

For the development of a successful method in the analysis of various drugs, some conditions have to be met to ensure accuracy, robustness, and reliability. As [11], method development involves a series of experiments aimed at optimizing the sample qualitative and quantitative analyses. Analytical pharmaceutical studies may be very lengthy and can take several months depending on the complexity of the method used and the expected results. This becomes even more challenging when analyzing complex formulations such as antidiabetic drugs used in treating type 2 diabetes. It is imperative to have a thorough understanding of some essential factors about the sample prior to developing an effective HPLC-UV/PDA method.

1.1.4 Physicochemical Properties of Drugs

To develop an appropriate method one needs to be familiar with the physicochemical properties of the individual drugs. This also encompasses information on solubility, stability, pH sensitivity, UV absorption and potential degradation products. It is essential to be aware of the chromatographic behaviour of antidiabetic agents such as metformin, linagliptin, empagliflozin, dapagliflozin, ertugliflozin, or sotagliflozin, to assure their separation and quantitation in the same method [12].

1.1.5 Sample Composition and Matrix Effects

Composition of the matrix affects the development of an appropriate technique. Ingredients such as excipients and stabilizers and other chemicals could make it challenging to analyze the active ingredients in the formulation. It is important to understand how the formulation works in order to predict and prevent matrix effects that could affect the analysis of the medicine [13]. Linearity and sensitivity of the method would be influenced by the concentration of the medicines in the formulation.

1.1.6 Instrumentation and Analytical Conditions

Adjustment of analytical parameters and equipment selection are essential elements to guarantee precise and reliable results. In HPLC-UV/PDA analysis, it is

essential to consider the column choice, mobile phase, flow rate, temperature, and wavelength of UV detection. In the simultaneous study of multiple antidiabetic medicines, which might possess different characteristics, adjustment of these parameters becomes necessary [14].

1.1.7 Stability Considerations and Forced Degradation Studies

Stability tests and forced degradation need to be done before the development of the method to determine the stability profile of the active pharmaceutical ingredients in the dosage form. Selection of the method will be important because it must be stability indicating and be able to differentiate between the drug substance and its degradation products. This is particularly vital for the drugs such as empagliflozin or dapagliflozin because their degradation products could interfere with detection or be toxic to patients.

1.1.8 Regulatory Requirements and Compliance

Requirements for regulation should be taken into account when designing an analytical procedure, especially where this is to be used in the quality control of a drug manufacturing process. The criteria issued by the competent authorities such as FDA, EMA and ICH are mandatory. This includes ensuring process validation for linearity, specificity, accuracy, precision and robustness. The process should follow industry guideline for pharmaceutical approval to ensure the safety and efficacy use of the combined drug for diabetes treatment.

1.2 Strategic Approach to HPLC Method Development for Multi-Drug Analysis

Creating an analytical method, especially one capable of simultaneous estimation of multiple drugs for antidiabetes, requires a systematic process to ensure reliability, precision and accuracy. The aim is to develop an effective method with the ability to separate and quantify each individual component in a pharmaceutical formulation. This is particularly important for HPLC-UV/PDA analysis of chemically diverse drugs like metformin, linagliptin, empagliflozin, dapagliflozin, ertugliflozin and sotagliflozin.

1.2.2 Selection of Analytical Technique

The first thing to consider is selecting an appropriate analytical method. HPLC with UV/PDA detection is commonly used for multiple analyses of drugs due to its sensitivity and reproducibility and its ability to deal with complex drug combinations [15].

1.2.3 Understanding Drug Chemistry

It is critical to have a detailed knowledge of the chemical content of each drug. Solubility, pKa, polarity, UV absorbance maxima and degradation behavior provide information for designing an appropriate method that will successfully resolve all of the components [16].

1.2.4 Mobile Phase and Column Selection

The composition, pH, buffer concentration of the mobile phase and selection of the stationary phase (such as C18 columns) are important factors in achieving good peak shape, resolution and minimal tailing. Gradient elution or isocratic elution can be employed, based on the physicochemical characteristics of the drug [17].

1.2.5 Method Optimization

During the trials, factors such as flow rate, column temperature, and detection wavelength are varied. This is done to facilitate the separation of all drugs in the most effective manner while ensuring proper retention and resolution times [18].

1.2.6 System Suitability Testing

To ensure the consistency and reliability of the methodology, system appropriateness parameters such as theoretical plates, tailing factor, resolution, and repeatability are evaluated prior to method finalization

1.2.7 Method Validation

Once the method is optimized, it must be validated according to regulatory guidelines (ICH/FDA). Validation parameters include specificity, linearity, accuracy, precision, limit of detection (LOD), limit of quantification (LOQ), robustness, and ruggedness [19].

1.3 Analytical Method Validation

Method validation, which provides an established evidence regarding whether a specific analytical method is suitable for a particular use, plays a pivotal role in drug analysis. Validation of a method ensures that each antidiabetic drug, including metformin, linagliptin, empagliflozin, dapagliflozin, ertugliflozin, and sotagliflozin, can be precisely, reliably, and repeatedly analyzed and quantified in the presence of other substances, including excipients, impurities, and degradants through simultaneous HPLC-UV/PDA analysis. In accordance with globally accepted guidelines such as ICH Q2(R1), which include important characteristics that should be evaluated, such as linearity, accuracy, precision, specificity, detection limit, quantification limit, and robustness, among others, validation of methods is carried

out. All these considerations help determine the applicability and reliability of the analytical procedure used in antidiabetic drugs.

1.3.2 Linearity

Linearity refers to the capability of the method to produce results that bear an exact relationship with the analyte concentration. In multi-drug HPLC analysis, there is a need to determine the linearity of each drug by drawing its calibration curve and then computing for its R^2 . The value should be nearer to 1.000, which denotes good linearity. In simultaneous estimation, the drugs should have independent linearity.

1.3.3 Accuracy

The extent of similarity between the value found and the true value is referred to as accuracy. The accuracy of an analysis is tested using recovery tests, where predetermined amounts of standards are introduced into the sample matrix. It should be proven in all drugs within a formulation at all ranges. An accuracy range between 98%-102% for pharmaceutical formulations is usually acceptable.

1.3.4 Precision

When this process is performed again under identical conditions, it is termed precision. Precision in an experiment is evaluated by its consistency in a laboratory with the same operator, apparatus, and a small period of time. Precision between different operators, instruments, and days within the laboratory. Accuracy between laboratories. Results are stated in terms of relative standard deviation or %RSD, and less than 2% RSD are acceptable for most pharmaceutical analyses [20].

1.3.5 Specificity

When this process is performed again under identical conditions, it is termed precision. Precision in an experiment is evaluated by its consistency in a laboratory with the same operator, apparatus, and a small period of time. Precision between different operators, instruments, and days within the laboratory. Accuracy between laboratories. Results are stated in terms of relative standard deviation or %RSD, and less than 2% RSD are acceptable for most pharmaceutical analyses.

1.3.6 Limit of Detection (LOD)

The lowest analyte concentration that is detectable but not necessarily quantifiable is known as LOD. It is computed using the calibration curve's slope and the response's standard deviation. A signal- to-noise ratio of 3:1 is typically regarded as the lowest detectable concentration for HPLC [21].

1.3.7 Limit of Quantification (LOQ)

The lowest concentration that can be accurately and precisely calculated quantitatively is known as the LOQ. A signal-to-noise ratio of 10:1 is commonly used to identify it. LOQ must be determined for every medication separately in simultaneous analysis [22].

1.3.8 Robustness

Robustness is the ability of the method to resist small, intentional changes in the analytical parameters, e.g., flow rate, temperature of the column, detection wavelength. This will ensure that the method can yield reliable results in different operational settings. For multi-drug analysis, robustness is essential to ensure that the precise amount of each drug is quantified [23].

1.4 Method Development on HPLC

One of the most common methods used in pharmaceutical analysis for identification and quantitation of active pharmaceutical ingredients (APIs) is High Performance Liquid Chromatography (HPLC). High resolution and sensitivity, it is particularly suitable for multi-component drug formulations (e.g., antidiabetic combinations) due to its high resolution, sensitivity and reproducibility. For simultaneous analysis of metformin, linagliptin, empagliflozin, dapagliflozin, ertugliflozin and sotagliflozin a careful method development is crucial to ensure the accurate separation of each drug component in a single run [24]. In HPLC, separation of components in a sample is effected by interaction between stationary and mobile phases. The choice of the chromatographic conditions (column type composition of the mobile phase, detection wavelength, flow rate, etc.) is part of the method development process temperature. All these parameters should be optimized for good resolution, peak shape, retention time and reproducibility.

1.4.2 Stationary Phase Selection

Because of their non-polar properties, reversed-phase columns (especially C18) are widely employed for the analysis of antidiabetic drugs. For good separation and peak resolution in the multi-drug analysis, an adequate surface area should be provided which is achieved by a C18 column with a particle size of 3–5 μm and length of 150–250 mm [25].

1.4.3 Mobile Phase Composition

Generally the mobile phase will be an organic solvent, e.g., methanol or acetonitrile, with an aqueous buffer. The important aspects of the buffer solution are its pH and ionic strength, which must be carefully controlled to ensure the stability

and solubility of different anti-diabetic drugs in the analysis. Gradient elution is advantageous over isocratic conditions for compounds of varying polarity and is a common method to use when multiple drugs are being analyzed [26].

1.4.4 Detection Wavelength

The wavelength of detection is chosen depending on the maximum absorption in the UV region (λ max) of the drug. For instance, if all the compounds have high absorption at a common wavelength (e.g., 225 nm), then all can be estimated simultaneously. (Swartz, 2010). The Photodiode Array (PDA) detector is beneficial because it can detect different wavelengths at the same time and can be used for peak purity analysis.

1.4.5 Flow Rate and Column Temperature

Resolution and retention time are both impacted by the rate of flow. For a reversed-phase HPLC, a flow rate 1.0 - 1.5 mL/min is generally optimum. Retention times can be reduced for higher flow rates can, however, affect resolution. The column temperature (usually 30 - 40°C) affects the viscosity of the mobile phase and diffusion of the analytes and should be optimized to improve the reproducibility [27].

1.4.6 Choice of Diluent

The appropriate diluent should be used that will allow all the active ingredients to be dissolved without decomposition or interaction. The diluent must also be compatible with the mobile phase so as to prevent peak distortion or baseline drift. For multi-drug HPLC analysis, diluent may contain a mixture of buffer (aqueous) with an organic solvent, similar to the mobile phase [28].

1.4.7 System Suitability Parameters

The appropriate diluent should be used that will allow all the active ingredients to be dissolved without decomposition or interaction. The diluent must also be compatible with the mobile phase so as to prevent peak distortion or baseline drift. Prior to finalizing the method, system suitability tests should be performed to assess the performance characteristics, including: Theoretical plates (column efficiency)

- Tailing factor
- Resolution between peaks
- Retention time reproducibility

The peak area is indicated by %RSD of peak areas. These parameters validate the reliability, repeatability and accuracy of this method for quality control [29].

1.5 Diabetes Mellitus

Diabetes mellitus is a collection of chronic metabolic disorders in which there is a high level of sugar in the blood due to defects in insulin production, insulin function or both. It is a significant public health problem around the world because it is a growing problem and is linked to potential complications, As well as cardiovascular disease, neuropathy, nephropathy and retinopathy [30].

Diabetes mellitus has been the impetus for the development of several classes of antidiabetic drugs to control blood glucose and prevent diabetes complications in the field of pharmaceuticals. Multimodal drug therapy requires powerful analytical methods for quality control, such as HPLC methods which are developed, reliable and validated for the analysis of multiple drug types at the same time [31].

Diabetes is on the rise worldwide. The IDF estimates that in 2021, there were 537 million adults (20–79 years old) living with diabetes, which is expected to rise to 643 million by 2030. Pakistan is one of the countries with high prevalence of diabetes, having more than 33 million diagnosed cases of diabetes (IDF, 2021). As a result of this emerging trend, there has been a wider use of combination therapy with agents like metformin, linagliptin, empagliflozin and others.

Because of the complexity of diabetes care, along with the advent of the fixed-dose combinations, quality and efficacy of the antidiabetic medications are required. Thus, the analytical procedures applied to the analysis of these combinations must be accurate, precise and stability indicating.

1.5.2 Complications of diabetes

Diabetic retinopathy, diabetic nephropathy, and diabetic neuropathy are among the many complications that can occur with diabetes if blood sugar levels are not controlled. These problems are caused by chronic injury to small blood vessels. Include cardiovascular disease – which includes coronary artery disease, stroke, and peripheral vascular disease – which are major causes of death in diabetics. Delayed gastric emptying, urinary incontinence and sexual dysfunction are also common in long-standing diabetes [32]. These complications highlight the need to control blood sugar levels closely and rely on good quality, well-controlled pharmaceutical treatments.

Diabetes Mellitus can be classified according to its etiology as follows:

There are several types of diabetes mellitus:

Type 1 Diabetes Mellitus: An autoimmune condition in which insulin-producing cells in the pancreas are destroyed resulting in complete insulin deficiency. Usually occurs in childhood or adolescence [33].

Type 2 Diabetes Mellitus: Most common type characterized by insulin resistance and relative insulin deficiency. It is tightly linked with obesity, lack of physical activity and genetic risk [34]. Hyperglycemia that occurs during pregnancy is called Gestational Diabetes Mellitus (GDM). Both mothers and children are at a higher risk of developing type 2 diabetes in the future with GDM.

Other specific types: These involve defects in beta-cell function (e.g., genetic), endocrinopathies, drug-induced (e.g., glucocorticoid-induced), and infections involving the function of the pancreas. It is important to diagnose correctly and to classify the patients so treatment is planned and monitored correctly. Diabetes Mellitus is treated by using medications. Diabetes Mellitus is treated with medicines.

Diabetes mellitus treatment consists of non-pharmacological and pharmacological methods to keep blood glucose levels at their best. Lifestyle changes, including healthy diet, exercise and weight management, is the basis. In oral antidiabetic drugs (OADs) are extensively used which includes their use in type 2 diabetes. These include biguanides, DPP-4 inhibitors and SGLT2 inhibitors many of which are prescribed in fixed dose combinations to increase compliance and therapeutic efficacy.

1.6.1 Biguanides

The initial treatment for type 2 diabetes is metformin, a biguanide. It reduces liver glucose production and increases insulin sensitivity. It is well tolerated and can be used in combination with other antidiabetic drugs, and has a low risk of hypoglycaemia [35].

1.6.2 DPP-4 Inhibitors

Sitagliptin and linagliptin are drugs that inhibit DPP-4, which increases the effects of the incretin hormones, which stimulate the release of insulin. They're safe and may be used in combination with metformin or SGLT2 inhibitors.

1.6.3 SGLT2 Inhibitors

Empagliflozin, dapagliflozin, ertugliflozin and sotagliflozin inhibit glucose reabsorption from the kidney, resulting in glycosuria and a reduction in plasma glucose levels. They have cardio- and reno-protective effects outside the glycemic range [36].

1.6.3.1 Goals of Diabetes Treatment

They main goals are to achieve target blood glucose levels, reduce the risk of complications, and improve quality of life. Fixed-dose combinations help by reducing pill burden and improving patient adherence [37].

AIMS & OBJECTIVES

- To develop a sensitive, specific, and accurate analytical method for simultaneous estimation of Metformin, Linagliptin, Empagliflozin, Dapagliflozin, Ertugliflozin, and Sotagliflozin in pharmaceutical dosage forms.
- To validate the developed method as per ICH guidelines for specificity, linearity, range, accuracy, precision, robustness, and system suitability.
- To determine the limit of detection (LOD) and limit of quantitation (LOQ) for each analyte.
- To verify that each component is accurately quantified while other components are present.

CHAPTER NO.2

LITERATURE REVIEW

A direct and stable liquid chromatographic procedure was developed and validated for the determination of metformin hydrochloride and its related substance, 1-cyanoguanidine, in tablet form by Al-Rimawi et al. (2009). In this study, liquid chromatography was done using a Nova-Pak silica column coupled with a UV detector at a wavelength of 232 nm. The mixture of ammonium hydrogen phosphate buffer-methanol is normally applied for isocratic evaluation. The standards that USP has for new assays in terms of accuracy, precision, specificity, linearity, and range have been met with the validation of this new procedure. Linearity of this technique ranges from $-0.01 - 0.03 \text{ mgmL}^{-1}$ of metformin hydrochloride dose, while it has an accuracy of 100.4%. For the testing of metformin hydrochloride, an uncomplicated stability-indicating liquid chromatography process was developed and validated. The Relative Standard Deviation of the duplicates, which equals 0.30%, can be employed to measure the accuracy of the test method. Additionally, validation criteria of the USP for quantitation of impurities, which include precision, accuracy, linearity and range, specificity, and LOQ, have been employed to validate the test method for the quantitation of 1-cyanoguanidine. Due to the low LOQ, the method enables the identification and quantitation of 1-cyanoguanidine in small quantities [38].

Reddy et al., (2015) analyzed and developed a new HPLC method for simultaneous analysis of metformin and canagliflozin in medicine dosage forms. The chromatographic analysis involved ODS, 250 mm X 4.6 mm, 5 particles size with mobile phase flow rate of 1 mL/minute using isocratic method comprising buffer, acetonitrile, and methanol. In this study, photodiode array detector at 212nm and 30°C column temperature was employed. ICH guidelines indicate that validation parameters such as system suitability, linearity, accuracy, precision, specificity, limit of quantitation, limit of detection (LOD) and robustness were analyzed. Retention time of metformin and canagliflozin were found to be 2.783 minutes and 3.781 minutes, respectively. Recovery of metformin and canagliflozin is found to be 100.1% and 100.2%, respectively. Thus, it is established that the relative standard deviation of the assay of tablet is less than 2% due to the efficiency of the technique, accuracy, and sensitivity which can be employed by the quality control laboratory of pharmaceutical industry [39].

In the study performed by Ardila et al., (2015), the cluster analysis was conducted on samples from commerce using HPLC-UV/PDA and MCR-ALS/PCA without peak alignment procedure. *Bauhinia forficata* Link has been accepted by Brazilian Health Ministry as an effective remedy against hypoglycemia and diabetes. The plant is full of various active ingredients, especially flavonoids that render it antidiabetic effects. Due to the high similarity between various representatives of the genus *Bauhinia*, it is necessary to have proper analytical methods. High-performance liquid chromatography coupled with UV or photodiode array detection (HPLC-UV/PDA) technique has been extensively used for this purpose. By using chemometric techniques, it presents an effective method for discrimination between pure products and those that have been adulterated or substituted. Nevertheless, variations in their chromatographic behavior, including changes in retention time, may pose difficulty in achieving this goal. Resolving this issue is important in ensuring the reproducibility and reliability of the methods that can be used for the identification of samples [40].

Srivastava et al. (2015) carried out a study on the reverse phase HPLC-ultraviolet photodiode array detector that confirmed the simultaneous quantitation of six phenolic acids/bioactives in *Roscoea purpurea* tubers, in addition to their cytotoxic effect *in vivo* against various cell lines. *Pharmacognosy Magazine*, 11(Suppl 3): S488. Identification of PAs from the whole tuber extract simultaneously was conducted through reversed phase HPLC-ultraviolet, and the cytotoxic effects as well as their antioxidant effect were assessed. Response activity guided fractionation was also tested for the same experiment [41].

A high-performance liquid chromatographic method was formulated by Naazneen et al. (2016) for maximal isocratic assessment to determine the formulation of combination tablet of linagliptin and empagliflozin. Isocratic HPLC method is a common procedure utilized for maximal isocratic assessment through separation by means of chromatography in a C18 column [BDS 250mm x 4.6-mm, 5 μ]. Acetonitrile and perchloric acid have a volume ratio of 60:40, flow at a speed of 1ml per minute, and are read using ultraviolet light at 230nm. The retention times for empagliflozin and linagliptin were determined to be 2.05 and 4.10 minutes, respectively. Both compounds had correlation coefficient values of 0.999 and 0.999, respectively. Linagliptin and empagliflozin were found to have percentage RSD of precision of 0.22 and 0.33, respectively. The results showed that the mean recovery percentage of empagliflozin was 100.96 – 101.48%, while that of linagliptin was 100.09 – 101.13%.

The values obtained for ruggedness, precision, LOD, LOQ [42].

Debata et al. (2017) proposed a method for the analysis of dapagliflozin, a brand-new reversed-phase high-performance liquid chromatographic according to ICH Guideline. This method is easy, accurate, fast, and precise. The column utilized in RP-HPLC is C18, 5 μ m with a particle size of 25 μ m $\times$ 4.6mm. Phosphate buffer and acetonitrile were mixed in the proportion of 60:40 v/v. with a flow rate of 1.0ml/min-1. The detection of UV was done at 237nm. Six minutes were spent on total run time. The Proposed method was verified according to ICH and USP guidelines. Validation of the method proved that the test is rapid, accurate, reliable, and reproducible. For dapagliflozin, linear calibration curves were obtained within a concentration range of 10–60 μ g/ml. For dapagliflozin, limits of detection were 0.02 μ g/ml and limits of quantification were 0.06 μ g/ml. Efficiency of the procedure in dosage form preparation was justified by good recovery rates and low coefficients of variation [43].

Nizami *et al.* (2018) developed cost-effective RP-HPL Method using the PDA detector at a wavelength of 224nm, which enables an exact quantification of metformin and ertugliflozin in the dose of medication. In the case of ERTU and METF, the analysis has been done between the concentration range of 50-150 μ g/mL as per ICH recommendations. In order to analyze the separated peaks, the ACE column C18 with the dimensions 150 x 4.6 x 5 μ m has been used. This process requires the mixture of acetonitrile with buffer potassium dihydric-orthophosphate. Samples after 30min of ultra-sonication have been passed through 47mm membrane filters with Nylon (N66). The PDA detection was carried out at 240 nm, flow rate is 1 mL/min and injection volume was 10 μ L. All calculations were made based on the temperature of the mobile phase in the column which was 30°C. The concentration range for ERTU is 3 – 15 μ g/ml and for METF 12.5 – 125 μ g/ml, where linearity exists. The ICH criteria have validated the system appropriateness, specificity, accuracy and robustness of the method. In METF and ERTU, the LOD and LOQ found to be 2.24, 0.43 and 0.74 μ g/mL [44].

Musmade et al. (2022) studied Method development, validation and estimation of relative response factor for the quantitation of known impurities in mometasone furoate nasal spray dosage form by RP-HPLC with UV/PDA detector. Mometasone furoate is a corticosteroid which is commonly used in various dosage forms such as nasal spray, nasal implant, inhalation powder, topical cream, lotion, and ointment due

to its wide range of therapeutic utilities. An extensive literature review showed that there was no existing literature available regarding the HPLC analysis of four toxic impurities of mometasone furoate. Hence, this research aimed at the development of a robust method for the quantitation of these impurities in the API and the nasal spray dosage form of mometasone furoate using simple RP-HPLC method with PDA detector. Since these impurities' reference standards are not available or are expensive, the relative response factors were calculated to ensure accurate quantification. The separation of these impurities by HPLC was conducted under an isocratic elution condition where the mobile phase consists of sodium octanesulfonate and phosphate buffer (pH 3.0) as mobile phase A, and acetonitrile as mobile phase B in a 45:55 (v/v) ratio. The flow rate of the mobile phase was kept constant at 0.8 mL/min. The separation of mometasone furoate and its known and Unknown impurities was achieved on a Bakerbond C18 column (250 × 4.6 mm, 5 μ m), while the temperature and wavelength were set at 50°C and 254 nm, respectively [45].

Chemo metric Pattern Recognition Techniques and Their Trends in the Analysis of Medicinal Plants was studied by Gaião Calixto et al., (2023). Medicinal plants have long been used and studied, from the time period when there were no written records to current Ethno pharmacology, including analytical chemistry, gastronomy, and pharmaceuticals. The need for product quality control to ensure detection of any impurities and biomarkers, chemical compositions, and other issues is expected in accordance with the international standards and government agencies related to health matters. In this sense, there needs to be better analytical techniques to uncover more interesting aspects and gather vast amounts of data. Chemo metrics refers to mathematical and statistical techniques [46]. Gonzaga et al. (2024) reported an investigation related to the development and validation of an innovative HPLC-PDA method for the determination of preservatives in milk. The current article discusses a validated analytical technique for identifying sodium benzoate and potassium sorbet in milk. Even though both preservatives are extensively used in the food industry, their inclusion in milk as antimicrobials is forbidden. The proposed method was validated according to ICH guidelines and successfully met all required validation parameters. For sample preparation, milk samples were mixed with acetonitrile in a 1:1 ratio to facilitate extraction. The prepared samples were then centrifuged and filtered to remove impurities before being analyzed using High-Performance Liquid Chromatography coupled with a Photodiode Array detector (HPLC–PDA). Detection and quantification limits for sodium benzoate were 0.204

mg/L and 0.618 mg/L, respectively, while for potassium sorbate, they were 0.108 mg/L and 0.328 mg/L. Accuracy was in the range of 92.67 to 99.53 percent, with pH 4.0 being recognized as the best pH value for separating the compounds. The method's environmental benefits lie in its simple procedure, which uses just one reagent in minimal amounts [47].

CHAPTER NO.3

MATERIAL AND METHOD

3.1 Material Required

3.1.1 Solvents and chemicals

Chemicals used were analytical grade from Merck Germany include sodium dihydrogen phosphate, sodium 1-heptanesulfonate and acetonitrile. PTFE filters having pore size 0.45 μ was used to filter the standard and sample.

3.1.2 Glass apparatus

Volumetric flask 100ml, volumetric flask 250ml, volumetric flask 20ml, bulb pipette 1ml, bulb pipette 2ml, bulb pipette 5ml, bulb pipette 10ml and glass beaker 100ml and pestle mortar.

3.1.3 Standards

Standards of Metformin hydrochloride, Linagliptin, Empagliflozin, Dapagliflozin propanediol monohydrate, Ertugliflozin L-pyroglutamic acid and Sotagliflozin with potency were arranged from Highnoon Laboratories Limited. Potencies of working standards are given in table 3.1.

Table 3.1 Potency of standards used for analysis

Name	LOD / Water	Potency	
		On dried anhydrous basis	On as is basis
Metformin Hydrochloride	0.40%	99.40%	99.00%
Linagliptin	0.17%	99.85%	99.68%
Empagliflozin	0.18%	99.90%	99.72%
Dapagliflozin Propanediol Monohydrate	0.14%	99.92%	99.78%
Ertugliflozin L-pyroglutamic Acid	0.25%	99.75%	99.50%
Sotagliflozin	0.43%	99.93%	99.50%

3.1.4 Products to be analyzed

Glucophage tablet 250mg containing Metformin was purchased from local medical store. Lina 5mg containing Linagliptin 5mg, Diajard Tablet 25mg containing Empagliflozin, Daploz Tablet 10mg containing Dapgliflozin and Inpefa Tablet 200mg containing Sotagliflozin was arranged from Highnoon Laboratories limited. Ertugliflozin Tablet 5mg was taken from CCL pharmaceuticals.

3.1.5 Instruments

The instrument was properly calibrated as per 21CFR compliance HPLC system of Shimadzu LC (Model No. 20AD) with autosampler (Model SIL-20AC), column oven (Model CTO-20A), and UV-Vis Diode Array Detector (Model SPD-M20A).

1. HPLC column Agilent Zorbax Eclipse Plus RP-C18 column (250 × 4.6)mm, 5µm particle size, Agilent Technologies)
2. Analytical balance (Mettler Toledo) Ultrasonic bath (XUBA1)
3. A glass vacuum filtration apparatus (Millipore) equipped with 0.45µm
4. Membrane filters was used for mobile phase preparation.
5. Filtration through 0.45 µm filters

3.1.6 HPLC column

Agilent zorbex eclipse column with stationary phase C18, length 150mm, diameter 4.6mm and particle size 3.5µm was used.

3.1.7 Chromatographic conditions

Flow Rate	1.5ml/min
Column oven temperature	40°C
Injection volume	10µl
Wavelength	225nm

3.1.8 Mobile phase preparation

A gradient mode was used to perform the analysis. Mobile phase A included buffer pH 4.0 which was prepared by dissolving the 1.56g sodium

dihydrogen phosphate and 500mg sodium n heptane sulfonic acid in 1000ml of water and pH is adjusted with diluted phosphoric acid or diluted sodium hydroxide to 4.0. Mobile phase B included the HPLC grade acetonitrile. Gradient program designed to perform the analysis is as followed.

Time (min)	Mobile Phase A (percent v/v)	Mobile Phase B (percent v/v)
0 – 1	90	10
1 – 5	90 – 60	10 - 40
5 – 10	60	40
10 – 16	60 – 40	40 - 60
16.1	90	10

3.1.9 Preparation of diluent

Mobile phase A and mobile phase B were mixed in ratio 70:30 to prepare the sample and standard.

3.1.10 Preparation of standard solution

100mg of Metformin hydrochloride, 99.9mg Linagliptin, 25mg of Empagliflozin, 35mg of Dapagliflozin Propanediol Monohydrate equivalent to 30mg of Dapagliflozin, 32mg Ertugliflozin L-pyroglutamic acid equivalent to 25mg Ertugliflozin and 100mg of Sotagliflozin was taken in 250ml volumetric and dissolved in diluent by sonication then made up volume to 250ml. Shake well to homogenize the standard. Final concentration was 0.4mg/ml Metformin hydrochloride, 0.39mg/ml Linagliptin, 0.1mg/ml Empagliflozin, 0.12mg/ml Dapagliflozin, 0.1mg/ml Ertugliflozin and 0.4mg/ml Sotagliflozin. Filtered the standard with PTFE 0.45µ filters into the HPLC vials.

3.1.11 Preparation of sample solution

Triturate the tablets of each brand selected mention in 2.2.4 in pestle mortar individually. Six individual sample preparations were prepared equivalent to standard concentration. 108.3mg of Glucophage tablet eq. to 100mg of Metformin, 310 mg of Lina Tablet eq. to 5 mg of Linagliptin, 280mg of Diajard Tablet eq. to 25 of Empagliflozin, 900mg of Daloz Tablet eq. to

30mg of Dapadliflozin, 525mg of Ertgliflozin Ttblet eq. to 25mg of Ertugliflozin, 208mg of Inpefa Tablet eq. to 100mg of Sotagliflozin was taken in 250 mg of volumetric flask, 150ml of diluent was added in volumetric flask and sonicate for 30min. Allowed to cool and made up volume with diluent to 250ml. Filtered the sample with PTFE 0.45 μ filters into the HPLC vials.

3.2 Validation

Validation was performed to verify the accuracy, precision, repeatability, LOD, LOQ, intermediate precision and robustness of analytical method according to ICH guideline Q2 (R1) (ICH, 2017).

3.2.1 Precision

The term precision refers to the agreement between observed measurements or succession attained from numerous analysis for the API run randomly under the optimized chromatographic conditions.

3.2.1.1 Repeatability

During repeatability one standard preparation and six individual sample preparations were prepared as described in 2.2.10 and 2.2.11 respectively. Then assay of all six prepared samples was performed through the developed method.

3.2.1.2 Intermediate precision

Same procedure was repeated as described in repeatability, but this time HPLC system was changed. During repeatability HPLC system was used as described in 2.2.5. Intermediate precision was performed on Shimadzu LC20 HPLC equipped with degassing unit DGU-20A5R, LC-20 AT pump, auto-sampler SIL-20ACHT, UV detector SPD-20A, column oven CTO-20AC and CFR 21 compliant software Lab Solutions version 6.83 was used to develop and validate the method. Standard and sample was prepared as mention in 2.2.10 and 2.2.11 respectively.

3.2.2 Linearity

It is defined as the test results are directly proportion to the concentration. Minimum five concentrations are recommended to perform the linearity test by the

guidelines. Stock solution of concentration 1.0mg/ml of each metformin, linagliptin. Empagliflozin, dapagliflozin, ertugliflozin and Sotaliflozin was prepared. Then stock solution was diluted in such way to form the dilutions 0.04mg/ml (40%), 0.06mg/ml (60%), 0.08mg/ml (80%), 0.1mg/ml (100%), 0.12mg/ml (120%), 0.14mg/ml (140%) and 0.16mg/ml (160%). 10µl injection of each dilution 40%, 60%, 80%, 100%, 120%, 140% and 160% was injected in HPLC.

3.2.3 Limit of Detection (LOD)

The standard deviation (SD) of the line of regression (response) and the slope (S) of the calibration curve at sites approaching LOD were used to calculate the detection limit.

Where,

$$\text{Limit of Detection} = \frac{3.3 \times \sigma}{S} \quad \text{----- eq (iii)}$$

σ = y-intercept / Standard Deviation of Regression line

S = Slope of Calibration Curve

3.2.4 Limit of Quantification (LOQ)

The standard deviation (SD) of the line of regression (response) and the slope (S) of the calibration curve at points near the LOQ were used to calculate the value of the quantification limit.

Where's,

σ = Standard Deviation of Regression line / y-intercept

S = Slope of the Calibration Curve

3.2.5 Accuracy

Accuracy of the analytical method used for the analysis of drug product is performed by recovery test. This test is executed by adding a known quantity of active substance to the placebo of the products. For preparation of standard weighed 98.90mg of metformin, 99.90 Linagliptin, 2ND.8mg of empagliflozin, 35.0mg of dapagliflozin, 32.0mg of ertugliflozin and 94.0mg of Sotagliflozin and dissolve in diluent in 100ml flask (dilution-1). Took 10ml from dilution-1 in to another 100ml volumetric flask and made up volume (dilution-2). For preparation of the sample solutions took nine 100ml volumetric flasks. To each of 100ml volumetric flask added

specified quantity of placebo. Took 6 ml of standard from dilution-1 and added to one of the nine flask and made up to the volume with diluent. Repeated this on another two flasks and identified these three flasks. In this way three flasks of 60% dilutions were prepared. In the end three 100% and three 140% dilutions were made by taking the 10ml and 14ml of standard from dilution-1 respectively in to remaining six 100ml volumetric flasks having specified quantity of 34 placebo and properly identified them. Three 60%, three 100% and three 140% dilutions were used to perform the accuracy.

3.2.6 Robustness

It is the capacity of an analytical method to remain unchanged when there is a small change in analytical parameters of a method. Robustness of this method was checked by changing the flow rate, wavelength and column oven temperature. Flow rate is changed from 1.5 to 1.3 and 1.7. Wavelength was changed from 225 to 223 and 225. Oven temperature was changed from 40°C to 38°C and 42°C.

3.2.7 Analytical solution

Stability Solution stability is performed to assess either solution of drugs is stable in diluent or not. Stability of solution also ensures that there is no any chemical reaction between sample and components of diluent used and there is no degradation of drugs during analysis on HPLC Because analysis on HPLC is sometimes lengthy due to longer run time. To perform this test six samples prepared for precision were again analyzed after 24 hours with new freshly prepared standard.

3.2.8 Range

Range defines the upper and lower limit of active substance that an analytical method can analyze in sample with accuracy and precision. Range is derived from linearity study. Range of any analytical method is depend upon the intended use. This particular method is validated for a range 40 to 160%.

3.2.9 Specificity

This test is performed to verify that there is no interference between any peak due to diluent and excipients used to manufacture the pharmaceutical dosage form and the eluted peaks of active substance during HPLC

CHAPTER NO.4

RESULTS

4.1 Method Development

4.1.1 Standard preparation

10 μ l of standard prepared was injected into HPLC. HPLC chromatogram of standard solution can be seen in fig. 4.1.1.1. Retention times of all the components are given in table 4.1.1.1.

<Sample information>

Sample Name	: Precision		
Sample ID	: Std-01		
Date File Name	: Sample 08-Feb-2025-02.lcd		
Method File Name	: AM Method.lcm		
Method File Name	: Sample 08-Feb-2025.lcb		
Vial #	: 1-2	Sample Type	: Unknown
Injection Volume	: 10 μ L		
Date Acquired	: 2/8/2025 3:14:18 PM	Acquired by	: Asif Malik
Date Processed	: 2/8/2025 3:39:19 PM	Processed by	: Asif Malik

<Chromatogram>

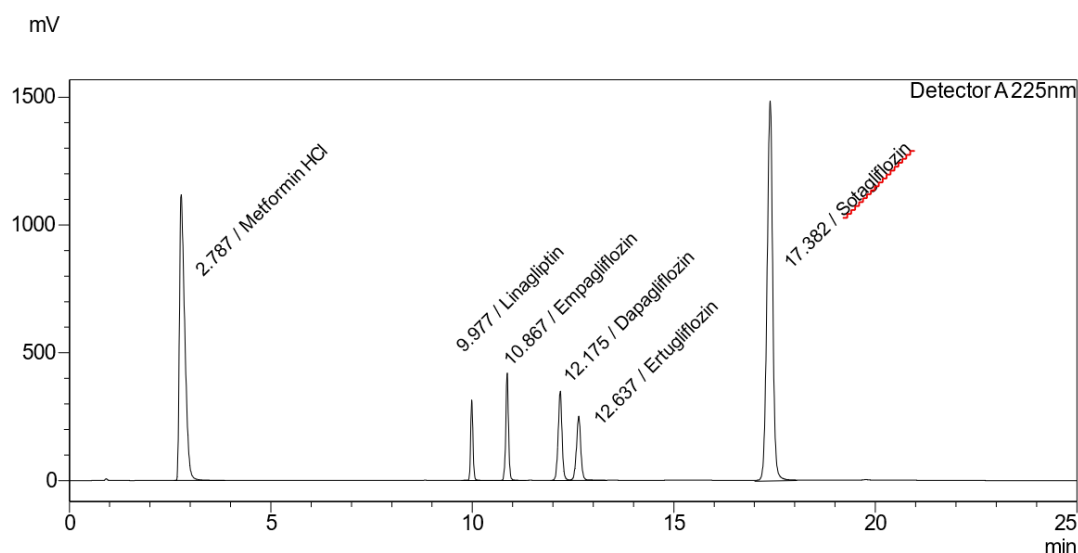


Figure 4.1.1.1 Chromatogram of standard

4.1.2 Sample preparation

Sample prepared from commercial products was injected into the HPLC. Like standard injection volume of sample was also 10 μ l. Chromatogram of sample can be seen in figure 4.2 and retention times of active substance in standard and sample can be seen in table 4.1.2.1.

<Sample information>

Sample Name : Precision
Sample ID : Sample-01
Date File Name : Sample 08-Feb-2025-08.lcd
Method File Name : AM Method.lcm
Method File Name : Sample 08-Feb-2025.lcb
Vial # : 1-3
Injection Volume : 10µL
Date Acquired : 2/8/2025 5:47:02 PM
Date Processed : 2/8/2025 6:12:06 PM

Sample Type : Unknown
Acquired by : Asif Malik
Processed by : Asif Malik

<Chromatogram>

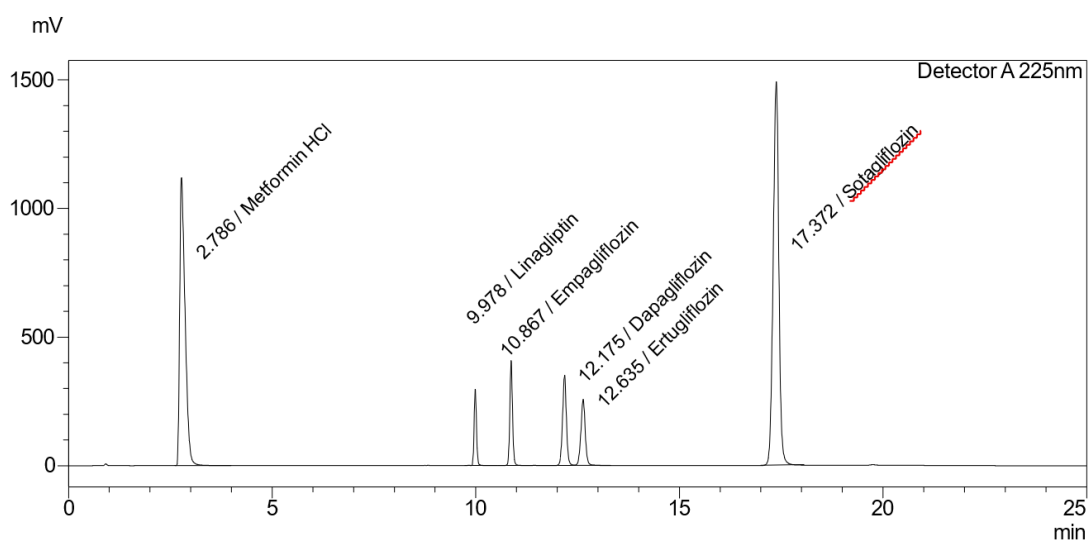


Figure 4.1.2.1 Chromatogram of sample

Table 4.1 Retention times of standard and sample

Name	Retention Time (min)	
	Standard	Sample
Metformin Hydrochloride	2.787	2.786
Linagliptin	9.997	9.978
Empagliflozin	10.867	10.867
Dapagliflozin Propanediol Monohydrate	12.175	12.175
Ertugliflozin L-pyroglutamic Acid	12.637	12.635
Sotagliflozin	17.382	17.372

4.2 Method Validation Results

4.2.1 System Suitability Parameters

This test is performed to check the chromatographic conditions are suitable and effective for further use in analysis. These parameters are tailing factor, number of theoretical plates, resolution, capacity factor and separation factor. Tailing factor (T) describes the symmetry of the peak eluting from the column. Tailing factor increases as the tailing of the peak increases (Ettre, 2003). Number of theoretical plates (N) provides information about the efficiency of the column used as stationary phase. Low number of theoretical plates mean column has lost efficiency of separation (Bose, 2014). Number of theoretical plates should not be less than 2000 but in Metformin HCl theoretical plates not less than 1500 (Patel *et al.*, 2021). Resolution is the separation between the peaks due to two different components eluting from the column at different retention times (Maryutina *et al.*, 2018). Capacity factor describes the time of interaction of active substance with the stationary phase. If the capacity factor is zero its mean there is no interaction between stationary phase and active substance. Ratio of capacity factor of two peaks due to different components is called separation factor. Resolution between the peaks of different components is only possible if rate of migration of these two components is different. All these system suitability parameters are calculated through software used to run the HPLC system (Ahuja & Dong, 2005). Results of these system suitability parameters are given in table 4.2.1.1.

Table 4.2.1.1 *System suitability parameters results*

Name	Number of Theoretical Plates (N)	Tailing Factor (T)	Resolution (Rs)	Capacity Factor (K)	Separation Factor
Metformin	1858	1.531	-	0.834	-
Linagliptin	124040	1.278	38.662	5.566	6.674
Empagliflozin	101999	1.024	7.131	6.124	1.116
Dapagliflozin	68378	1.037	8.116	7.045	1.134
Ertugliflozin	60137	1.003	2.359	7.333	1.041
Sotagliflozin	69943	1.006	20.232	9.362	1.277

4.2.1 Precision

4.2.1.1 Repeatability

Six 10µl injections of standard preparation was injected into HPLC to fulfil the requirement of system suitability. Single injection of 10 µl injection volume of each sample was injected and assay of all the six sample preparations was calculated. Relative standard deviation (RSD) of percentage assay of six sample should not be more than 2.0 (Ethiraj *et al.*, 2011). Summary of precision results can be seen in table 4.2.2.1.7. Individual results of precision of each molecule can be seen in table 4.2.2.1.1 for metformin, table 4.2.2.1.2 for linagliptin, table 4.2.2.1.3 empagliflozin, table 4.2.2.1.4 for dapgliflozin, table 4.2.2.1.5 for ertugliflozin and table 4.2.2.1.6 for Sotagliflozin. RSD of areas of standards should not be more than 2 (Bhatt & Rane, 2011). RSD of standards was 0.08 for metformin, 0.08 for linagliptin, 0.12 for empagliflozin, 0.11 for dapagliflozin, 0.09 for ertugliflozin and 0.06 for Sotagliflozin.

Table 4.2.2.1.1 Precision results of Metformin Hydrochloride

		WS (mg)	P	LC (mg)	MW (mg)	F
		100.00	99.00%	250	268.1	-
Standard (As)		Sample	AT	WT (mg)	Results (%)	Difference
1	10326437	1	10339355	105.10	101.02	1.46
2	10338436	2	10324959	107.30	98.81	-0.76
3	10342032	3	10327189	106.90	99.21	-0.37
4	10350210	4	10338961	107.30	98.95	-0.63
5	10337805	5	10329646	106.50	99.60	0.03
6	10336218	6	10374587	106.70	99.85	0.28
AVG	10338523	AVG	99.57			
STDEV	7756.4	MIN	98.81			
%RSD	0.08	MAX	101.02			
		STDEV	0.81			
		%RSD	0.81			

Table 4.2.2.1.2 Precision results of Linagliptin

		WS (mg)	P	LC (mg)	MW (mg)	F
		99.90	99.68	5	155	0.8060
Standard (As)		Sample	AT	WT (mg)	Results (%)	Difference
1	1227472	1	1252724	310.10	101.50	1.82
2	1228689	2	1230159	311.50	99.23	-0.46
3	1230093	3	1151331	290.10	99.72	0.03
4	1229297	4	1228969	310.90	99.32	-0.37
5	1227485	5	1209751	305.10	99.63	-0.06
6	1228462	6	1217788	309.90	98.74	-0.96
AVG	1228583	AVG	99.69			
STDEV	1025.3	MIN	98.74			
%RSD	0.08	MAX	101.50			
		STDEV	0.95			
		%RSD	0.96			

Table 4.2.2.1.3 Precision results of Empagliflozin

		WS (mg)	P	LC (mg)	MW (mg)	F
		25.00	99.72%	25	282	-
Standard (As)		Sample	AT	WT (mg)	Results (%)	Difference
1	1949732	1	1882933	275.60	98.32	-1.40
2	1955181	2	1954724	277.90	101.22	1.51
3	1956636	3	1882669	275.50	98.34	-1.38
4	1955396	4	1954040	280.10	100.39	0.68
5	1953398	5	1938054	278.60	100.11	0.39
6	1954499	6	1945583	280.20	99.92	0.21
AVG	1954140.3	AVG	99.72			
STDEV	2408.4	MIN	98.32			
%RSD	0.12	MAX	101.22			
		STDEV	1.16			
		%RSD	1.17			

Table 4.2.2.1.4 Precision results of Dapagliflozin Propanediol Monohydrate

		WS (mg)	P	LC (mg)	MW (mg)	F
		35.00	99.78%	10	301.2	0.8486
Standard (As)		Sample	AT	WT (mg)	Results (%)	Difference
1	2350417	1	2338919	900.10	98.49	-0.33
2	2357910	2	2354051	900.90	99.04	0.22
3	2355129	3	2338731	900.00	98.49	-0.33
4	2355747	4	2348145	900.40	98.85	0.03
5	2355572	5	2351579	900.10	99.02	0.21
6	2355511	6	2352537	900.50	99.02	0.20
AVG	2355047.7	AVG	98.82			
STDEV	2474.7	MIN	98.49			
%RSD	0.11	MAX	99.04			
		STDEV	0.26			
		%RSD	0.27			

Table 4.2.2.1.5 Precision results of Ertugliflozin L-pyrogutamine Acid

		WS (mg)	P	LC (mg)	MW (mg)	F
		32.00	99.50%	5	101.9	0.7716
Standard (As)		Sample	AT	WT (mg)	Results (%)	Difference
1	1892046	1	1920516	500.20	101.65	1.58
2	1891853	2	1891244	500.90	99.96	-0.11
3	1893119	3	1916933	505.30	100.44	0.37
4	1891636	4	1895436	507.10	98.96	-1.11
5	1888131	5	1899065	503.90	99.78	-0.29
6	1890468	6	1889869	502.20	99.63	-0.44
AVG	1891208.8	AVG	100.07			
STDEV	1730.3	MIN	98.96			
%RSD	0.09	MAX	101.65			
		STDEV	0.91			
		%RSD	0.91			

Table 4.2.2.1.6 Precision results of Sotagliflozin

		WS (mg)	P	LC (mg)	MW (mg)	F
		100.00	99.50%	200	420.1	-
Standard (As)		Sample	AT	WT (mg)	Results (%)	Difference
1	14488365	1	14501223	210.60	99.27	0.02
2	14505763	2	14474826	210.00	99.37	0.12
3	14493058	3	14499320	211.20	98.98	-0.28
4	14508122	4	14449276	208.60	99.86	0.62
5	14487992	5	14512009	210.10	99.58	0.33
6	14495938	6	14489277	212.20	98.44	-0.82
AVG	14496540	AVG	99.25			
STDEV	8620.8	MIN	98.44			
%RSD	0.06	MAX	99.86			
		STDEV	0.50			
		%RSD	0.50			

Table 4.2.2.1.7 Summary of repeatability results

Name of Drugs	1	2	3	4	5	6	Avg	RSD
Metformin	101.02	98.81	99.21	98.95	99.60	99.85	99.57	0.81
Linagliptin	101.50	99.23	99.72	99.32	99.63	98.74	99.69	0.96
Empagliflozin	98.23	101.22	98.34	100.39	100.11	99.92	99.72	1.17
Dapagliflozin	98.49	99.04	98.49	98.85	99.02	99.02	98.82	0.27
Ertugliflozin	101.65	99.96	100.44	98.96	99.78	99.63	100.07	0.91
Sotagliflozin	99.27	99.37	99.98	99.86	99.58	98.44	99.25	0.50

Note: As per ICH Q2(R2) guidelines Precision results RSD not more than 2.

4.2.1.1 Intermediate precision

Same repeatability procedure was proceeded in intermediate precision by different analyst on different HPLC system. Results of intermediate precision can be seen in table 4.2.2.2.1.1 for metformin, table 4.2.2.2.2 for Linagliptin, table 4.2.2.2.3 empagliflozin, table 4.2.2.2.4 for dapgliflozin, table 4.2.2.2.5 for Ertugliflozin and table 4.2.2.2.6 for Sotagliflozin.

Table 4.2.2.2.1 Intermediate precision results of Metformin

		WS (mg)	P	LC (mg)	MW (mg)	F
		100.23	99.00%	250	268.1	-
Standard (As)		Sample	AT	WT (mg)	Results (%)	Difference
1	10714090	1	10528651	108.00	99.50	-0.32
2	10719738	2	10617749	109.70	98.78	-1.03
3	10681240	3	10570351	107.20	100.64	0.82
4	10701574	4	10613649	108.40	99.93	0.11
5	10701552	5	10576684	108.10	99.86	0.04
6	10695567	6	10631890	108.30	100.19	0.38
AVG	10702294	AVG	99.82			
STDEV	13660.4	MIN	98.78			
%RSD	0.13	MAX	100.64			
		STDEV	0.63			
		%RSD	0.63			

Table 4.2.2.2.2 Intermediate precision results of Linagliptin

		WS (mg)	P	LC (mg)	MW (mg)	F
Standard (As)		Sample	AT	WT (mg)	Results (%)	Difference
1	1252472	1	1250724	310.10	98.83	-0.18
2	1253689	2	1251159	311.50	99.57	0.57
3	1252093	3	1250331	290.10	98.50	-0.52
4	1251297	4	1250969	310.90	98.80	-0.21
5	1253485	5	1252751	305.10	99.64	0.64
6	1258462	6	1250788	309.90	98.71	-0.30
AVG	1252472	AVG	99.01			
STDEV	1025.3	MIN	98.50			
%RSD	0.08	MAX	99.64			
		STDEV	0.48			
		%RSD	0.48			

Table 4.2.2.2.3 Intermediate precision results of Empagliflozin

		WS (mg)	P	LC (mg)	MW (mg)	F
		25.08	99.72%	25	282	-
Standard (As)		Sample	AT	WT (mg)	Results (%)	Difference
1	1982962	1	1940038	279.30	99.49	-0.05
2	1994420	2	1967728	281.20	100.22	0.69
3	1987087	3	1940782	280.00	99.28	-0.27
4	1991021	4	1936444	278.20	99.69	0.15
5	1991726	5	1936097	279.00	99.39	-0.15
6	1989512	6	1946354	281.10	99.17	-0.37
AVG	1989454.7	AVG	99.54			
STDEV	4000.8	MIN	99.17			
%RSD	0.20	MAX	100.22			
		STDEV	0.38			
		%RSD	0.38			

Table 4.2.2.2.4 Intermediate precision results of Dapagliflozin

		WS (mg)	P	LC (mg)	MW (mg)	F
		35.9	99.78%	10	301.2	0.8486
Standard (As)		Sample	AT	WT (mg)	Results (%)	Difference
1	2411460	1	2350367	900.10	98.59	-0.84
2	2412581	2	2390087	900.90	100.05	0.63
3	2402456	3	2363243	900.00	99.45	0.02
4	2406161	4	2368226	900.40	99.19	-0.24
5	2407139	5	2363502	900.10	99.13	-0.29
6	2404840	6	2382587	900.50	100.14	0.72
AVG	2407439.5	AVG	99.43			
STDEV	3896.6	MIN	98.59			
%RSD	0.16	MAX	100.14			
		STDEV	0.59			
		%RSD	0.59			

Table 4.2.2.2.5 Intermediate precision results of Ertugliflozin

		WS (mg)	P	LC (mg)	MW (mg)	F
		32.42	99.50%	5	101.9	0.7716
Standard (As)		Sample	AT	WT (mg)	Results (%)	Difference
1	1891646	1	1838529	500.20	99.46	-0.36
2	1893390	2	1877238	500.90	100.72	0.91
3	1890557	3	1850099	505.30	99.81	0.00
4	1892264	4	1852875	507.10	99.45	-0.36
5	1891615	5	1847991	503.90	99.51	-0.30
6	1891646	6	1862983	502.20	99.92	0.11
AVG	1891853	AVG	99.81			
STDEV	933.1	MIN	99.45			
%RSD	0.05	MAX	100.72			
		STDEV	0.49			
		%RSD	0.49			

Table 4.2.2.2.6 Intermediate precision results of Sotagliflozin

		WS (mg)	P	LC (mg)	MW (mg)	F
		100.02	98.50%	200	420.1	-
Standard (As)		Sample	AT	WT (mg)	Results (%)	Difference
1	14498365	1	14507223	210.60	100.00	0.12
2	14505763	2	14476826	210.00	99.78	-0.10
3	14493058	3	14497320	211.20	100.72	0.85
4	14508122	4	14448276	208.60	100.12	0.25
5	14497992	5	14515009	210.10	99.31	-0.57
6	14495938	6	14486277	212.20	99.33	-0.55
AVG	14496540	AVG	99.88			
STDEV	8620.8	MIN	99.31			
%RSD	0.11	MAX	100.72			
		STDEV	0.53			
		%RSD	0.53			

Table 4.2.2.2.7 Summary of intermediate repeatability results

Name of Drugs	1	2	3	4	5	6	Avg	RSD
Metformin	99.50	98.78	100.64	99.93	99.86	100.19	99.82	0.63
Linagliptin	98.83	99.57	98.50	98.80	99.64	98.71	99.01	0.48
Empagliflozin	99.49	100.22	99.28	99.69	99.39	99.17	99.54	0.38
Dapagliflozin	98.59	100.05	99.45	99.19	99.13	100.14	99.43	0.59
Ertugliflozin	99.46	100.72	99.81	99.45	99.51	99.92	99.81	0.49
Sotagliflozin	100.00	99.78	100.72	100.12	99.31	99.33	99.88	0.53

Note: As per ICH Q2(R2) guidelines Intermediate Precision results RSD not more than 2.

4.2.2 Linearity

Slope was drawn between area of each dilution prepared for linearity and concentration 40%, 60%, 80%, 100%, 120%, 140% and 160%. A linear curve was obtained for each molecule which can be seen in figures 4.3 for metformin, 4.4 for linagliptin, 4.5 for empagliflozin, 4.6 for dapagliflozin, 4.5 for ertugliflozin and 4.6 for Sotagliflozin. Correlation co-efficient was calculated for each curve. Values of correlation co-efficient were 0.9998, 1.0000, 1.0000, 1.0000, 0.9998 and 0.9943 for metformin, Linagliptin, empagliflozin, dapagliflozin, ertugliflozin and Sotagliflozin respectively.

Concentration ppm (x)	Area (y)	Linest (y=mx+b)
40	4247215	4377745.1
60	6357938	6327531.0
80	8335399	8277316.9
100	10341456	10227102.7
120	12243141	12176888.6
140	14049927	14126674.4
160	16014643	16076460.3
Correlation co- efficient	0.9998	
Y- Intercept (b)	478173.4	
% Y- Intercept	5.74	
Slope (m)	97489.3	
Linest equation	y = 11467.6x + (-35294.4)	
STEYX	4155.66	
LOD	0.51	
LOQ	1.55	

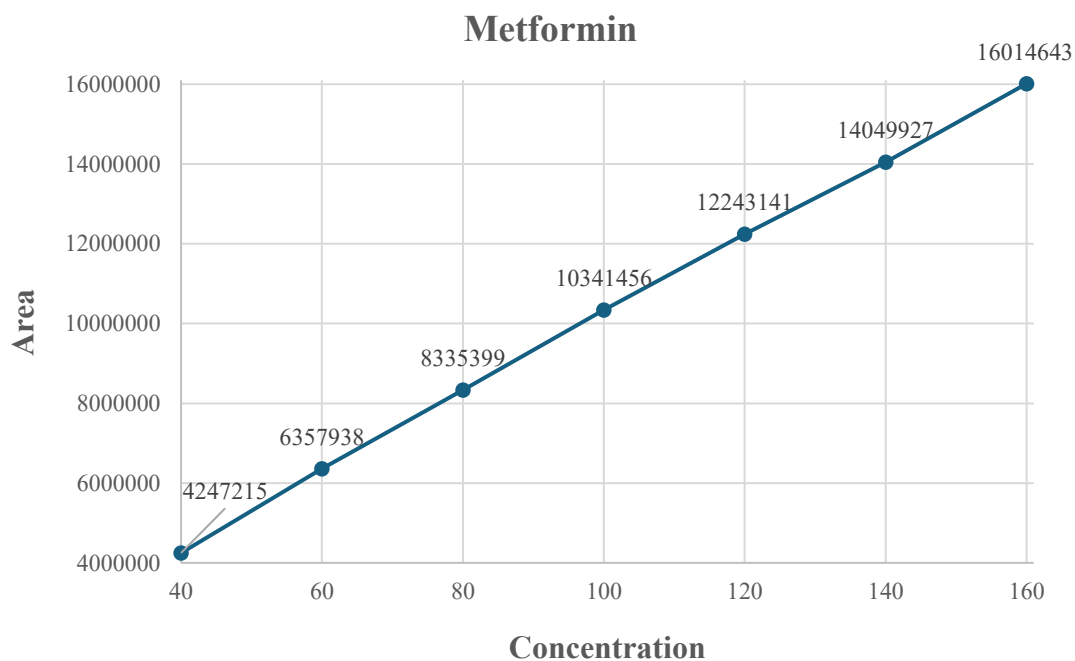


Figure 4.3.1 Linearity curve of Metformin

Concentration ppm (x)	Area (y)	Linest (y=mx+b)
40	498227	501033.9
60	745646	743520.6
80	988771	986007.4
100	1230034	1228494.1
120	1470818	1470980.9
140	1704334	1713467.6
160	1961629	1955954.4
Correlation co- efficient	1.0000	
Y- Intercept (b)	16060.4	
% Y- Intercept	1.62	
Slope (m)	12124.3	
Linest equation	$y = 11467.6x + (-35294.4)$	
STEYX	1481.64	
LOD	5.52	
LOQ	16.73	

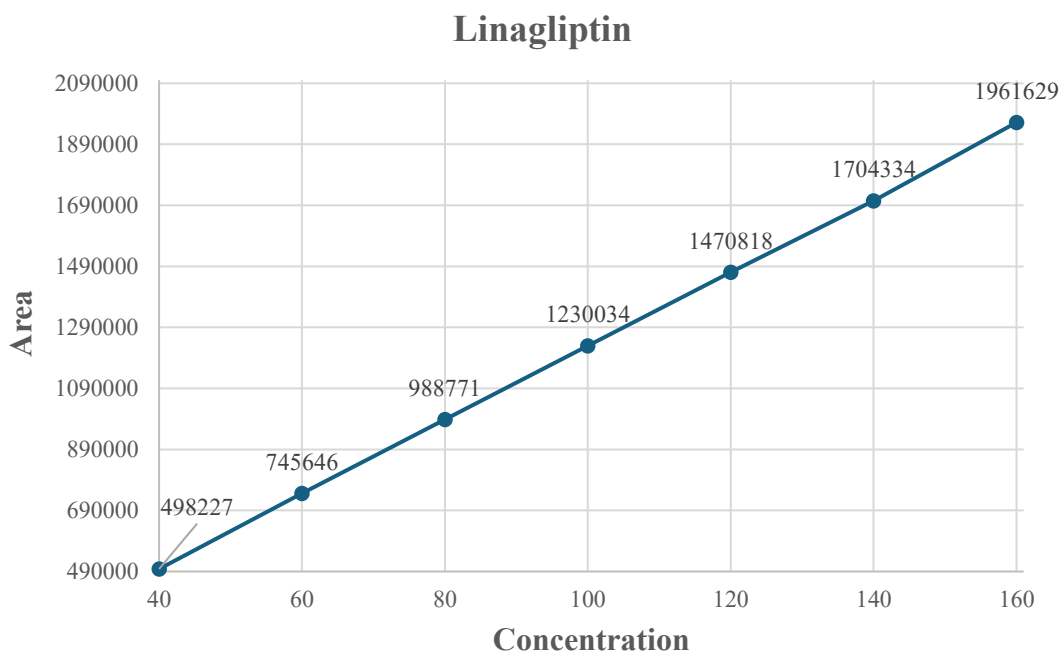


Figure 4.3.2 Linearity curve of Linagliptin

Concentration ppm (x)	Area (y)	Linest (y=mx+b)
40	790310	795101.4
60	1187146	1180814.1
80	1565818	1566526.8
100	1956532	1952239.4
120	2339139	2337952.1
140	2708337	2723664.8
160	3118394	3109377.5
Correlation co- efficient	1.0000	
Y- Intercept (b)	23676.0	
% Y- Intercept	1.51	
Slope (m)	19285.6	
Linest equation	y = 11467.6x + (-35294.4)	
STEYX	17321.15	
LOD	2.93	
LOQ	8.89	

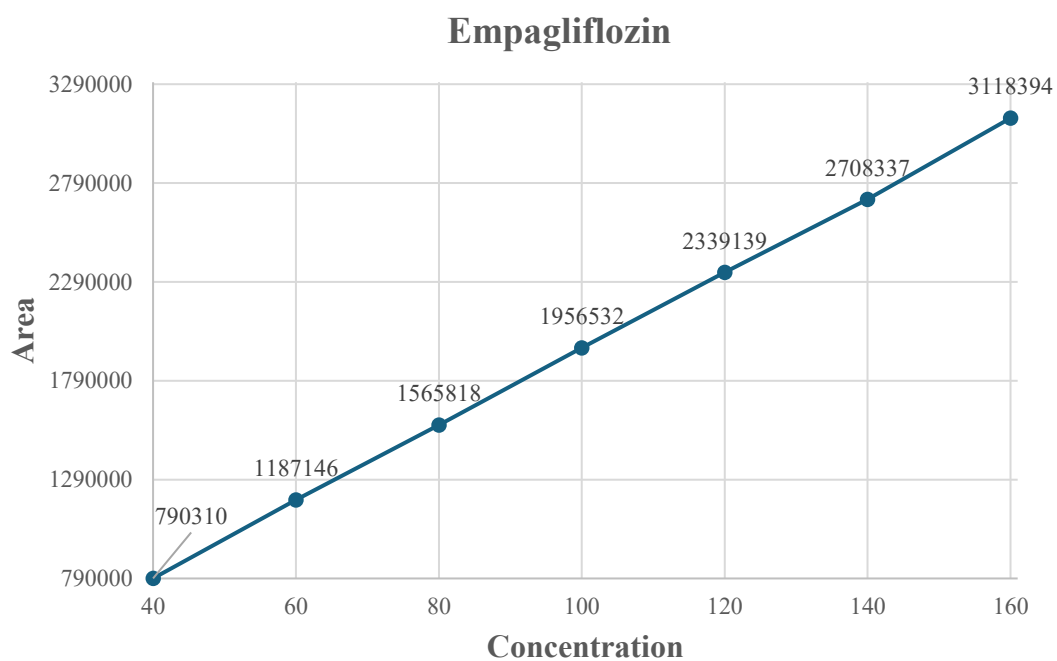


Figure 4.3.3 Linearity curve of Empagliflozin

Concentration ppm (x)	Area (y)	Linest (y=mx+b)
40	943161	944378.8
60	1419170	1411938.2
80	1877045	1879497.6
100	2347420	2347057.0
120	2811384	2814616.4
140	3268510	3282175.8
160	3762709	3749735.2
Correlation co- efficient	1.0000	
Y- Intercept (b)	9260.0	
% Y- Intercept	0.49	
Slope (m)	23378.0	
Linest equation	y = 11467.6x + (-35294.4)	
STEYX	4627.17	
LOD	0.90	
LOQ	2.72	

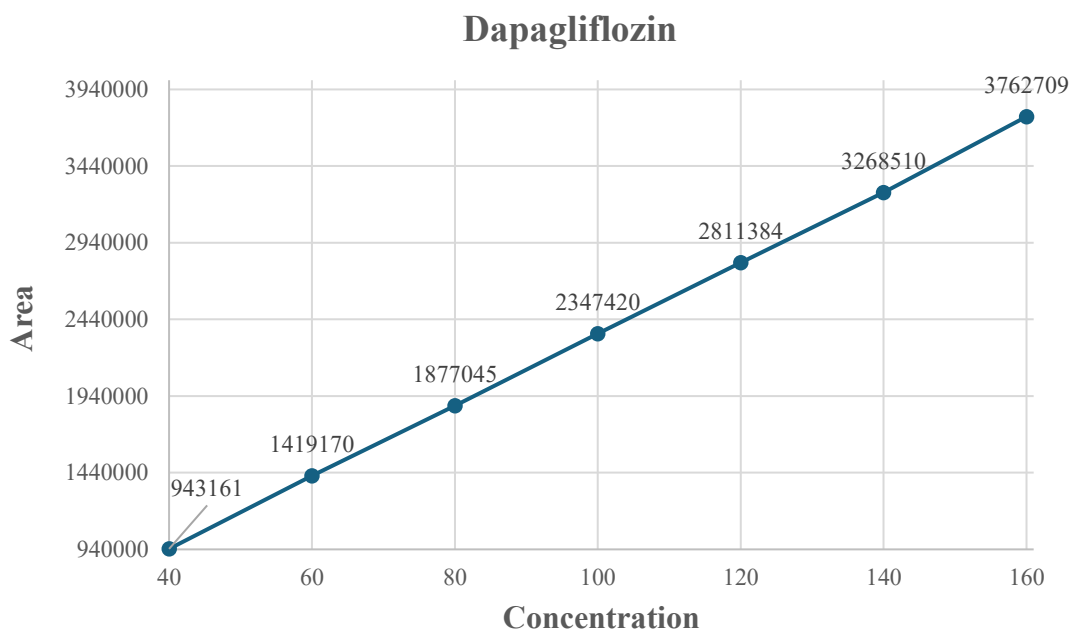


Figure 4.3.4 Linearity curve of Dapagliflozin

Concentration ppm (x)	Area (y)	Linest (y=mx+b)
40	763790	765282.4
60	1147355	1141357.1
80	1517132	1517431.8
100	1897488	1893506.4
120	2270057	2269581.1
140	2612923	2645655.8
160	3045800	3021730.5
Correlation co- efficient	0.9998	
Y- Intercept (b)	13133.0	
% Y- Intercept	0.87	
Slope (m)	18803.7	
Linest equation	y = 11467.6x + (-35294.4)	
STEYX	4799.87	
LOD	1.09	
LOQ	3.32	

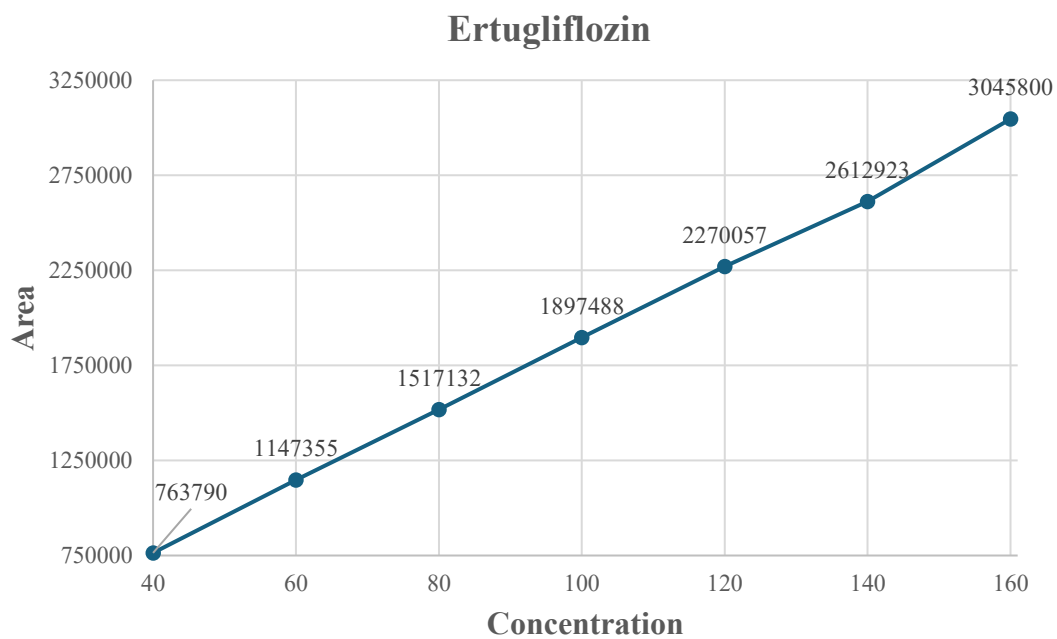


Figure 4.3.5 Linearity curve of Ertugliflozin

Concentration ppm (x)	Area (y)	Linest (y=mx+b)
40	6243722	6948431.5
60	9255143	9268072.3
80	11999260	11586713.1
100	14486868	13905353.9
120	16705793	16223994.6
140	18483212	18542635.4
160	20163479	20861276.2
Correlation co- efficient	0.9943	
Y- Intercept (b)	2312149.9	
% Y- Intercept	19.27	
Slope (m)	115932.0	
Linest equation	y = 11467.6x + (-35294.4)	
STEYX	1633.85	
LOD	0.46	
LOQ	1.38	

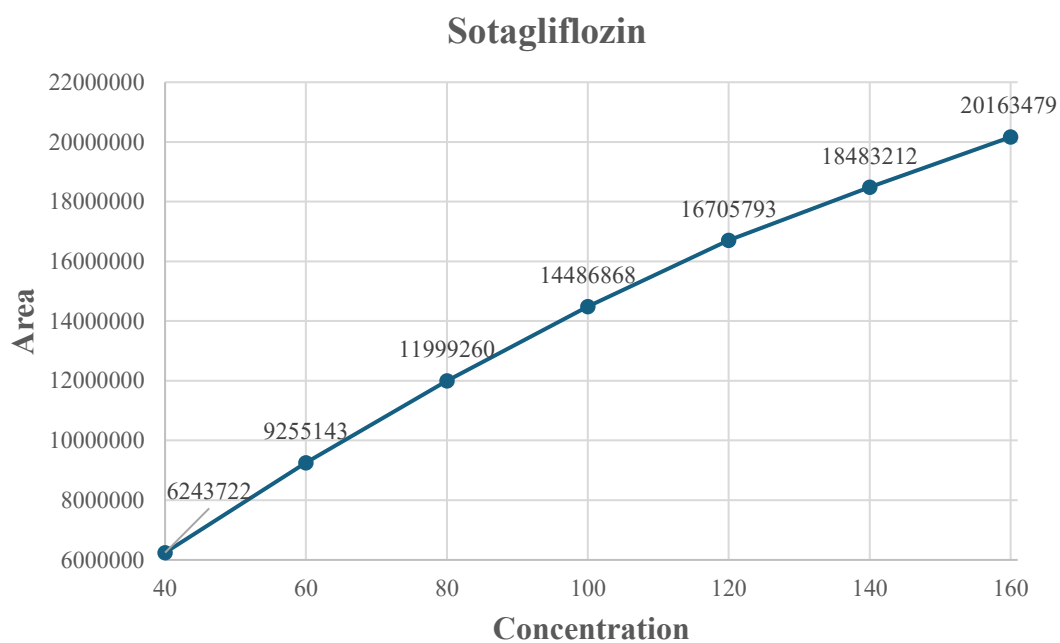


Figure 4.3.6 Linearity curve of Sotagliflozin

4.2.3 LOD/LOQ

Values of LOQ and LOD of all the components can be seen in table 4.17.

Table 4.2.4.1 LOD and LOQ values

Name of Drugs	Concentration	Y-Intercept	Slope	LOD (µg/ml)	LOQ (µg/ml)
Metformin	40-160	478173.4	97489.3	0.51	1.55
Linagliptin	40-160	16060.4	12124.3	5.52	16.73
Empagliflozin	40-160	23676.0	19285.6	2.93	8.89
Dapagliflozin	40-160	9260.0	23378.0	0.90	2.72
Ertugliflozin	40-160	13133.0	18803.7	1.09	3.32
Sotagliflozin	40-160	2312149.9	115932.0	0.46	1.38

Note: As per ICH Q2 (R2) guidelines Correlation co-efficient Value is never less than 0.99.

4.2.4 Accuracy

Six 10µl injection of standard from dilution-2 and one 10µl injection from each of nine samples were injected into the HPLC. Results of percentage recovery can be seen in table 4.2.5.1, 4.2.5.2, 4.2.5.3, 4.2.5.4, 4.2.5.5 and 4.2.5.6 for metformin, Linagliptin, empagliflozin, dapagliflozin, ertugliflozin and Sotagliflozin respectively. Summary of recovery results is summarized in table 4.2.5.7.

Table 4.2.5.1 Recovery results of Metformin

Standard		WS (mg)	98.90
1	10310593	P	99.00%
2	10308525	F	-
3	10311644		
4	10306568		
5	10320363		
6	10320765		
AVG	10313076.3		
STDEV	6058.1		
% RSD	0.06		

Sample	Standard Average Area	Sample Area	Actual Content (ppm)	Weight of Std. for Stock Solution (mg)	Theoretical Content (ppm)	% Recovery
60%	10313076.3	6355536	301.69	100.00	297.00	101.58
		6358796	301.85	100.00	297.00	101.63
		6289713	298.57	100.00	297.00	100.53
Average Recovery						101.25

Sample	Standard Average Area	Sample Area	Actual Content (ppm)	Weight of Std. for Stock Solution (mg)	Theoretical Content (ppm)	% Recovery
100%	10313076.3	10318710	489.82	100.00	495.00	98.95
		10328967	490.31	100.00	495.00	99.05
		10322222	489.99	100.00	495.00	98.99
Average Recovery						99.00

Sample	Standard Average Area	Sample Area	Actual Content (ppm)	Weight of Std. for Stock Solution (mg)	Theoretical Content (ppm)	% Recovery
140%	10313076.3	14321508	679.83	100.00	693.00	98.10
		14417217	684.38	100.00	693.00	98.76
		14427719	684.87	100.00	693.00	98.83
Average Recovery						98.43

Table 4.2.5.2 Recovery results of Linagliptin

Standard		WS (mg)	99.90
1	1228361	P	99.68%
2	1228387	F	-
3	1227480		
4	1227283		
5	1227163		
6	1227009		
AVG	1227613.8		
STDEV	608.7		
% RSD	0.05		

Sample	Standard Average Area	Sample Area	Actual Content (ppm)	Weight of Std. for Stock Solution (mg)	Theoretical Content (ppm)	% Recovery
60%	1227613.8	722814	293.16	100.00	299.04	98.03
		741522	300.75	100.00	299.04	100.57
		737179	298.99	100.00	299.04	99.98
Average Recovery						99.53

Sample	Standard Average Area	Sample Area	Actual Content (ppm)	Weight of Std. for Stock Solution (mg)	Theoretical Content (ppm)	% Recovery
100%	1227613.8	1232174	499.75	100.00	498.40	100.27
		1249464	506.76	100.00	498.40	101.68
		1220275	494.93	100.00	498.40	99.30
Average Recovery						100.97

Sample	Standard Average Area	Sample Area	Actual Content (ppm)	Weight of Std. for Stock Solution (mg)	Theoretical Content (ppm)	% Recovery
140%	1227613.8	1784816	723.89	100.00	697.76	103.75
		1711435	694.13	100.00	697.76	99.48
		1727861	700.79	100.00	697.76	100.43
Average Recovery						101.61

Table 4.2.5.3 Recovery results of Empagliflozin

Standard		WS (mg)	24.80
1	1951101	P	99.72%
2	1952042	F	-
3	1951185		
4	1951998		
5	1951376		
6	1952106		
AVG	1951634.7		
STDEV	463.5		
% RSD	0.02		

Sample	Standard Average Area	Sample Area	Actual Content (ppm)	Weight of Std. for Stock Solution (mg)	Theoretical Content (ppm)	% Recovery
60%	1951634.7	1163943	73.75	25.00	74.79	98.60
		1166622	73.92	25.00	74.79	98.83
		1199788	76.02	25.00	74.79	101.64
Average Recovery						99.69

Sample	Standard Average Area	Sample Area	Actual Content (ppm)	Weight of Std. for Stock Solution (mg)	Theoretical Content (ppm)	% Recovery
100%	1951634.7	1959280	124.14	25.00	124.65	99.59
		1969713	124.80	25.00	124.65	100.12
		1982257	125.59	25.00	124.65	100.76
Average Recovery						100.15

Sample	Standard Average Area	Sample Area	Actual Content (ppm)	Weight of Std. for Stock Solution (mg)	Theoretical Content (ppm)	% Recovery
140%	1951634.7	2722162	172.47	25.00	174.51	98.83
		2721006	172.40	25.00	174.51	98.79
		2744228	173.87	25.00	174.51	99.63
Average Recovery						99.09

Table 4.2.5.4 Recovery results of Dapagliflozin

Standard		WS (mg)	35.0
1	2343352	P	99.78%
2	2344460	F	0.8486
3	2343563		
4	2343424		
5	2342669		
6	2349443		
AVG	2344485.2		
STDEV	2495.6		
% RSD	0.11		

Sample	Standard Average Area	Sample Area	Actual Content (ppm)	Weight of Std. for Stock Solution (mg)	Theoretical Content (ppm)	% Recovery
60%	2344485.2	1417802	89.61	30.00	89.80	99.79
		1416785	89.54	30.00	89.80	99.71
		1484103	93.80	30.00	89.80	104.45
Average Recovery						101.32

Sample	Standard Average Area	Sample Area	Actual Content (ppm)	Weight of Std. for Stock Solution (mg)	Theoretical Content (ppm)	% Recovery
100%	2344485.2	2343957	148.14	30.00	149.67	98.98
		2337292	147.72	30.00	149.67	98.70
		2328430	147.16	30.00	149.67	98.33
Average Recovery						98.67

Sample	Standard Average Area	Sample Area	Actual Content (ppm)	Weight of Std. for Stock Solution (mg)	Theoretical Content (ppm)	% Recovery
140%	2344485.2	3250742	205.46	30.00	209.54	98.05
		3274122	206.93	30.00	209.54	98.76
		3302028	208.70	30.00	209.54	99.60
Average Recovery						98.80

Table 4.2.5.5 Recovery results of Ertugliflozin

Standard		WS (mg)	32.0
1	1892769	P	99.20%
2	1891934	F	0.7716
3	1891176		
4	1890594		
5	1891864		
6	1883526		
AVG	1890310.5		
STDEV	3404.5		
% RSD	0.18		

Sample	Standard Average Area	Sample Area	Actual Content (ppm)	Weight of Std. for Stock Solution (mg)	Theoretical Content (ppm)	% Recovery
60%	1890310.5	1151277	74.59	25.00	74.40	100.25
		1159726	75.14	25.00	74.40	100.99
		1154871	74.82	25.00	74.40	100.57
Average Recovery						100.60

Sample	Standard Average Area	Sample Area	Actual Content (ppm)	Weight of Std. for Stock Solution (mg)	Theoretical Content (ppm)	% Recovery
100%	1890310.5	2343957	148.14	30.00	149.67	98.98
		2337292	147.72	30.00	149.67	98.70
		2328430	147.16	30.00	149.67	98.33
Average Recovery						99.93

Sample	Standard Average Area	Sample Area	Actual Content (ppm)	Weight of Std. for Stock Solution (mg)	Theoretical Content (ppm)	% Recovery
140%	1890310.5	2686308	174.04	25.00	173.60	100.25
		2645974	171.43	25.00	173.60	98.75
		2670784	173.03	25.00	173.60	99.67
Average Recovery						99.56

Table 4.2.5.6 Recovery results of Sotagliflozin

Standard		WS (mg)	94.00
1	14491214	P	99.50%
2	14501121	F	-
3	14468565		
4	14510279		
5	14424739		
6	14453106		
AVG	14474837		
STDEV	32362.2		
% RSD	0.22		

Sample	Standard Average Area	Sample Area	Actual Content (ppm)	Weight of Std. for Stock Solution (mg)	Theoretical Content (ppm)	% Recovery
60%	14474837	9250206	298.85	100.00	298.50	100.12
		9258815	299.13	100.00	298.50	100.21
		9374110	302.86	100.00	298.50	101.46
Average Recovery						100.60

Sample	Standard Average Area	Sample Area	Actual Content (ppm)	Weight of Std. for Stock Solution (mg)	Theoretical Content (ppm)	% Recovery
100%	14474837	15268281	493.28	100.00	497.50	99.15
		15486092	500.32	100.00	497.50	100.57
		15287788	493.91	100.00	497.50	99.28
Average Recovery						99.67

Sample	Standard Average Area	Sample Area	Actual Content (ppm)	Weight of Std. for Stock Solution (mg)	Theoretical Content (ppm)	% Recovery
140%	14474837	21446110	692.88	100.00	696.50	99.48
		21430552	692.37	100.00	696.50	99.41
		21488559	694.25	100.00	696.50	99.68
Average Recovery						99.52

Table 4.2.5.7 Summary of recovery study results

Name of drug	Spiked concentration	% Recovery	Average % Recovery
Metformin	60%	101.58	101.25
		101.63	
		100.53	
	100%	98.95	99.00
		99.05	
		98.99	
	140%	98.10	98.43
		98.76	
		98.83	
Linagliptin	60%	98.03	99.53
		100.57	
		99.98	
	100%	100.27	100.97
		101.68	
		99.30	
	140%	103.75	101.61
		99.48	
		100.43	
Empagliflozin	60%	98.60	99.69
		98.83	
		101.64	
	100%	99.59	100.15
		100.12	
		100.76	
	140%	98.83	99.09
		98.79	
		99.63	

Name of drug	Spiked concentration	% Recovery	Average % Recovery
Dapagliflozin	60%	99.79	101.32
		99.71	
		104.45	
	100%	98.98	98.67
		98.70	
		98.33	
	140%	98.05	98.80
		98.76	
		99.60	
Ertugliflozin	60%	100.25	100.60
		100.99	
		100.57	
	100%	99.36	99.93
		99.96	
		100.47	
	140%	100.25	99.56
		98.75	
		99.67	
Sotagliflozin	60%	100.12	100.60
		100.21	
		101.46	
	100%	99.15	99.67
		100.57	
		99.28	
	140%	99.48	99.52
		99.41	
		99.68	

4.2.5 Robustness

Five injections of 10µl of standard prepared for precision was injected in HPLC during each change. RSD of retention time and areas of standards was calculated. RSD of retention time (RT) and area should not be more than 2.0 according to guidelines. Values of RSD are shown in table 4.2.6.1. Resolution between the peaks of drugs was closely observed during each change in parameters. Comparison of resolution during normal parameters and changed parameters is presented in table Chromatograms of standard under normal and robust conditions can be seen in figure 4.2.6.1 under normal condition, figure 4.2.6.2 at wavelength 223nm, figure 4.2.6.3 at wavelength 227nm, figure 4.2.6.4 at column oven 38°C, figure 4.2.6.5 at column oven 42°C, figure 4.2.6.6 at flow rate 1.3min/ml and figure 4.2.6.7 at flow rate 1.7ml/min.

Table 4.2.6.1 Resolution comparison of peaks during robustness study

Chromatographic Parameters	Resolution						
	Normal Conditions	Wavelength		Flow Rate		Oven Temperature	
		223	227	1.3	1.7	38°C	40°C
Metformin	-	-	-	-	-	-	-
Linagliptin	28.916	29.668	28.712	28.620	30.879	28.691	30.320
Empagliflozin	8.332	8.148	8.022	8.083	8.243	7.959	8.391
Dapagliflozin	9.508	9.279	9.164	9.634	9.133	9.240	9.279
Ertugliflozin	2.723	2.639	2.610	2.743	2.595	2.648	2.578
Sotagliflozin	15.250	14.459	14.434	16.094	13.830	14.511	14.392

Table 4.2.6.2 *RSD of area and retention time (RT) during robustness study*

Chromatographic Parameters	Wavelength 223 (RSD)		Wavelength 227 (RSD)		Flow Rate 1.3ml/min (RSD)		Flow Rate 1.7ml/min (RSD)		Oven Temperature 38°C (RSD)		Oven Temperature 42°C (RSD)	
	Area	RT	Area	RT	Area	RT	Area	RT	Area	RT	Area	RT
Metformin	0.48	0.46	0.97	0.45	1.30	0.51	0.48	0.27	0.64	0.36	0.99	0.46
Linagliptin	0.41	0.04	0.61	0.05	0.40	0.42	0.41	0.01	0.70	0.13	0.75	0.03
Empagliflozin	0.43	0.02	0.64	0.02	0.37	0.19	0.42	0.08	0.78	0.05	0.79	0.03
Dapagliflozin	0.42	0.06	0.67	0.04	0.37	0.16	0.44	0.03	0.70	0.06	0.79	0.06
Ertugliflozin	0.43	0.07	0.08	0.06	0.38	0.14	0.44	0.03	0.74	0.06	0.77	0.03
Sotagliflozin	0.37	0.03	0.61	0.02	0.38	0.12	0.38	0.04	0.61	0.03	0.71	0.03

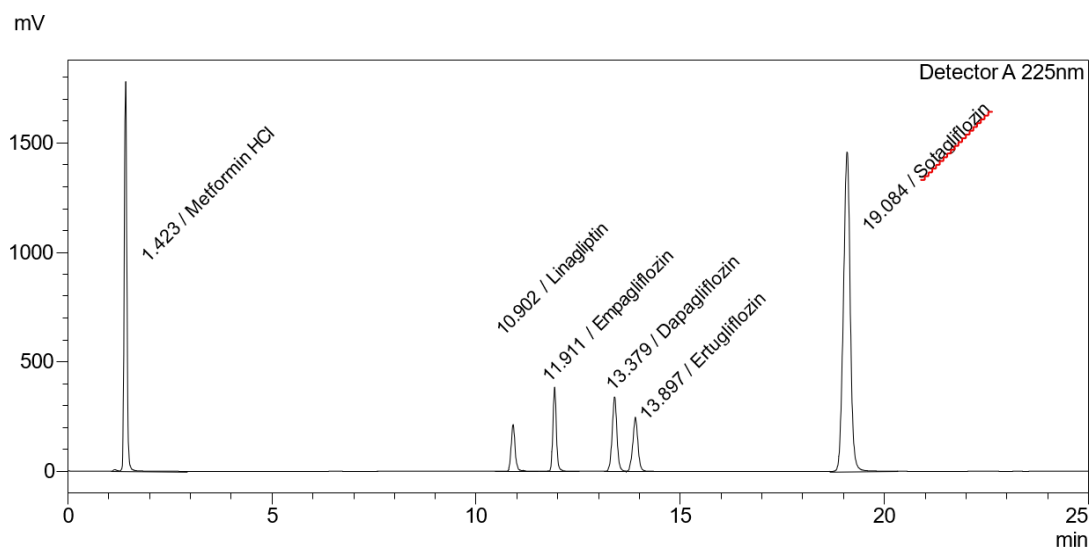


Figure 4.2.6.1 Standard chromatogram under normal chromatographic condition (Robustness)

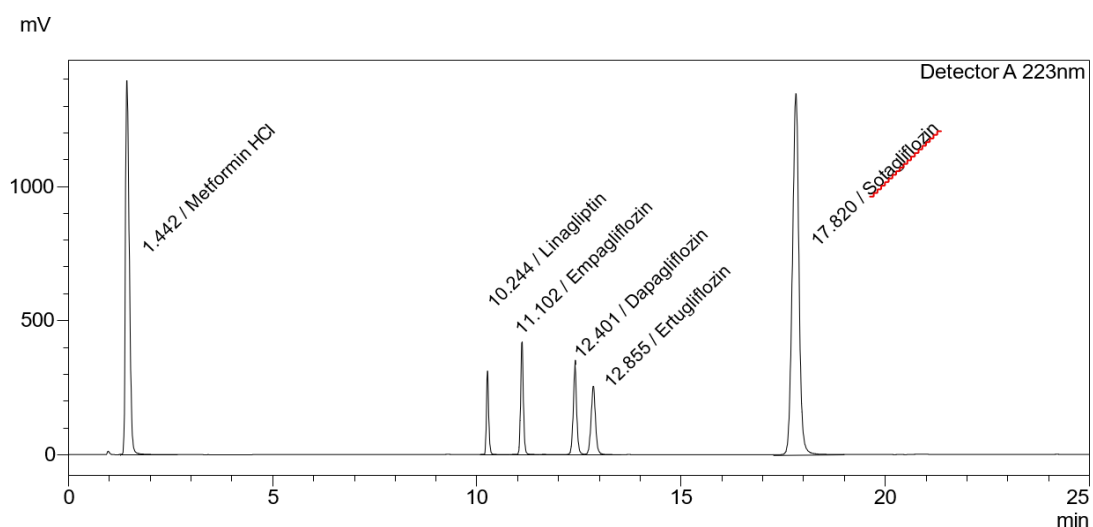


Figure 4.2.6.2 Standard chromatogram at wavelength 223nm (Robustness)

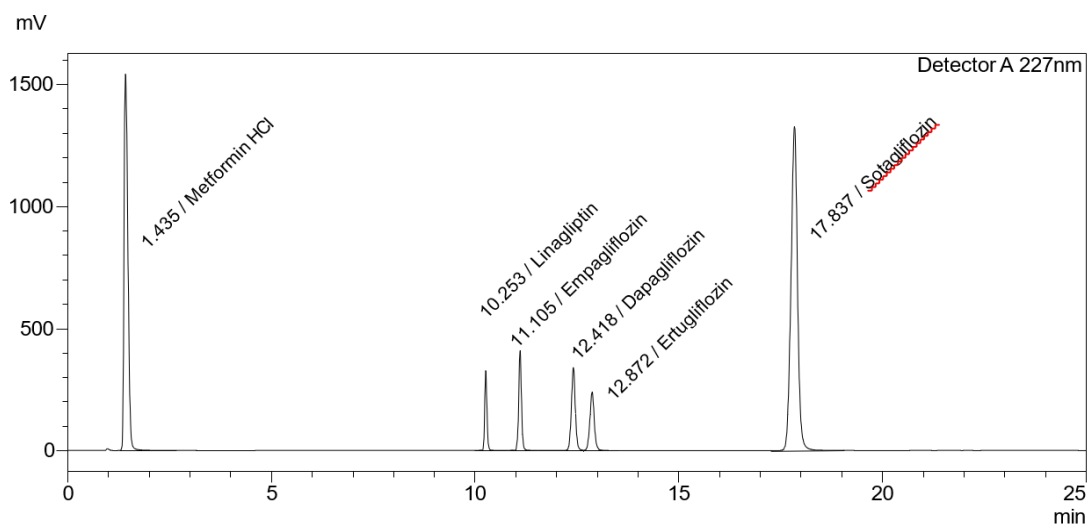


Figure 4.2.6.3 Standard chromatogram at wavelength 227nm (Robustness)

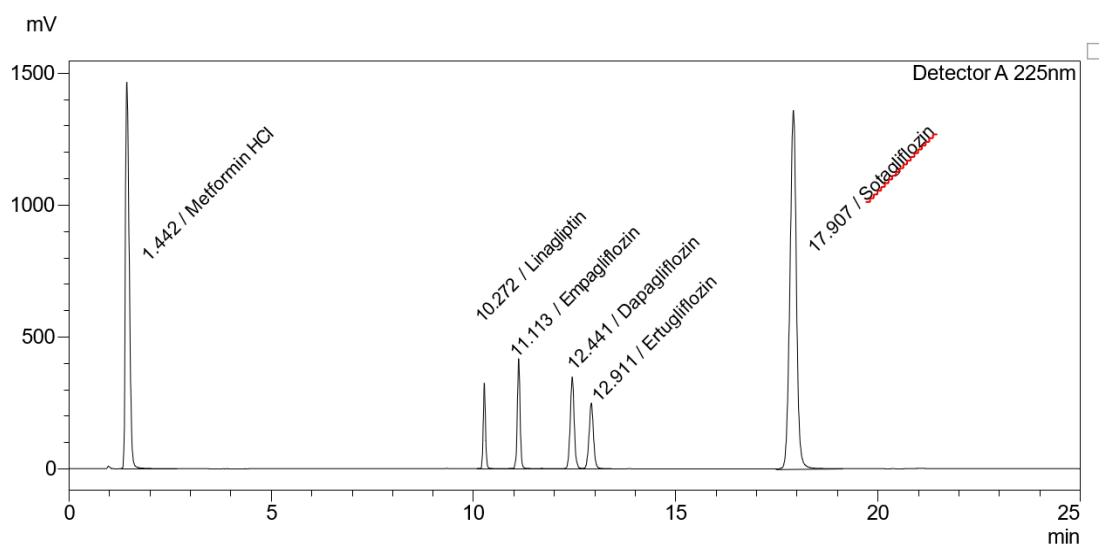


Figure 4.2.6.4 Standard chromatogram at column oven 38°C (Robustness)

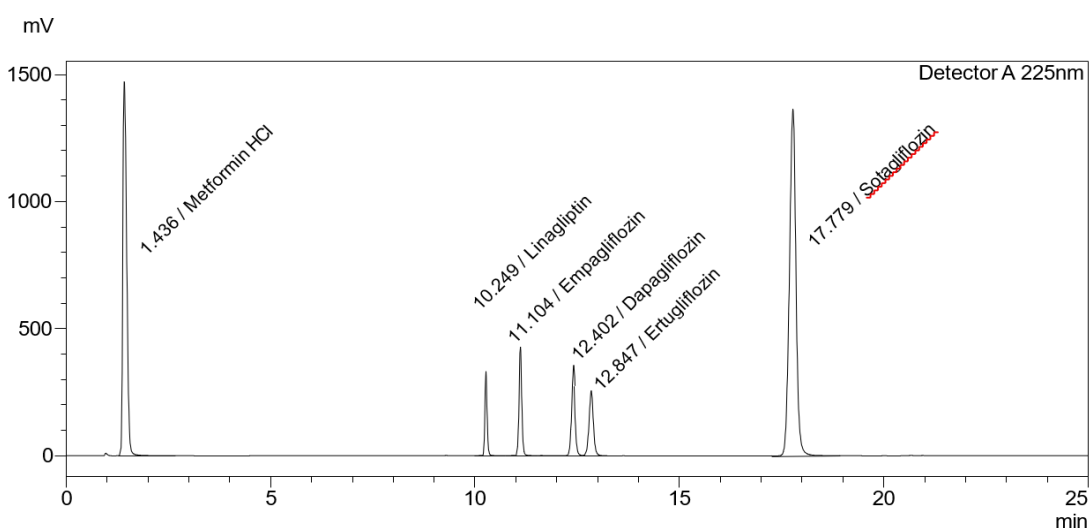


Figure 4.2.6.5 Standard chromatogram at column oven 42°C (Robustness)

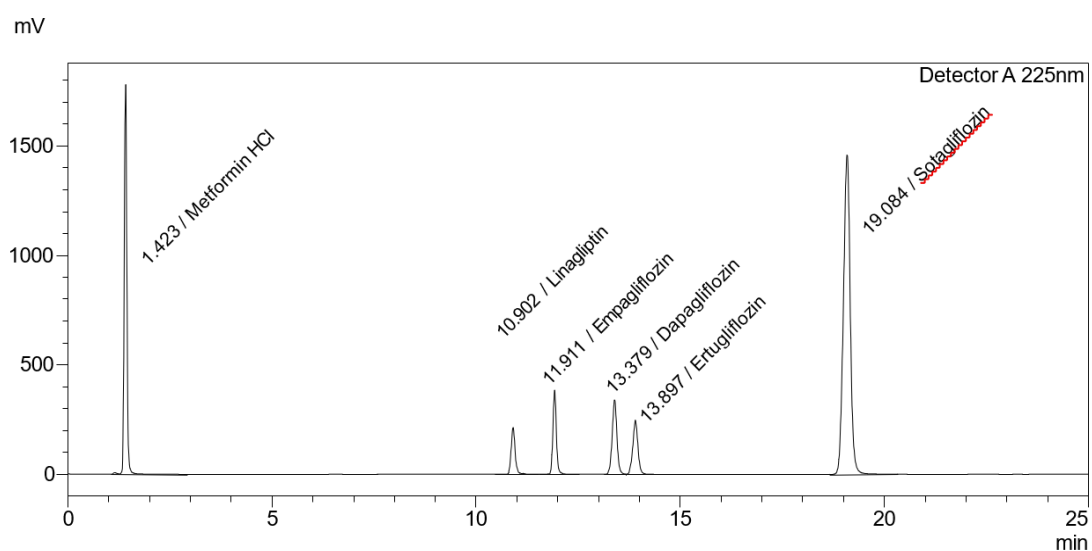


Figure 4.2.6.6 Standard chromatogram at flow rate 1.3min/min (Robustness)

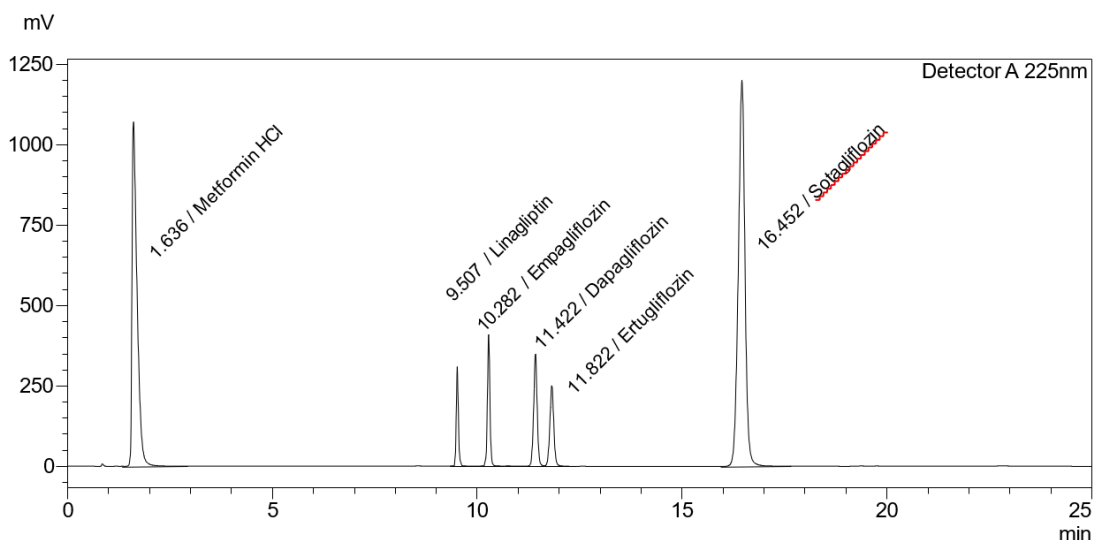


Figure 4.2.6.7 Standard chromatogram at flow rate 1.7min/min (Robustness)

4.2.6 Analytical Solution Stability

Results of six samples prepared for precision were again analyzed by freshly prepared standard after 24 hours. Results of each molecule can be seen in table 4.2.7.1, 4.2.7.2, 4.2.7.3, 4.2.7.4, 4.2.7.5, and 4.2.7.6 for Metformin, Linagliptin, Empagliflozin, Dapagliflozin, ertugliflozin and Sotagliflozin respectively. Comparison of average results of six samples for precision and analytical solution stability are presented in table 4.2.7.1.

Table 4.2.7.1 Analytical solution stability results of Metformin

		WS (mg)	P	LC (mg)	MW (mg)	F
		100.90	99.00%	250	268.1	-
Standard (As)		Sample	AT	WT (mg)	Results (%)	Difference
1	10459435	1	10362505	105.10	100.93	1.22
2	10470434	2	10360705	107.30	98.84	-0.87
3	10469564	3	10351541	106.90	99.13	-0.59
4	10454025	4	10353947	107.30	98.78	-0.93
5	10459870	5	10495644	106.50	100.88	1.18
6	10474212	6	10391557	106.70	99.70	-0.01
AVG	10464590	AVG	99.71			
STDEV	7898.7	MIN	98.78			
%RSD	0.08	MAX	100.93			
		STDEV	0.98			
		%RSD	0.99			

Table 4.2.7.2 Analytical solution stability results of Linagliptin

		WS (mg)	P	LC (mg)	MW (mg)	F
		100.50	99.68%	5	280	-
Standard (As)		Sample	AT	WT (mg)	Results (%)	Difference
1	1247313	1	1231740	310.10	98.89	-0.64
2	1246860	2	1236277	311.50	98.81	-0.72
3	1246814	3	1154277	290.10	99.06	-0.46
4	1246202	4	1233349	310.90	98.77	-0.76
5	1247774	5	1247781	305.10	101.82	2.31
6	1249191	6	1242177	309.90	99.79	0.27
AVG	1247359	AVG	99.52			
STDEV	1040.7	MIN	98.77			
%RSD	0.08	MAX	101.82			
		STDEV	1.19			
		%RSD	1.19			

Table 4.2.7.3 Analytical solution stability results of Empagliflozin

		WS (mg)	P	LC (mg)	MW (mg)	F
		25.00	99.72%	25	282	-
Standard (As)		Sample	AT	WT (mg)	Results (%)	Difference
1	1982208	1	1960841	275.60	100.88	0.61
2	1983362	2	1952228	277.90	99.60	-0.66
3	1983993	3	1962323	275.50	100.99	0.72
4	1980700	4	1962041	280.10	99.32	-0.95
5	1983220	5	1983533	278.60	100.95	0.68
6	1986688	6	1973438	280.20	99.86	-0.41
AVG	1983361.8	AVG	100.27			
STDEV	1994.9	MIN	99.32			
%RSD	0.10	MAX	100.99			
		STDEV	0.76			
		%RSD	0.76			

Table 4.2.7.4 Analytical solution stability results of Dapagliflozin

		WS (mg)	P	LC (mg)	MW (mg)	F
		35.70	99.78%	10	301.2	0.8486
Standard (As)		Sample	AT	WT (mg)	Results (%)	Difference
1	1982208	1	1960841	275.60	100.88	0.61
2	1983362	2	1952228	277.90	99.60	-0.66
3	1983993	3	1962323	275.50	100.99	0.72
4	1980700	4	1962041	280.10	99.32	-0.95
5	1983220	5	1983533	278.60	100.95	0.68
6	1986688	6	1973438	280.20	99.86	-0.41
AVG	2388275.2	AVG	100.13			
STDEV	6386.8	MIN	99.35			
%RSD	0.27	MAX	101.11			
		STDEV	0.61			
		%RSD	0.61			

Table 4.2.7.5 Analytical solution stability results of Ertugliflozin

		WS (mg)	P	LC (mg)	MW (mg)	F
		32.50	99.20%	5	104.99	0.7116
Standard (As)		Sample	AT	WT (mg)	Results (%)	Difference
1	1921810	1	1893157	500.20	100.05	0.04
2	1914008	2	1894063	500.90	99.95	-0.05
3	1916193	3	1903408	505.30	99.57	-0.43
4	1922301	4	1901585	507.10	99.12	-0.88
5	1914802	5	1922127	503.90	100.83	0.83
6	1918496	6	1909465	502.20	100.51	0.50
AVG	1917935	AVG	100.01			
STDEV	3540.2	MIN	99.12			
%RSD	0.18	MAX	100.83			
		STDEV	0.62			
		%RSD	0.62			

Table 4.2.7.6 Analytical solution stability results of Sotagliflozin

		WS (mg)	P	LC (mg)	MW (mg)	F
		101.10	99.50%	200	420.1	-
Standard (As)		Sample	AT	WT (mg)	Results (%)	Difference
1	14523334	1	14396067	210.60	99.41	-0.50
2	14509705	2	14379219	210.00	99.57	-0.34
3	14535256	3	14423557	211.20	99.31	-0.60
4	14525592	4	14429850	208.60	100.59	0.69
5	14543457	5	14600631	210.10	101.06	1.15
6	14543617	6	14520029	212.20	99.51	-0.40
AVG	14530160	AVG	99.91			
STDEV	13188.9	MIN	99.31			
%RSD	0.09	MAX	101.06			
		STDEV	0.73			
		%RSD	0.73			

Table 4.2.7.7 Analytical solution stability result

Name of Drugs	Average Results	
	Freshly prepared sample solution $\pm$ RSD	Sample solution after 24 hours $\pm$ RSD
Metformin	99.57 $\pm$ 0.81	99.71 $\pm$ 0.99
Liaglipitin	99.69 $\pm$ 0.96	99.52 $\pm$ 1.19
Empagliflozin	98.72 $\pm$ 1.17	100.27 $\pm$ 0.76
Dapagliflozin	99.82 $\pm$ 0.27	100.13 $\pm$ 0.61
Ertugliflozin	100.07 $\pm$ 0.91	100.01 $\pm$ 0.62
Sotaflozin	99.25 $\pm$ 0.50	99.91 $\pm$ 0.73

4.2.8 Range

Linearity test defines the upper and lower limit of the test. 40 to 160% is the range which this validated method can precisely and accurately analyze in a sample.

4.2.9 Specificity

To check the interference of diluent used and excipients 10 μ l injection volume of blank and available placebo of products were injected into HPLC. Chromatograph of blank can be seen in figure 4.2.9.1

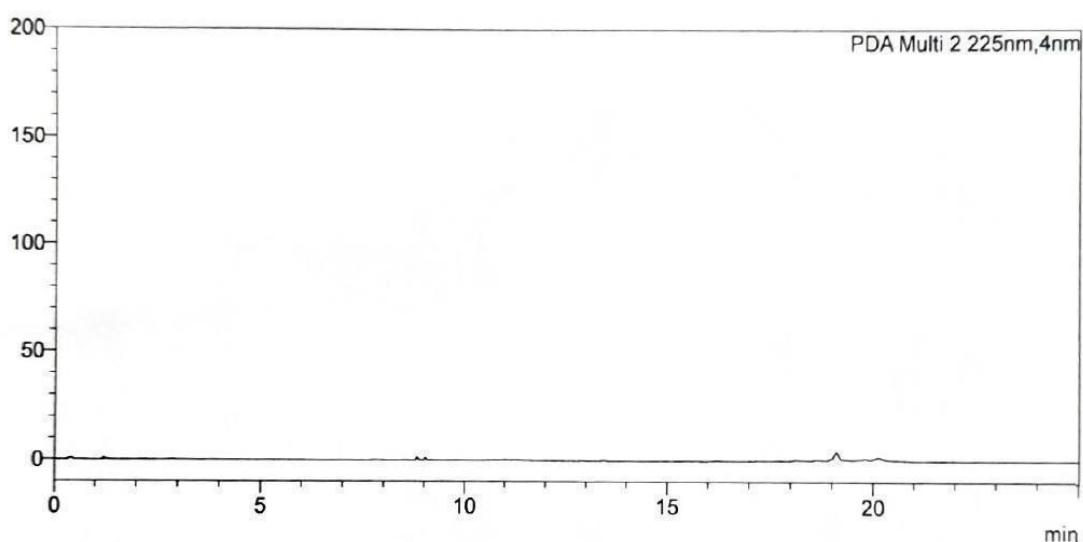


Figure 4.2.9.1 Chromatogram of blank

4.2.10 Results of selected formulations

Glucophage Tablet 250mg, Diajard-L Tablet 25mg, Diajard Tablet 25mg, Daploz Tablet 10mg and Inpefa Tablet 200mg and Ertugliflozin Tablet 5mg were arranged to check the accuracy and precision of the proposed method. Assay results of all commercial products can be seen in table, which showed that this method had capacity to analyze the active ingredients in products in the presence of excipients without any interference. Diluent used had the ability to extract the active completely from the products. These results are average of results of repeatability, intermediate precision and analytical solution stability.

Table 4.2.10.1 Results of commercial products

Product Name	Active Substances	Assay Result
Glucophage Tablet 250mg	Metformin	99.57 %
Diajard-L Tablet 25mg	Linagliptin	99.69 %
Diajard Tablet 25mg	Empagliflozin	99.72 %
Daploz Tablet 10mg	Dapagliflozin	98.82 %
Ertugliflozin Tablet 5mg	Ertugliflozin	100.07 %
Inpefa Tablet 200mg	Sotagliflozin	99.25 %

CHAPTER NO.5

DISCUSSION

5.1 Method Development and Optimization

The present study successfully developed and validated a reverse-phase HPLC method for the simultaneous determination of six antidiabetic drugs, namely Metformin, Linagliptin, Empagliflozin, Dapagliflozin, Ertugliflozin, and Sotagliflozin in pharmaceutical dosage forms. As fixed-dose combination therapies are increasingly used in diabetes management, there is a growing need for analytical methods that are accurate, efficient, and suitable for routine quality assessment. The developed method addresses these requirements by providing reliable performance with acceptable accuracy, precision, and robustness.

Chromatographic conditions were optimized using a C18 column and a gradient mobile phase composed of phosphate buffer (pH 4.0) and acetonitrile. Under these conditions, complete separation of all analytes was achieved within a total run time of 16 minutes (Fig. 4.1.1.1 and Fig. 4.1.2.1). The chromatographic peaks were well defined and symmetrical, with stable retention times and minimal peak tailing, indicating good selectivity and method performance. The retention times presented in Table 4.1 demonstrated consistent reproducibility between standard and sample analyses.

System suitability testing further confirmed the reliability of the developed method. As shown in Table 4.2.1.1, all evaluated parameters met the established acceptance criteria. Tailing factor values remained close to unity, while the number of theoretical plates exceeded 2,000 for all analytes, indicating satisfactory column efficiency. In addition, resolution values greater than 2 confirmed effective separation of each drug under the optimized chromatographic conditions.

5.2 Method Validation

Method validation was carried out in accordance with ICH Q2(R1) guidelines and the results demonstrated satisfactory analytical performance. Linearity was evaluated for all six antidiabetic drugs (Figs. 4.3–4.6), and the calibration curves

showed correlation coefficients (R^2) greater than 0.999, indicating a strong linear relationship between concentration and detector response within the selected working range. Accuracy assessment produced recovery values between 98–102%, confirming that the method was capable of providing reliable and accurate measurements.

Precision was assessed through repeatability and intermediate precision studies (Tables 4.2.2.1.7 and 4.2.2.2.7). The obtained %RSD values remained below 2.0, demonstrating good consistency and reproducibility of the analytical procedure. Robustness testing further confirmed the reliability of the method, as minor intentional changes in flow rate, wavelength, and column temperature did not produce any significant effect on assay performance.

In addition, the low values obtained for the limit of detection (LOD) and limit of quantification (LOQ) indicated that the method possessed adequate sensitivity for detecting and quantifying low concentrations of analytes. Specificity studies showed that excipients and diluent components did not interfere with the chromatographic peaks of the target compounds, ensuring accurate determination of the active pharmaceutical ingredients.

Overall, the developed method demonstrated a balance of simplicity, reliability, and analytical efficiency. Compared with conventional single-drug analysis, the proposed approach offers reduced analysis time, lower solvent consumption, and improved laboratory productivity, making it suitable for routine quality control, stability assessment, and pharmaceutical formulation analysis.

5.3 Conclusion

This study successfully developed and validated a reverse-phase HPLC method for the simultaneous determination of six antidiabetic drugs, including Metformin, Linagliptin, Empagliflozin, Dapagliflozin, Ertugliflozin, and Sotagliflozin in pharmaceutical dosage forms. The chromatographic conditions were optimized using a C18 column and gradient elution with phosphate buffer (pH 4.0) and acetonitrile, resulting in effective separation of all analytes within a total run time of 16 minutes.

Method validation was performed according to ICH Q2(R1) guidelines and demonstrated satisfactory analytical performance. The method showed excellent linearity ($R^2 > 0.999$), acceptable accuracy with recovery values ranging from 98–102%, and good precision with %RSD values below 2.0. In addition, low LOD and LOQ values confirmed the sensitivity of the method, while robustness and specificity studies indicated that minor variations in operating conditions did not significantly influence the results.

Overall, the developed method proved to be simple, reliable, and suitable for routine pharmaceutical analysis and quality control applications. The ability to simultaneously quantify multiple active pharmaceutical ingredients reduces analysis time, solvent consumption, and laboratory workload, improving overall analytical efficiency. The findings of this study provide a practical and validated analytical approach that supports pharmaceutical quality assurance, promotes consistency in drug performance, and contributes to ensuring patient safety. This work may also serve as a useful reference for future studies involving multi-component pharmaceutical formulations and analytical method development.

5.4 Future Recommendations

Future studies may focus on further improving and broadening the applicability of the developed HPLC method for both research and industrial purposes. One possible extension is its application to biological samples such as plasma, serum, and urine, which would allow its use in pharmacokinetic, bioavailability, and clinical investigations. In addition, the method may be employed in stability and forced degradation studies under different stress conditions, including heat, light exposure, oxidation, and hydrolysis, to evaluate degradation behavior and estimate the shelf life of pharmaceutical products. Further improvements could include coupling the method with advanced analytical techniques such as LC–MS/MS to achieve greater sensitivity, improved selectivity, and more reliable detection of trace impurities. Future optimization may also consider the principles of green analytical chemistry by reducing solvent usage and selecting environmentally safer mobile phases. Moreover, incorporation into automated or high-throughput analytical platforms could improve efficiency and reproducibility in routine industrial analysis. With additional inter-laboratory validation, the method may eventually support regulatory applications for the simultaneous determination of multiple antidiabetic drugs and contribute to more consistent pharmaceutical quality control practices.

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