Development and validation of analytical methods for estimation of selected anti-diabetic drugs: Quality by design approach

Thesis submitted to

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(Accredited 'A+' Grade by NAAC) (3rd Cycle) [Placed in Category 'A' by MoE (GoI)}

For the award of the degree of

Doctor of Philosophy

In the Faculty of

Pharmacy

by

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(Registration No: **KLEU/Ph.D./2019-20/D01219022**)

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MARCH 2024

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Shailendra S. Suryawanshi

CONTENTS

ABBREVIATIONS

%	Percentage
%CV	Percentage Coefficient of Variance
%RSD	Percentage Residual Standard Deviation
2D	Two Dimensional
3D	Three Dimensional
ACN	Acetonitrile
AGREE	Analytical Greenness
ANOVA	Analysis of Variance
AQbD	Analytical Quality by Design
ATP	Analytical Target Profile
AUC	Area Under Curve
BBD	Box-Behnken design
CAAs	Critical Analytical Attributes
CCD	Central Composite Design
CQAs	Critical Quality Attributes
CV	Coefficient of Variance
DAPA	Dapagliflozin
DM	Diabetes Mellitus
DMF	Dimethyl Formamide
DMSO	Dimethyl Sulfoxide
DoE	Design of Experiment
DPP-4	Dipeptidyl Peptidase-4
DW	Distilled Water
EMPA	Empagliflozin
F	Value of F-Distribution
g	Gram
GAC	Green Analytical Chemistry
HCL	Hydrochloride
HPLC	High Performance Liquid Chromatography
ICH	International Conference on Harmonization
IFB	Ishikawa Fishbone
LC	Liquid Chromatography
LINA	Linagliptin
LOD	Limit of Detection
LOQ	Limit of Quantification
MeoH	Methanol
MET	Metformin HCl

ABBREVIATIONS

min	Minute
ml	Milliliter
mM	Millimolar
MODR	Method Operational Design Region
MW	Milli-Q-Water
nm	nano meter
OPA	Ortho Phosphoric Acid
p value	Probability value
PA	Peak Area
PDA	Photo Diode Array
QbD	Quality by Design
QRM	Quality Risk Management
r2	Correlation Coefficient
RA	Risk Assessment
RP-HPLC	Reverse Phase-High Performance Liquid Chromatography
RP-HPLC	Reverse Phase
RSD	Residual Standard Deviation
RT	Retention Time
S	Slope
SD	Standard Deviation
SGLT-1	Sodium-Glucose Co-Transporter Subtype -1
SGLT-2	Sodium-Glucose Co-Transporter Subtype -2
SITA	Sitagliptin
TF	Tailing Factor
TP	Theoretical Plates
UV	Ultra Violet
USFDA	United States Food and Drug Administration
v/v	Volume by Volume
VF	Volumetric Flask
WHO	Word Health Organization
µ	Micron
µg	Microgram
µg/ml	Micro Gram Per Milli Liter
µl	Microliter
σ	Standard Deviation of y-intercept of calibration curve

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ABSTRACT

Background: Quality by Design is a methodical approach to development that emphasizes on excellent investigation and quality risk management in the creation of pharmaceutical products. It begins with defined goals and places an emphasis on comprehension and control. The development, optimization, and validation of reliable analytical techniques are all greatly affected by the concept of analytical quality by design. There are many anti-diabetic drug formulations available in the market for the treatment of diabetes mellitus; therefore, it is essential to perform quality testing of these pharmaceuticals using appropriate analytical techniques.

Objectives: The main objectives of proposed research work are to develop, optimize and validate quality by design assisted analytical methods for estimation of selected anti-diabetic drugs.

Methodology: In the proposed research work selected anti-diabetic drugs Metformin HCL, Dapagliflozin, Empagliflozin, Linagliptin and Sitagliptin were used as study drugs. A simple, specific, selective, sensitive, linear, precise, robust, rugged and accurate analytical methods have been developed and validated for estimation of selected drugs in their pharmaceuticals. Various steps involved in quality by design were followed. High Performance Liquid Chromatographic, UV-Spectrophotometric and Conductometric methods were designed and validated for estimation of selected drugs. The validation of methods was performed by using International Conference on Harmonization guidelines. In order to verify the applicability of proposed methods, marketed formulations loaded with anti-diabetic drugs were estimated.

Results: In the proposed research work, five chromatographic methods were developed for estimation of Metformin HCL, Dapagliflozin, Empagliflozin, Linagliptin and Sitagliptin using Methanol, Acetonitrile, Millipore water and o-phosphoric acid buffer system as mobile phase using C-18 columns as stationary phase. Four UV-Spectrophotometric methods were developed for estimation of Dapagliflozin, Empagliflozin, and Linagliptin using quality by design approach. Additionally, a conductometric method was developed for the estimation of Metformin HCl. The Box-Behnken and Central Composite designs were found to be effective for optimization of proposed spectrophotometric and chromatographic methods. The validation results showed that all the methods were found to be specific, exhibited good linearity with r^2 0.999 with high sensitivity. All the methods were found to be precise, robust, rugged and accurate as the % RSD values obtained for each response were found to be less than 2% with good recovery values ranging from 97.37-102.33%.

Conclusion: The newly designed and validated HPLC, UV-Spectrophotometric and Conductometric techniques based on the quality by design approach were found to be simple, precise, and accurate for estimation of selected anti-diabetic drugs and can be used for routine quality control analysis in pharmaceutical industries.

Keywords: Analytical quality by design, Anti-diabetic drugs, Dapagliflozin, Empagliflozin, Linagliptin, Metformin HCl, Sitagliptin, Pharmaceutical industries, Quality control.

Chapter 1

Introduction

1.1: BACKGROUND

A drug is described as an active chemical substance derived from diverse sources and used to treat, mitigate, diagnose, prevent, and cure various diseases and disorders in humans and animals, either taken internally or administered externally in appropriate dose forms. Drug companies produce pharmaceuticals in a variety of dosage forms, and before any product is released into the market, it must be tested for quality. The quality testing of pharmaceuticals in bulk and pharmaceutical goods is essential and it can be performed using a variety of techniques in industrial research and development and Quality Control (QC) departments, including titrimetric, spectrophotometric, chromatographic, and biological procedures. [1]

The evolution of pharmaceuticals has brought about a transformative impact on human health. However, the effectiveness of these medications' pivots on their purity and accurate dosage. In order to guarantee that pharmaceuticals fulfil their intended purpose, a series of chemical and instrumental methods for drug assessment have been consistently developed. Contaminants can potentially emerge at different points in the pharmaceutical life cycle, including during development, transit, and storage. Detecting and quantifying these contaminants is essential to assure the quality of pharmaceuticals. Therefore, a continuous commitment to identifying and measuring contaminants is crucial to maintaining the integrity of pharmaceuticals and safeguarding public health. Analytical instrumentation and methods play a role in these circumstances. Analytical procedures are useful in every stage in case of pharmaceutical product life cycle, drug discovery, clinical research analysis, and post-market testing. New methods of analysis are constantly being invented, while a few are old and have been available for over a century. [2]

Pharmaceutical Drug Analysis

Drug analysis is essential to the pharmaceutical industry. The qualitative and quantitative components are primarily involved in drug QC analysis. Utilizing various analytical techniques and modern technology is the main way of ensuring the QC of drugs and medications originating from various sources. [3-4] Monitoring the quality of drugs and medications is achievable through the utilization of diverse contemporary equipment and analytical methods. Below are some categories into which the analytical procedures are divided. [5]

- Optical or spectral techniques
- Chromatographic procedures
- Electrochemical techniques
- Physical or thermoanalytical techniques
- Biological and microbiological assays
- Radioactive techniques
- Titration techniques; and other methods.

Spectral Analysis

The light that the drug or medications absorb or emit is examined in spectral methods of investigation. We examine the samples using various types of radiation. In the present day, spectrum analysis holds considerable importance in the pharmaceutical and medicinal product industries. It is an excellent tool for determining chemical structure of drugs. Drug analysis, both qualitative and quantitative, can be performed and recorded in spectral analysis with accuracy. [1]

UV Spectrophotometry is a significant analytical instrument utilized in the pharmaceutical industry. When an analyte or molecular solution is exposed to electromagnetic waves, a form of characteristic spectrum termed as the UV and Visible spectrum. Electromagnetic radiations have wavelengths between 200 and 800 nm. In general, the visible range is 400 to 800 nm, and the UV range is 200 to 400 nm. The distinctive UV or visible spectrum of a drug or analyte is produced when a solution of the analyte or drug molecule is subjected to wavelengths at which the valence electrons in the analyte absorb light of particular wavelengths and undergo excitation. [5] Many fields, including analytical chemistry, biochemistry, environmental science, and pharmaceuticals, use UV-Visible spectroscopy. [1]

It can be used to quantify the concentration of molecules in solution, identify and classify molecules, and assess the purity of a material. An effective analytical method to gain information about the composition, behavior, and dynamics of molecules is UV-visible spectroscopy. UV-Visible spectroscopy can also be used to find impurities in a sample and track the development of a reaction.[1]

Chromatographic Analysis

Chromatographic techniques are a class of separation methods mostly employed in quantitative and qualitative applications. This approach for separation can also be used to separate a combination of components into their individual components by employing a stationary and a mobile phase. The pharmaceutical sector makes considerable use of chromatography to analyze and detect the presence of trace levels of chemicals and components in a particular sample. Chromatography is an important step in the discovery of novel pharmaceuticals. [6,7]

Chromatographic technique like high pressure liquid chromatography (HPLC) is very much useful in quantitative analysis. It is used to separate and isolate complicated mixtures or molecules that have a chemical, biological, or natural origin. Its analytical precision, accuracy, and specificity are all quite excellent. HPLC is one of the most used chromatographic methods and systems for pharmacological and natural product analysis and QC, according to a large body of literature. [1]

Electrochemical Methods

In current days, a notable rise in the utility of electrochemical techniques for the examination of pharmaceuticals and therapeutics have been observed. This resurgence of interest can be attributed, in part, to the advancements in instruments and a deeper understanding of electrochemical methods. Electrochemical methods, as a category of analytical techniques, are employed to quantify the electrochemical characteristics of medicinal compounds. Examples of these methods include Conductometry, Potentiometry, and Amperometry.[1]

Conductometric analysis, a quantitative laboratory technique, is utilized for measuring amount of analyte in a solvent. This involves assessing the conductivity of the solute in solution. The electrical conductivity is influenced by factors such as the number of free charges in the solvent, and concentration of the analyte.[1,5]

Conductometric titration, as a specific application, offers several advantages. It is capable of measuring very dilute solutions and weak acids, analysing coloured or turbid solutions, and serving diverse utility in acid and base titrations, redox-titrations, precipitation-titrations, and complexometric titrations. This versatility makes conductometric analysis a valuable tool in pharmaceutical and therapeutic research.[1,5]

Quality by Design (QbD)

QbD is an organized development method which commences with defined objectives, placing emphasis on comprehension, control, robust research, and effective quality risk management. Within the pharmaceutical industry, QbD serves as a scientific methodology for the ongoing assessment and enhancement of product quality. The International Conference on Harmonization (ICH) introduced the concept of QbD through a series of guidelines. Regulatory agencies and the pharmaceutical sector presently prioritize the integration of QbD into pharmaceutical product development processes.[8]

The incorporation of QbD is pivotal for pharmaceutical companies in upholding and ensuring product quality. Functioning as a link between industry and drug regulatory bodies, QbD facilitates the changeover to a risk-based, complete, technical, and practical tactic in medicinal product development. The pharmaceutical sector, governed by a rigorous quality policy, is deeply rooted in the principles of risk management within the quality assurance system to guarantee pharmaceutical purity and patient safety. In the QC labs the identification of contaminants in both raw form and final products is a significant focus. The ICH Q8 guideline specifically addresses the QbD concept, emphasizing that superiority should be inherently integrated in the design. QbD principles, encompassing risk and knowledge-based decision-making, a systematic approach to development, and a commitment to continuous improvement, contribute to the establishment of a robust and capable pharmaceutical process. This approach aligns with the overarching goal of ensuring the superiority, security, and effectiveness of pharmaceutical products throughout their development and manufacturing processes. [9]

Adhering to the codes of QbD is imperative to establish practical and systematic processes, particularly in mitigating risks associated with analytical approaches. When the concept and methods of QbD are applied to the analytical development then this approach can also be referred as Analytical QbD (AQbD). AQbD serves as a framework primarily focused on constructing robust analytical methods that consistently exhibit the expected performance throughout their lifecycle. AQbD is a scientific approach to developing simple and reliable analytical tools for critical analysis. AQbD seeks excellence in measurement. The primary goals are to illustrate the many phases involved in designing an analytical technique utilizing a QbD methodology, as well as to demonstrate how QbD is used to validate analytical procedures. [10]

The AQbD strategy is increasingly being used since it provides for an early knowledge of the procedure and ensures the determination of a broader range of experimental conditions. It permits the analytical process to transfer inside the method operable strategy region while also producing fewer out-of-trend and out-of-spec findings due to the method's robustness within the region. The chapters on analytical QbD have been amended by the US Pharmacopoeia and the European Pharmacopoeia, and flexibility is allowed for an investigative process that can be changed without the requirement for revalidation if the AQbD methodology is used. [11] The primary use of QbD ideologies for analytical method optimization is the concept of incorporating quality into the analytical technique as it is developed. As a result, the creation of an analytical QbD method should take an organized approach. The goal of the QbD method development is to meet set objectives. [11, 12]

The steps involved in AQbD are listed as below (Figure 1):

- Analytical Target Profile (ATP)
- Critical quality/analytical attributes (CQAs/CAAs)
- Critical Method Parameters/Variables (CMPs/CMVs)
- Risk Assessment (RA)
- Experimental Design
- Method Operational Design Region (MODR)
- Control Strategy
- Lifecycle Management

Advantages of QBD

Utilizing QbD principles in analytical procedures offers benefits such as pinpointing and mitigating sources of variability that could compromise method robustness. This approach ensures that the analytical process consistently meets the specified performance necessities across the entire lifecycle of both the invention and the technique. Several advantages of the QbD concept are outlined as follows:[13]

- Improves decision-making flexibility.
- Increases life cycle approval chances.
- Reduce batch errors.
- Improved understanding of the process.
- Improved quality of reviews.
- Enables continual improvement till method completion.
- Reduced deviation and costly investigations.
- It impacts product design, process development, and improving the method's robustness by reducing variability in analytical attributes.

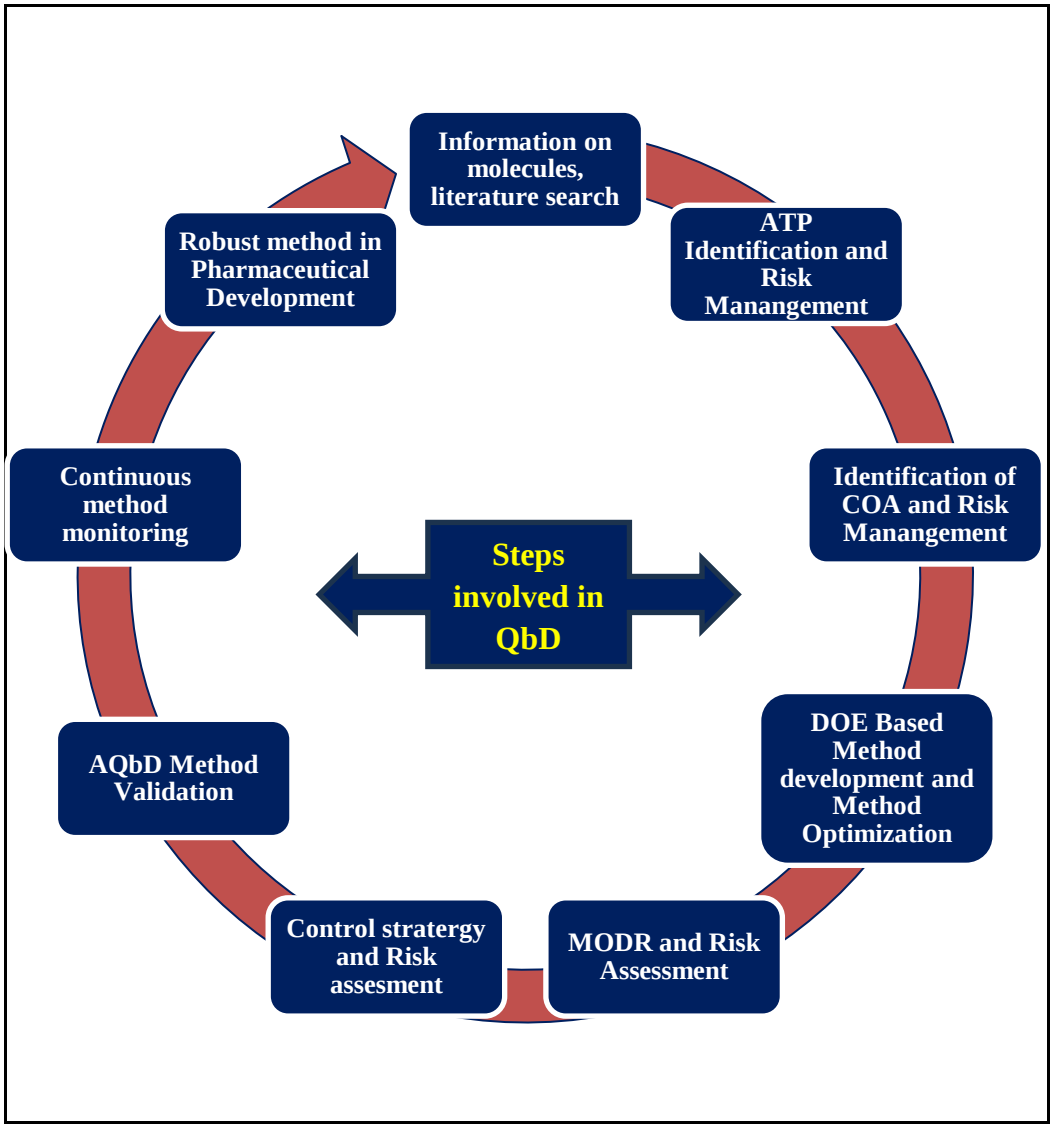

Figure 1: Phases of analytical quality by design approach

Diabetes Mellitus

Diabetes mellitus (DM) comprises a set of metabolic disorders marked through raised blood sugar, resultant from factors such as insulin resistance, inadequate insulin production, or heightened secretion of glucagon. Type 1 diabetes is an autoimmune condition that causes the loss of pancreatic beta cells. Type 2 diabetes is characterized by progressive poor glucose regulation caused by malfunctioning pancreatic beta cells and insulin resistance. [14]

Globally, an estimated 422 million people are diabetic. In India, 69.2 million individuals have diabetes, and 1.6 million deaths are directly caused by it in 2019. In 2021, 537 million adults, or one in ten, had diabetes. The projected figures indicate that the figure is expected to rise to 643 million on 2030 and further to 783 million by 2045. Approximately 44% of individuals with diabetes, totalling 240 million, remain undiagnosed. Diabetes caused 6.7 million deaths in 2021. Diabetes was responsible for at least $966 billion in health expenditures in 2021, accounting for 9% of total healthcare spending globally. [15-17]

Anti-diabetics Drugs

Anti-diabetic drugs are active substances used to treat diabetes mellitus. Diabetes medicines are divided into 2 categories: insulin preparations and oral anti-diabetic medications. Many marketed formulations loaded with oral anti-diabetic agents are available in the market for effective management of diabetes mellitus. Insulin preparations are the primary treatment for both type 1 and type 2 diabetes. Insulin preparations are either short-acting, long-acting, or a combination to simulate natural insulin secretion. Type II diabetes is frequently managed with oral antihyperglycemic medications. [18-20]

1.2: NEED FOR THE STUDY

The development of drugs revolutionized human health. These drugs will only perform their intended function if they are free of contaminants and provided in the suitable dose. To ensure that pharmaceuticals perform their intended purpose, several chemical and instrumental methods for drug assessment have been developed at regular intervals. These medications may vary in therapeutic dose at various stages of development, transportation, and storage, which renders them unsafe to use, hence they must be assessed. Analytical instrumentation and methods play an important role in these circumstances.

Quality control is an essential operation of the pharmaceutical industry. Drugs must be marketed as safe and therapeutically active formulations whose performance is consistent and predictable. New and better medicinal agents are being produced at an accelerated rate. At the same time more exacting and sophisticated analytical methods are being developed for their evaluation. Quality control of drugs in its bulk and pharmaceutical dosage forms is very much essential in the pharmaceutical industries. Estimating the amount of drug and determining its % purity in pharmaceuticals are the two important parameters used for quality control. Analytical methods play essential and important role in the quality control activities.

Anti-diabetic drugs are pharmaceuticals or active chemical substances used to treat diabetes mellitus. Oral anti-diabetic medications are commonly administered for patients with type 2 diabetes. In the proposed study, we selected Metformin, Dapagliflozin, Empagliflozin, Linagliptin, and Sitagliptin as study drugs. These drugs are manufactured in a variety of pharmaceutical preparations, therefore determining their quality is essential employing quality analytical methods.

A literature search revealed that there are few spectroscopic and chromatographic methods available for estimating the bulk and pharmaceutical preparations of the selected study drugs. The reported analytical methods have limitations and disadvantages such as the use of costly solvents, longer analysis times, the use of hazardous solvents, non-robust methods, method failure during analysis resulting in out-of-trend and out-of-spec results, and non-compliance with regulatory requirements, among others. Apart from the constraints mentioned above, it was discovered that a lack of quality by design method was used for the quantification of chosen anti-diabetic medications in their formulations.

QbD is an organized approach to development that starts with established goals and stresses comprehension and control while relying on strong research and quality risk management. It is a science-based method used extensively in the pharmaceutical business to continuously check and improve product quality. Analytical Quality by Design is primarily used to create robust analytical methods that consistently deliver the expected performance throughout their lifecycle. AQbD is a scientific approach to developing simple and reliable analytical tools for critical analysis.

Hence in order to overcome one or other limitations associated with reported method there is need for development, optimization and validation of new analytical methods for estimation of selected antidiabetic drugs as per current regulatory requirements using QbD approach.

1.3. AIM AND OBJECTIVES

Aim

The main aim of proposed research work is to estimate the selected anti-diabetic drugs in bulk and pharmaceutical products using analytical methods.

Objectives

The main objectives of proposed research work are:

1. To develop suitable analytical methods for selected Anti-diabetic drugs using Quality by Design Approach.

2. To validate the analytical method as per current regulatory guidelines using Quality by Design Approach.

3. To estimate the selected anti-diabetic drugs in pharmaceutical product.

Chapter 2

Review of Literature

2.1: Drug Profile

2.2: QbD analysis of drugs and pharmaceuticals

2.3: Analytical methods for estimation of selected drugs

2.1. DRUGS PROFILE

The list of anti-diabetic drugs selected in proposed research work are presented in **Table 1**. The drug profile of selected drugs is described as below:

Table 1: List of selected anti-diabetic drug in proposed research

Sl. No.	Drugs	Category	Mechanism of action
1	Metformin HCl	Biguanide	Improves the action of insulin
2	Dapagliflozin	Sodium glucose cotransport inhibitor	Act by suppressing sodium glucose co-transport
3	Empagliflozin	Sodium glucose cotransport inhibitor	Act by suppressing sodium glucose co-transport
4	Linagliptin	Dipeptidyl Peptidase Inhibitor	Inhibits GLP-1 breakdown and stimulates glucose-dependent insulin secretion.
5	Sitagliptin	Dipeptidyl Peptidase Inhibitor	Inhibits GLP-1 breakdown and stimulates glucose-dependent insulin secretion.

Metformin HCL

Metformin HCl, depicted in **Figure 2**, an orally administered anti-diabetic medication belonging to the biguanide class, effectively employed in treatment of type 2 diabetes. Its primary mechanism involves the reduction of glucose secretion, and it has demonstrated additional benefits such as lowering cholesterol levels and aiding in weight loss under certain conditions. Metformin HCl is available in both single and combination dose forms, often in conjunction with other antidiabetic medications. Metformin HCl may exhibit side effects such as lactic acidosis and occasional gastrointestinal issues. It is contraindicated for individuals with liver, renal, lung, or cardiac conditions.[21-24]

Chemically Name: 3-(diamino methylidene)-1,1-dimethylguanidine; hydrochloride

Molecular Formula: $C_4H_{12}ClN_5$

Molecular Mass: 165.62g/mol

Pka: 12.4

Solubility: It is soluble very freely in H_2O

Appearance: It available as a crystalline powder with a color white to off-white.

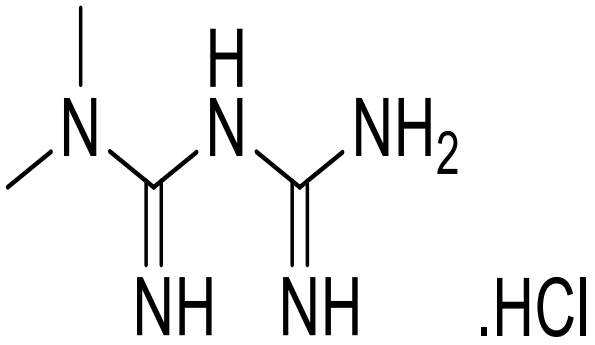

Figure 2: Chemical structure of Metformin HCL

Dapagliflozin

Dapagliflozin, illustrated in **Figure 3,** is a inhibitor of sodium-glucose co transporter sub type 2 (SGLT2) with anti-diabetic properties. Notably, in patients with established atherosclerosis, dapagliflozin has demonstrated a reduction in cardiac death and heart failure rates. It has shown no adverse impact on major adverse cardiovascular events and has exhibited a capacity to impede the development of kidney disease. The overall tolerability profile has been favorable, characterized by a low risk of hypo-glycaemia. [25-27]

Chemically Name: (2S 3R, 4R, 5S, 6R)-2-[4-chloro-3-[(4-ethoxyphenyl) methyl] phenyl]-6-(hydroxy methyl) oxane-3,4,5-triol

Molecular Formula: $C_{21}H_{25}ClO_6$

Molecular Mass: 408.9 g/mol

Pka: 12.7

Solubility: Organic diluters like, ethanol, DMF and DMSO, sparingly soluble in aqueous buffers.

Appearance: White to off-white crystalline powder.

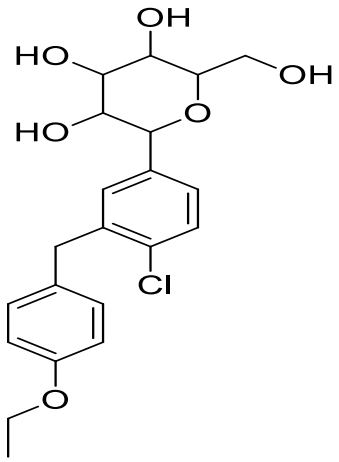

Figure 3: Chemical structure of Dapagliflozin

Empagliflozin

Empagliflozin, depicted in **Figure 4**, functions as an inhibitor of SGLT2, primarily responsible for glucose reabsorption in the kidney. Professionally prescribed for treatment of type- DM. Empagliflozin proves beneficial in improving glycaemic control in patients aged 10 and older with type 2 diabetes, either as a standalone treatment or in combination with metformin or linagliptin. Additionally, it is indicated to decrease the risk of cardiac death in adult patients with both type 2 DM and existing cardiac ailment, whether used alone or in combination with Metformin. [28-30]

Chemically Name: 2*S*,3*R*,4*R*,5*S*,6*R*)-2-[4-chloro-3-[[4-[(3*S*)-oxolan-3-yl] oxy phenyl] methyl] phenyl]-6-(hydroxy methyl) oxane-3,4,5-triol

Molecular Formula: $C_{23}H_{27}ClO_7$

Molecular Mass: 450.9 g/mol

Pka: 12.5

Solubility: Organic solvents like, methanol, ethanol, DMF and DMSO and sparingly soluble in aqueous buffers.

Appearance: Appears as white to off white crystalline powder

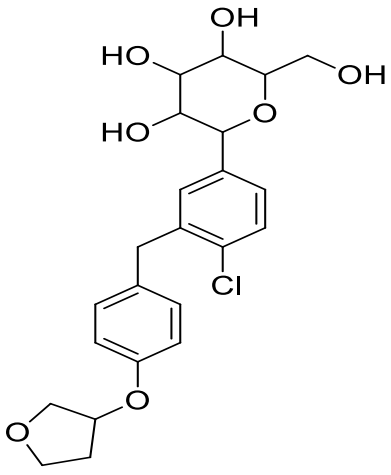

Figure 4: Chemical structure of Empagliflozin

Linagliptin

Linagliptin (**Figure 5**) is a recently approved oral antidiabetic medication that works by blocking the enzyme dipeptidyl peptidase-4. Unlike other DPP-4 inhibitors, linagliptin is primarily excreted through the enterohepatic system and can be administered without dose change in patients with renal or hepatic impairment. Linagliptin is associated with substantial enhancements in glycosylated haemoglobin, fasting plasma glucose, and postprandial glucose. A greater number of patients receiving linagliptin exhibited meaningful improvements, successfully meeting glycosylated haemoglobin objectives. The tolerability of linagliptin was favorable, displaying an adverse event profile similar to that of placebo, and a modest incidence of hypoglycemic episodes.[31-33]

Chemically Name: 8-[(3R)-3-aminopiperidin-1-yl]-7-but-2-ynyl-3-methyl-1-[(4-methylquinazolin-2-yl) methyl] purine-2,6-dione

Molecular Formula: $C_{25}H_{28}N_8O_2$

Molecular Mass: 472.5g/mol

Pka: 8.6

Solubility: Organic solvents such as ethanol, DMSO, and dimethyl formamide.

Appearance: Available as white to off white powder

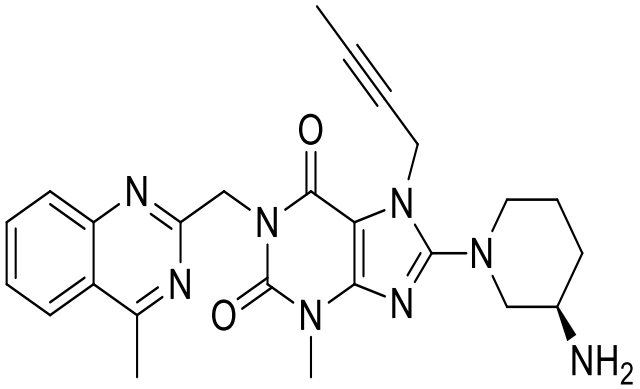

Figure 5: Chemical structure of Linagliptin

Sitagliptin

Sitagliptin, illustrated in **Figure 6**, a recently approved DPP-4 inhibitor used in the treatment of DM (Type-2). Its mechanism of action involves elevating the levels of incretin-hormones, specifically glucagon like peptide-1 and stomach repressive polypeptide. Sitagliptin is efficient in decreasing HbA1c, fasting and postprandial sugar, both alone and in combination with other oral antidiabetic medications. When there is hyperglycemia, it promotes insulin secretion while inhibiting glucagon secretion. Sitagliptin is typically well tolerated, with most side events being mild to moderate in severity and few individuals terminating treatment because to these events. Sitagliptin medication did not raise the incidence of hypoglycemia or bodyweight gain, which are known cardiovascular disorder risk factors.

Chemically Name: (3R)-3-amino-1-[3-(trifluoromethyl)-6,8-dihydro-5H-[1,2,4] triazolo [4,3-a]pyrazin-7-yl]-4-(2,4,5-trifluorophenyl)butan-1-one

Molecular Formula: $C_{16}H_{15}F_6N_5O$

Molecular Mass: 407.31g/mol

Pka: 8.7

Solubility: Freely soluble in water.

Appearance: White to off-white, crystalline, non-hygroscopic powder.

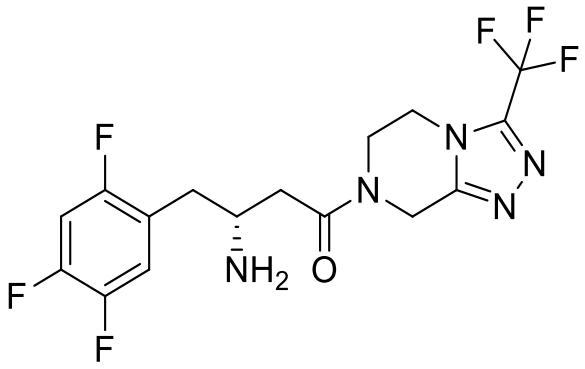

Figure 6: Chemical structure of Sitagliptin

2.2. QBD ANALYSIS OF PHARMACEUTICALS

In recent years, the QbD approach has been implemented in the optimization and validation of analytical techniques for estimating drugs in pharmaceuticals. The following examination provides an in-depth analysis of research work, focusing on the examination of drugs and pharmaceuticals through the application of the QbD approach:

Suryawanshi D et al. reported that analytical QbD technique was utilized to construct and optimize chromatographic conditions for estimating cholecalciferol levels. The Taguchi-orthogonal array and the Box-Behnken Design (BBD) were hired for the screening and optimization of vital parameters in the technique. The separation was conducted on a Eurosphere, C8 column using a solvent solution of methanol: acetonitrile at a flow rate (FR) of 1ml/minute. [36]

Muheem A, et al., conducted a study focusing on the chromatographic estimation of Sofosbuvir bulk, which was developed and validated in line with analytical QbD principles. The analysis utilized an Agilent LC 1200 equipped with a photodiode array detector, employing an Inersil ODS stationary phase. The solvent solution with a composition 65:35 methanol and water. The identification of the analyte was performed at 261 nm, and the FR was maintained at 1 mL/minute. [37]

Vijayaraj S et al. reported a BBD assisted RP-HPL-chromatographic process for the determination of gliclazide and GI-A. The design facilitated the optimization of FR, volume of injection, and buffer strength to reduce the retention period of gliclazide and GI-A. The optimized conditions included a 25mM ortho phosphoric acid (OPA) buffer in a mixture of Acetonitrile (ACN) (50:50 v/v), a FR of 1 mL/minute. [38]

Pranali Yeram et al. conducted a stability representative chromatographic process for quantification of sofosbuvir and ledipasvir in pharmaceutical dosage forms, employing a QbD methodology. The BBD within the QbD framework was utilized to screen and optimize buffer pH, solvent composition, and FR. The impact on response variables such as RT, TP, and TF were assessed through 3D response graphs. [39]

Shah U et al. applied the QbD approach to validate a stability representing chromatographic method for quantification of ezetimibe and glimepiride. They utilized Shimadzu's high-performance liquid chromatograph and a Phenomenex analytical C-18 column. The solvent comprised ACN: ammonium acetate buffer: methanol with a FR of 1.5 mL/minute and a detecting at 232 nm. The approach was validated according to ICH recommendations. [40]

Patel K. et al. devised chromatographic process to estimating evogliptin in marketed formulations, employing a QbD-based approach. This facilitated the creation of a design area and operational area by comprehensively considering all performance parameters. The developed method proves to be faster and suitable for routine QC of both API and sold formulations. [41]

Alruwaili NK et al. optimized and analyzed the atorvastatin technique using a BBD. Three parameters solvent phase, FR, and wavelength act as self-regulating factors. The optimized HPLC conditions included acetonitrile: water (50:50), a FR of 0.6 ml/minute, and a UV detection at 235 nm. [42]

Patel KY et al. investigated an Central Composite Design (CCD) based HPLC method. Chromatographic settings were optimized using Phenomenex column C18, a solvent phase of ACN: water (70:30, v/v), and a FR of 1 ml/minute, with a RT of 4.1

minute. The QbD technique contributed to analytical method development by enhancing the considerate of method variables. [43]

Haque MA et al. reported on spectrophotometric methods for dolutegravir estimation developed using the QbD approach, aligning with ICH Q8(R2) recommendations. Multiple parameters were altered and organized into an Ishikawa diagram. The study focused on creating a sensitive, accurate, and cost-effective Ultra violet method for determining dolutegravir in pharmaceuticals, with critical parameters established using principal component analysis and observation. [44]

Panda SS et al. explored the creation spectral technique aimed at estimating Vilazodone based on QbD. Fractional factorial strategy was initially working for screening, and screened factors were then exposed to a CCD. Vilazodone exhibited highest absorption at 285 nm in methanol and the process was identified as robust for determining vilazodone in pharmaceutical dosage forms. [45]

2.3. ANALYTICAL PROCEDURES FOR ESTIMATION OF SELECTED ANTI-DIABETIC DRUGS

The existing literature has documented numerous analytical processes for quantification of selected drugs in pharmaceutical preparations. These methods encompass spectrophotometric, chromatographic, and electrochemical approaches for single and combined formulations. However, the reported quantification procedures exhibit several limitations and drawbacks, such as:

- Reliance on traditional approaches for the development.
- Lack in QbD-based approach for the estimation of selected drugs using analytical methods.
- Utilization of complex solvent system compositions.
- Prolonged retention times in chromatographic methods.
- Deployment of expensive solvents, contributing to higher operational costs.
- Non-robust development of analytical methods.

These identified limitations underscore the need for advancements in analytical methodologies, emphasizing the adoption of QbD principles, simplicity in solvent systems, reduced retention times, and cost-effective practices to enhance the overall efficacy and efficiency of drug quantification processes. The detailed review of literature on selected drugs have been described in detail as below:

2.3.1. Analytical procedures for estimation of Metformin HCl

2.3.2. Analytical procedures for estimation of Dapagliflozin

2.3.3. Analytical procedures for estimation of Empagliflozin

2.3.4. Analytical procedures for estimation of Linagliptin

2.3.5. Analytical procedures for estimation of Sitagliptin

2.3.1. Analytical Procedures for estimation of Metformin HCL

Arayne SN et al. devised an RP-HPLC technique utilizing a C18 column for accurate detection of metformin hydrochloride in both raw materials and pharmaceutical products, with diazepam as an internal reference. The method detects concentrations 0.3 to 5 µg per milli liter with a linear correlation 0.9995. Well-formed peaks appear at 4.4 minutes of retention time, demonstrating sensitivity and consistency. [46]

Nikam N et al. established a chromatographical approach for testing MET using a C18 column and a phosphate buffer/methanol mixture. Isocratic elution with a phosphate buffer and MeOH at a FR of 1 ml/minute yielded consistent results. Statistical examination confirmed excellent linearity, accuracy, and precision, with Metformin concentrations falling between 98% and 102%. This approach has potential widespread use in assessing the quality of Metformin formulations. [47]

Rimawa AF et al. devised chromatographic tool to assess Metformin and its connected molecule, cyanoguanidine, in formulation. Using Ultra Violet detection at 232 nm and a Nova-Pak silica column, the procedure involved isocratic elution and MeoH, producing effective results. [48]

Praveen KA et al. established a new chromatographic technology for assessment of Alogliptin and Metformin in tablets. Accurate findings are obtained using a buffer system and MeoH with detection at 254 nm and a 10-minute duration. Validation shows excellent linearity (25-150 µg/ml) for both medications, with high correlation coefficients. [49]

Neelima K. et al. published a gradient chromatographical approach for simultaneous measurement of Metformin, Voglibose, and Glimepiride in formulations. Using an Inertsil ODS 3V column and gradient elution with phosphate buffer and ACN, the procedure consistently produces precise and accurate findings. Linear calibration curves for all three medications, together with LOQ and LOD, demonstrate the method's robustness, meeting ICH criteria for routine QC assessments. [50]

Godasu SK, et al. reported a liquid chromatographical approach for measurement of Metfor min and Empagliflozin. The stationary phase was a symmetry C18 column, and the solvents phase consisted of MeoH and buffer of phosphate (70:30 v/v). The approach was validated using ICH recommendations. [51]

Attimarad M et al. investigated a analytical approach for measurement of three novel Gliptin anti-diabetic drugs in a binary mixture with Metformin. The elution was done by means of a monolithic C-18 stationary phase with a mobile phase composed of phosphate buffering system and ACN. The FR employed for analysis was 2.5 millilitre per minute. Eluents were detected using a UV detector. [52]

Elkady EF et al. conducted chromatographic analysis using the HPLC process for the quantification of Metformin sitagliptin phosphate pioglitazone gliclazide, glibenclamide, and repaglinide in bulk, laboratory-prepared mixtures, and pharmaceutical formulations. The separation was accomplished using CAN and mixtures of potassium dihydrogen phosphate, and sodium octane sulphonate buffers at a FR of 0.85 ml/min on a Kromasil C18. Ultra Violet detection was conducted at 220nm, and the approach was standardizing according ICH criteria. [53]

Gedawy A et al. investigated and validated a LC technique for the detection of gliclazide and MET in commercially products. The mobile phase utilized for the runs was composed of formate buffer and AAN. The elution was done using isocratic mode on an Alltima CN stationary phase. [54]

Vasanthi R et al. investigated the RP-UFLC approach for the measurement of gliptin and analyte metformin. A C18 Phenomenex Kinetex column was utilized for stationary. To elute the analytes, a solvent phase of MeoH, ACN, and phosphate buffer in the concentration of 40:20:40 was used at the FR of 1 mL/minute, and the volume was 10 micro liter. The eluent was noticed at 250 nm using a PDAUV detector. [55]

Dange DY et al. analyzed metformin using a simple, rapid, accurate, economical, and precise ultra violet approach in bulk and tablet formulations. Water was employed as a solvent, and metformin had the highest absorbance at 234 nm. Metformin recovery from dosage forms was reported to be 99-101%. [56]

Adolfo et al. reported conductometric titration of MET using modeling and experimental. quantification was accomplished by evaluating the chloride with silver nitrate. The titration and endpoint were followed by conductometric titration and potentiometric titration. Theoretical conductivities of the MET HCl solution were determined when known quantity of titrant were added, utilizing limit conductivity data for each ion found in literature. MET HCl has an equivalency point of 0.99 based on RSD. The conductometric titration is the most effective instrument for quantification and can be used in routine analysis. [57]

Elen RS et al. investigated the conductometric measurement of MET in pharmaceutical dosage consuming silver nitrate titrant. The process was based on reaction between MET HCl and Ag(I) ions, producing the precipitate Ag and Cl. The approach was used to analyzed MET in three different medications. [58]

2.3.2. Analytical procedures for estimation of Dapagliflozin

Debata J et al. introduced a novel process for quantifying DAPA using rp-hplc in accordance with ICH requirements. Employing a Waters C18 stationary phase and a solvent phase comprising phosphate buffer and ACN, the process exhibited speed, accuracy, precision, dependability, and reproducibility, with a 6-minute runtime and a 3.461-minute retention time for DAPA. Validation demonstrated linear calibration curves ranging from 10-60 μg/ml. The process's efficacy was further confirmed through its successful application in measuring DAPA in commercial preparation.[59]

Sanagapati M. et al. devised a RP chromatographical tool for analysing DAPA in its API. This approach utilized a BDS stationary phase and gradient elution with ACN and OPA. The process underwent validation following ICH principles. It demonstrated efficiency in assessing the stability and purity of DAPA under various environano meterental conditions, including acidic, alkaline, peroxide, neutral, heat, and UV exposures. [60]

Mante G V et al. have studied the hplc tool for measuring DAPA in pharmaceutical preparation. formulations that followed ICH requirements. On a Princeton C18 stationary phase, an isocratic mode was used with a combination of ACN and 0.1percentage triethylamine to achieve effective separation. The process had a

retention time of 5.163 minutes Validation study fulfilled acceptable standards, while stress testing demonstrated process reliability under a variety of situations. [61]

Singh N et al. reported a stability-testing rp-hplc tool for measuring DAPA and saxagliptin in pharmaceutical preparations. The tool used an Xterra C18 stationary phase and a buffer ACN solvent phase, resulting in good linearity (2-14 µg mL/ml) and high correlation coefficients for both pharmaceuticals. Stress testing verified tool specificity, demonstrating its suitability for pharmaceutical analysis and QC. [62]

Sarkar S. et al. devised and validated a hplc tool for estimating DAPA in pharmaceutical preparation. The analysis was done on Symmetry C18 stationary phase with a solvent mixture of MeOH: ACN: OPA in a amount of 75:25:05 v/v/v at a FR of 1.0 milli liter per minute. The analyte was detected at 246 nm. The retention time of DAPA. [63]

Aswini M et al. reported a rp-hplc tool for the identification of DAPA and saxagliptin. Analytes were separated using an Inertsi C18 stationary phase. The solvent phase was a 45:55 v/v combination of MeOH and phosphate buffer supplied at a FR of 1 milli liter per minute. The eluents are monitored using a PDA at 210 nms were measured. [64]

Verma et al. developed a process for estimating DAPA in API and pharmaceutical dose forms. The stationary phase was Agilent C18, and the solvent phase was ACN: phosphate buffer, 40:60 v/v with a FR of 1 milli liter per minute. The effluent was monitored at 222 nano meter using a photo diode detector. The proposed process found appropriate for estimating DAPA in API and pharmaceutical forms. [65]

Padmaja et al. explored the assessment of DAPA and saxagliptin in medicinal preparation. The chromatographic analysis was conducted using a standard BDS stationary phase. The solvent phase consisted of phosphate buffer and ACN with the pH 3.8. The solution was pushed through the stationary phase at a FR of 1 ml per minute, while the stationary phase. The optimized wavelength for identification was 210 nm. The process was effectively applied to estimate both DAPA and Saxagliptin in API and combination pharmaceutical preparation. [66]

Jeyabaskaran et al. presented an chromatography process for the estimation of DAPA in API and pharmaceutical preparations. They employed a Hypersil BDS stationary phase with a solvent phase 0.1 percentage OPA buffer and ACN in a 50: 50 percentage v/v ratio, flowing at a rate of 1milli liter per minute. [67]

Gundala et al. developed an HPLC process for the quantification of saxagliptin and DAPA in pharmaceuticals. The optimized conditions, as indicated by the contour diagram, included a 50:50 ratio of ACN and OPA, a FR of 0.9 milli liter per minute, with a runtime of less than 6 minutes were achieved. [68]

Vaghela et al. described a stability representative process for estimating DAPA in pharmaceutical preparation. They employed a Zorbax Eclips XDB C18 stationary phase in with a solvent phase of Buffer: ACN: MeOH (60:37:03 percentage v/v/v). The FR was 1.0 milli liter per minute, and the detecting wavelength was 220 nano meter. DAPA was subjected to a forced degradation study using the rp-hplc process, which included acid, base, oxidative, photo, and thermal degradation, and the results were found to be within the limits. [69]

Deepan et al. devised an hplc process for determining DAPA and saxagliptin in API and pharmaceutical preparation. The separation was done on Xterra C18 stationary phase with ACN: water. The analytes were measured with 248 nano meter using a FR of 1 milli liter per minute. [70]

K. Bhavyasri and colleagues used UV-Spectroscopy to conduct stress tests on DAPA and MET in API and marketed formulations. DAPA and MET were estimated using the Q absorption process at 222 and 232 nano meters with water as the diluent. The degradation investigations with acid, base in peroxide, temperature, and light was expored. The discovered process can be applied to routine QC analyses.[71]

Karuna Priya Chitra and colleagues have presented a novel UV process for quantifying DAPA in both API and pharmaceuticals. The absorption maxima were identified at 233 nm. DAPA exhibited conformity with concentration range of 10 to 35 µg/ml. The suggested process is uncomplicated and suitable for routine quality control analyses.[72]

Jani BR et al. outlined UV first derivative process for the quantification of DAPA and MET in a synthetic combination. This process involves employing the first derivative process based on absorbance measurements at 235 and 272 nms by UV and visible spectrophotometer with MeOH as the solvent. [73]

2.3.3. Analytical Process for Estimation of Empagliflozin

Patil SD et al. documented a validated stability-Demonstrating rp-hplc process for quantifying EMPA, employing a Phenomenex C18 stationary phase and a solvent consisting of MeOH: Water,70:30 percentage v/v. The approach showed significant

linearity (2 to 14 μg/ml). This approach provides a reliable way to analyse EMPA in pharmaceutical formulations.[74]

Siridevi MP et al. developed an accurate hplc tool for measuring EMPA in API and pharmaceutical preparation. Using C18G stationary phase with a MeOH-water solvent phase. The approach demonstrated excellent linearity (10-90μg/ml), with a r^2 of 0.99 and a retention duration of 6.2 minutes. The application to EMPA pills resulted in recovery rates ranging from 99.65percentage to 99.89percentage, demonstrating its precision and reliability for routine QC testing.[75]

Hanif MA et al. created a validated analytical process for assessing EMPA using a C 18 stationary phase using 0.1 percentage trifluoroacetic acid solution and ACN. The process demonstrated high linearity in the range of 0.025-30 μg/ml.[76]

Shirisha V. et al. established a successful RP-HPLC approach for analyzing EMPA in both API and pharmaceutical formulations, using a ODS HG-5 RP C18 stationary phase with UV identification at 228 nm. The process displayed excellent linearity within the range of 0 to 50 μg/ml, attaining recovery rates of 98 percentage to 102 percentage. [77]

Naga TK et al. developed a new technology for concurrently determining LINA, EMPA, and MET in solid formulations. The process, which used a Kromosil stationary phase and buffer: ACN elution, demonstrated robustness and specificity, with retention durations of around 2.370, 2.787, and 3.419 minutes, respectively. [78]

Sharmila Donepudi et al. reported their work on the creation of an isocratic tool for the quantification of LINA and EMPA pharmaceuticals and in biological matrix. The elution was obtained by employing the stationary phase system Discovery C18 and the solvent system consisted of buffer and ACN with a FR of 1 milli liter per minute. The analysis was monitored at 218 nm. The RTs for LINA and EMPA were 6.421 and 4.696 minutes, respectively. They determined that the proposed approach used for quantifying both medications in human plasma samples. [79]

Manojkumar KM et al. introduced four novel UV spectrophotometric process for the instantaneous quantification of EMPA and MET HCL. These approaches included a concurrent analysis at 224 and 232 nm, using different tools. This process demonstrated effectiveness in the concurrent assessment of EMPA and MET for routine QC tests. [80]

Padmaja N. et al. reported a UV spectrophotometric tool for simultaneously estimating EMPA and LINA in API and pharmaceutical preparations. Simultaneous analysis was conducted using Vierodt's tool. The absorption was analysed at 233 nano meter and 277 nm for EMPA and LINA. Linearity was tested for EMPA at concentrations ranging from 5-15µg/ml and LINA at 2-6 µg/ml. Both EMPA and LINA had accuracy levels ranging from 98 to 101 percentage and validated in accordance with Q2 R1 ICH requirements. [81]

Jyothirmai N et al. have described easy and spectrophotometric process for determining the quality and quantity of EMPA in pharmaceutical preparations. All three procedures were confirmed in accordance with the ICH guiding principles. The suggested procedures were effective for evaluating EMPA in pharmaceutical preparation. dosage forms. The established process was straightforward, accurate,

dependable, and affordable. The proposed approach can be utilized for repeated examination of EMPA in API and in marketed formulations. [82]

Bassam M A et al. reported on computer-assisted analysis of pharmaceutical samples by developing simultaneous estimation of EMPA and MET using their ratio spectra, which they applied to a recently approved and marketed product. The validation of the proposed approach followed ICH criteria. They determined that the optimized tool was suitable for manufacturing QC laboratories. [83]

Dina et al. reported green analytical chemistry-assisted chromatographic tools for the simultaneous measurement of EMPA, LINA, MET, and the Pharmacopoeia contaminants cyanoguanidine and melamine. The established methods greenness was assessed using the Analytical Eco-Scale, as well as their eco-friendliness, and compared to previously reported approaches utilizing NEMI, GAPI, and the innovative AGREE tool. [84]

Ishita M. Patel et al. have developed a hp-tlc process for the concurrent assessment of EMPA, LINA, and MET in synthetic combinations and API forms. The process utilized TLC stationary phase and a solvent phase consisting of n butanol, water, and glacial acetic acid. The retention factor for EMPA, LINA, and MET were determined as 0. 7, 0. 5, and 0. 3, respectively. [85]

Manoj Kumar KM et al. have reported a novel stability-based analytical approach based on advanced TLC and DoE tool. The planned tool is valuable for estimating MET and EMPA in API and dose forms simultaneously. The procedure was devised and carried out on a silica gel-coated plate using a solvent phase of ammonium acetate, isopropyl alcohol, and triethylamine. [86]

Amin, Khanda FM, et al. studied the process using UV for the assessment of MET, EMPA, and glimepiride in their organized research laboratory blends and pharmaceutical preparation. The absorption spectra of the aforementioned medicines ranged from 200 to 400 nano meter. The approaches were linear across concentration of 1 to 10, 2.5 to 30, and 1 to 10 μg/ml of MET, EMPA, and glimepiride, respectively. [87]

Jyoti J. Vikhe et al. have published a comprehensive update on various analytical processs for determining EMPA and MET hydrochloride in API materials and pharmaceutical preparations. The review discusses analytical processes for quantifying them. This study includes thorough information on the development of EMPA and MET in API and pharmaceutical formulations. [88]

2.3.4. Analytical procedures for estimation of Linagliptin

Badugu RL et al. have validated a robust HPLC approach to precisely detect LINA levels in pharmaceutical formulations, overcoming analytical obstacles. The process, which used a symmetric 18 stationary phase and elution with MeOH: Water (83:17 v/v) at pH 4.1, was simple, specific, and accurate. Validation in accordance with ICH requirements confirmed its dependability, with a linear range of 5-30 ppm and a retention duration of 5.85 minutes for LINA, making it a useful approach for routine LINA measurement in pharmaceutical goods. [89]

B Lakshmi, TV Reddy, et al. published hplc approach for the rapid assessment of LINA in pharmaceutical preparation. form. The procedure used isocratic elution on a symmetric Chromosil C18 stationary phase at room temperature. LINA retention duration was 7 minutes while using a solvent phase of ACN, water, MeOH with UV

identification at 238 nm. Validation according to ICH requirements ensured process dependability, with precision and accuracy percentage RSD values of less than 2percentage, making it appropriate for regular LINA analysis in both pharmaceutical preparations. and API drug forms. [90]

Rajbangshi JC et al. established a validated chromatograph process for sensitively analysing LINA in API with pharmaceutical formulations. Using a C18 stationary phase and PDA detector, the approach obtained a retention time of 2.8 minutes and showed high linearity over concentrations of 40 to 60µg/ml. The approach was reported appropriate for precisely measuring LINA in a variety of pharmacological situations. [91]

Rahshekaran et al., have reported cost-effective hptlc process has been recognized for the concurrent assessment of MET and LINA in a formulation. The separation was carried out on precoated silica 60 GF254 plate using acetone MeOH-toluene formic acid in 4: 3: 2: 1, v/v/v/v with analyte detection at 259 nm. [92]

Donepudi and S. Achanta et al. devised a process for the concurrent measurement of LINA and EMPA in human plasma. Using a Waters HPLC setup with a C18 stationary phase and a solvent phase of buffer and ACN, the approach achieved accurate identification at 218 nm. There was no interference with retention times of 6.4 minute for LINA and 4.6 minute for EMPA. [93]

2.3.5. Analytical Procedures for Estimation of Sitagliptin

R Lavanya, M Yunoos, et al. reported hplc technology for precisely measuring SITA in API and pharmaceuticals. The process uses a Zorbax Eclipse XDB C18 stationary phase with a solvent phase of Phosphate buffer: MeOH and observed good linear response at 5 to 30 µg/ml with a r2 0.999. [94]

Raza A et al. described a validated a quick, cost-effective, and environment-friendly HPLC tool for simultaneously assessing MET and SITA in commercial pharmaceutical preparation. Using a Shimadzu C18 stationary phase with a solvent phase of acidified water and with retention periods of 1.9 and 3.7 minutes for MET and SIT, respectively. [95]

Babu CD et al. established a sensitive HPLC process for concurrently measuring Ertugliflozin and SITA in both API and pharmaceutical forms. It used a Waters C18 stationary phase with retention periods of 2.39 and 4.60 minutes for ETR and SGT, respectively, with a solvent phase orthophosphate buffer and MeOH. Using PDA identification at 215nano meter." [96]

Sankar ASK et al. reported a precise and simple chromatography approach for determining the SITA Phosphate and MET in pharmaceutical formulations using a Phenomenex Luna stationary phase, with identification at 252nano meter. 2.7 and 1.9 minutes are the elution time for SITA and MET respectively. [97]

B Krishnan, K Mishra, et al. established a new HPLC approach based on QbD principles for the measurement of SITA and MET in API and pharmaceutical formulations. Using a Monolithic C18 stationary phase with PDAUV identification, ideal conditions were achieved with MeOH, ACN, and a pH adjusted with OPA. [98]

Laxmi M et al. proposed an analytical approach based on reverse phase LC with PDA identification for the assessment of ertugliflozin and SITA in pharmaceutical preparation. Analytes were separated using a Cosmicsil C8 stationary phase as the stationary phase. The solvent phase was optimized with 0.1 mol dipotassium hydrogen phosphate and MeOH. The eluted analytes are measured at 225 nm. [99]

Anjali M, et al. have reported the assessment of SITA and ertugliflozin in medicinal preparation. dosage forms by Vierodt's approach. Absorbance was measured at 210 and 221 nms for SITA and Ertugliflozin, respectively. The solvent system consisted of a 0.1percentage ortho phosphoric acid buffer and ACN solution. SITA and Ertugliflozin have linear concentration ranges of 7 to 42 μg/ml and 4 to 6 micro grams per milli liter. [100]

Chapter 3

Materials and Methods

Active Pharmaceutical Ingredients (API)

Metformin HCL, Dapagliflozin, Empagliflozin, Linagliptin, and Sitagliptin were selected as anti-diabetic medicines for the proposed study. In order to develop and validate the analytical approaches, APIs have been used. Metformin HCl (99%) was got as a free API from FDC Limited, Goa. Dapagliflozin (100%) and Empagliflozin (99.0%) were provided as a free sample by MSN Laboratories Private Limited. Hyderabad Linagliptin (99%) and Sitagliptin available as Sitagliptin phosphate monohydrate (99%) received from Dr. Reddy's.

Chemicals and reagents

The chemicals and reagents utilized for analytical purposes remained of analytical grade and acquired from the chemical store of college. HPLC grade Methanol, Acetonitrile, o-phosphoric acid was analytical grade and sourced from Merck India Limited for the analytical work.

Marketed formulations

For verifying the suitability of developed technique, a marketed formulations loaded with selected anti-diabetic drugs were procured from local Pharmacy market. Metformin HCl tablets (Label claim 500 mg), Dapagliflozin tablets (Label claim 10 mg), Empagliflozin tablets (Labelclaim 10 mg), Linagliptin tablets (Labelclaim 5 mg), and Sitagliptin tablets (Labelclaim 50 mg) are used for the analysis.

Instruments and apparatus

An electronic analytical weighing machine of sartorius was used to weigh the analyte, an Ultrasonic bath Sonicator-Ultrasonic cleaner was used to sonicate the solutions, an "Elico CM 183EC-TDS" analyser was used to measure the conductance of the MET, and Millipore Water was collected for analysis using a Direct Q UV aquatic distillation system. UV-spectrophotometric methods were developed using a Shimadzu 1800 spectrophotometer. For the optimization and validation of HPLC techniques, an Agilent 1220 HPLC system with Chemstation software, an Agilent 1100 with Chemstation software, and a Shimadzu-Prominence-i series LC-2030c 3D plus HPLC system with Shimadzu Prominence software, Lab solutions Version 5.97 SP were employed.

General methodology

In the proposed research methodology, the general steps involved in analytical method development and validation were followed. The optimization of analytical process was carried out by following principles and approaches of QbD before the method validation. The newly developed and optimized method also subjected for the validation protocol as per the ICH. To ensure the process performance and applicability, marketed dosage forms containing selected anti-diabetic drugs were analyzed. **Figure 7** represents the general steps involved in proposed research. [101-103]

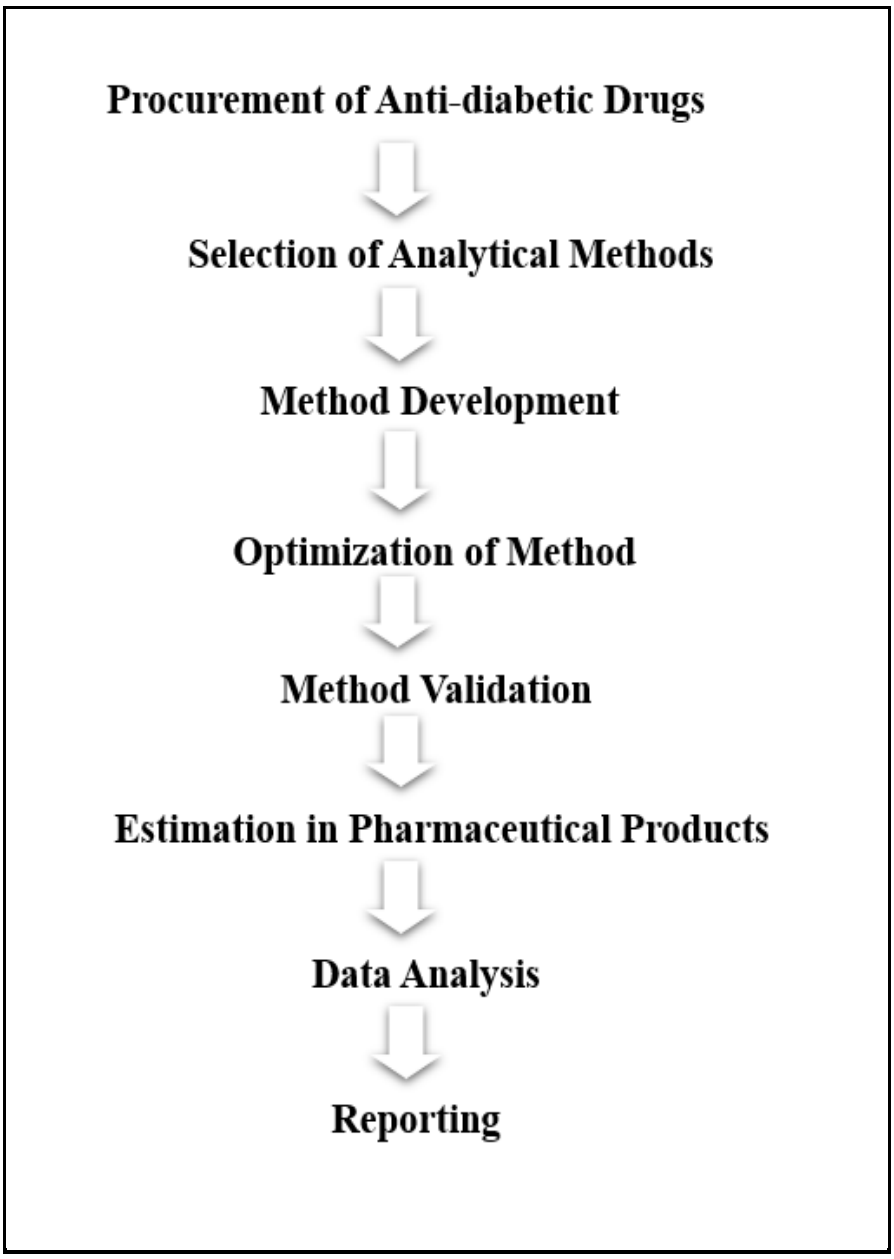

Figure 7: General steps involved in proposed research methodology

Step-I: Procurement of selected Anti-diabetic drugs

In the proposed research work following anti-diabetic drugs were selected based on the review of literature:

1. Metformin HCl
2. Dapagliflozin
3. Empagliflozin
4. Linagliptin
5. Sitagliptin

Step-II: Selection of analytical methods

Analytical methods for estimation of selected Antidiabetic drugs were selected founded on the nature and assets of API molecules. Based on current review of literature and regulatory requirement QbD analytical methods were planned to develop. In the proposed research work chromatographic, Spectrophotometric and electrochemical based methods were adopted. The details of methods planned for analysis of selected anti-diabetic drugs were listed in **Table 2.** [101-103]

- High-performance LC process was planned for the analysis of Metformin HCL, Dapagliflozin, Empagliflozin, Linagliptin and Sitagliptin.

- UV-Spectrophotometric tool was planned for the analysis of Metformin HCL, Dapagliflozin, Empagliflozin, Linagliptin and Sitagliptin.

- Conductometric method was planned for the examination of Metformin HCL.

Table 2: Planned analytical methods for selected drugs

Sl. No.	Analyte Name	Analytical Methods
1	Metformin HCL	• HPLC • Conductometric method
2	Dapagliflozin	• HPLC • UV spectrophotometric method
3	Empagliflozin	• HPLC • UV spectrophotometric method
4	Linagliptin	• HPLC • UV spectrophotometric method
5	Sitagliptin	• HPLC

Step-III: Method development

A. Creation of high-performance liquid chromatographic method

The development of HPLC method was started with selection of following parameters:

- Stationary phase/column system
- Mobile phase/solvent system
- Flow rate
- Temperature condition
- Detection wavelength
- Injection volume

To determine the appropriate stationary phase and other experimental conditions, preliminary experimental runs were carried out to identify viable combinations and select the stationary and mobile phase. Initial trials were undertaken out employing a literature review. The chromatographic technique parameters were initially determined by taking into account characteristics such as minimum retention duration, maximum theoretical plates, minimum tailing aspect, and good peak symmetry of the chromatogram. Following the development and identification of basic chromatographic conditions, an optimization strategy based on QbD principles was used to validate the chromatographic results. [104-110]

B. Development of U-Spectrophotometric tool

The development of the UV process began with the selection of a solvent solution and the identification of the wavelength for detection. Initially, the solubility of selected medicines was assessed in various solvents. The analyte solutions were then produced in the appropriate solvents and analyzed using wavelength identification. The solvent system employed in the development study was optimized using DoE software based on QbD principles. The UV-Spectrophotometer Shimadzu 1800 was used to analyse specific medications. [111-121]

C. Development of conductometric method

The conductometric tool was developed using a conductivity meter and Millipore Water (MW). Different concentrations of Metformin HCl in MW were prepared, and their conductivity was analysed. Every different analyte concentration led to varying conductance values, with a rise in amount corresponding to an increase in the electrical conductance of the solution. The suitability of the developed process was assessed by analysing the commercial tablet formulation of Metformin HCl. The electrical conductivity meter Elico was employed in analysis. [122]

Step-IV: Method optimization (QbD approach)

The QbD method useful and effective in optimizing the proposed procedures. The approaches that were created were optimized utilizing quality-by-design principles. This strategy has helped identify analytical target profiles, analytical attributes, critical method variables, risk analysis, experimental optimization, control plan execution, and continuous improvement for analytical lifecycle management. Expert software with a variety of designs was used to optimize experimental variables. QbD is characterized as a method to progress that initiate with explicitly defined objectives and emphasizes a comprehensive considerate of both product and process through robust scientific principles and effective quality risk management. The QbD framework involves enhancing scientific insights into critical product parameters and attributes, devising tests and controls grounded in scientific comprehension during the developmental phase, and leveraging knowledge and data acquired throughout the entire lifecycle of the product for continuous improvement. In the context of optimizing HPLC methods, our focus extended to various aspects of AQbD, seamlessly integrating them into the practical implementation. This process commenced with formulating an analytical target profile, proceeded to scrutinizing critical quality and analytical aspects, and involved risk assessment utilizing the fishbone method. Subsequently, DoE was employed to facilitate the optimization of factors in the RP-HPLC technology. [123-139]

Following steps were followed during the method optimization of analytical methods based on QbD: development of ATP, dentification of AAs. identification of CMPs, risk Valuation, DoE based method optimization, implementation of control strategy and continuous improvement of analytical life cycle.

Creation of ATP

The initial and decisive stage in formulating a QbD-based analytical method is the identification and establishment of the ATP. The ATP encompasses the essential information needed to achieve the desired quality attributes in method development. This ensures the analytical method's reliability, quality, and practical applicability when employed in the pharmaceutical industry for commercial purposes. [123]

Documentation of critical analytical characteristics

To meet the requirements of the analytical procedure's target profile, distinct CAAs must be selected, which is a critical stage in the development of AQbD-based methods. The CAAs can be described as the quantifiable properties of the analyte chromatogram that must fall inside an acceptable boundary, range, or threshold to ensure the analytical procedure's anticipated quality and capability. [151]

- Critical quality attributes for chromatographic development include peak area (PA), tailing factor (TF), retention time (RT), theoretical plates (TP), peak spectral purity, and assay limit.

- In case of UV-Spectrophotometric method absorbance was used as critical quality analytical attributes.

- In case of conductometric method conductance was used as critical quality analytical attributes.

Identification of critical method variables

CMPs are the sensitivity levels associated with an analytical technique. CMPs can be categorized using a variety of analytical techniques, including UV, GC, HPLC, and HPTLC.

In the proposed research work following parameters were identified as CMPs:

- **HPLC Method:** The organic solvent concentration in mobile system, flow rate, wavelength of identification.

- **UV-Spectrophotometric method:** Scanning speed, solvent ratio, wavelength of detection

Risk estimation

Risk assessment is a crucial phase in the development of QBD-based methods. In this investigation, Risk estimation was employed to evaluate the likelihood of operational failure. To achieve this, a cause-and-effect model linking serious material attributes and CMPs with CAAs was recognized and applied through "Ishikawa Fish-Bone" (IFB) figure. [151]

a. Risk valuation in chromatographic method

For chromatographic analysis, an IFB diagram (**Figure 8**) was built to highlight important risk variables that could impact the method's performance. The identified risk factors may encompass elements such as the sample extraction method, extraction duration, solvent employed for extraction, and instrumental parameters like solvent system ratio, chromatographical mode, FR, and volume used for injection. The risky method variables were identified and subsequently explored utilizing a suitable experimental strategy. The selected risky method variables included the mobile phase's flow rate, the organic phase percentage composition (MeoH, ACN in the solvent phase) and the detection wavelength.[142-146]

b. Risk assessment in UV-Spectrophotometric method

An IFB diagram (**Figure 9**) was created to identify potential dangers and root causes that affect the performance of the UV method by emphasizing several method variables that may influence the method attributes of the UV spectrophotometer technique. Variations in solvent type, detection wavelength, scan speed, sampling interval, sample integrity, and slit width were discovered to be associated with high final scores, suggesting high risk variables.[148,159]

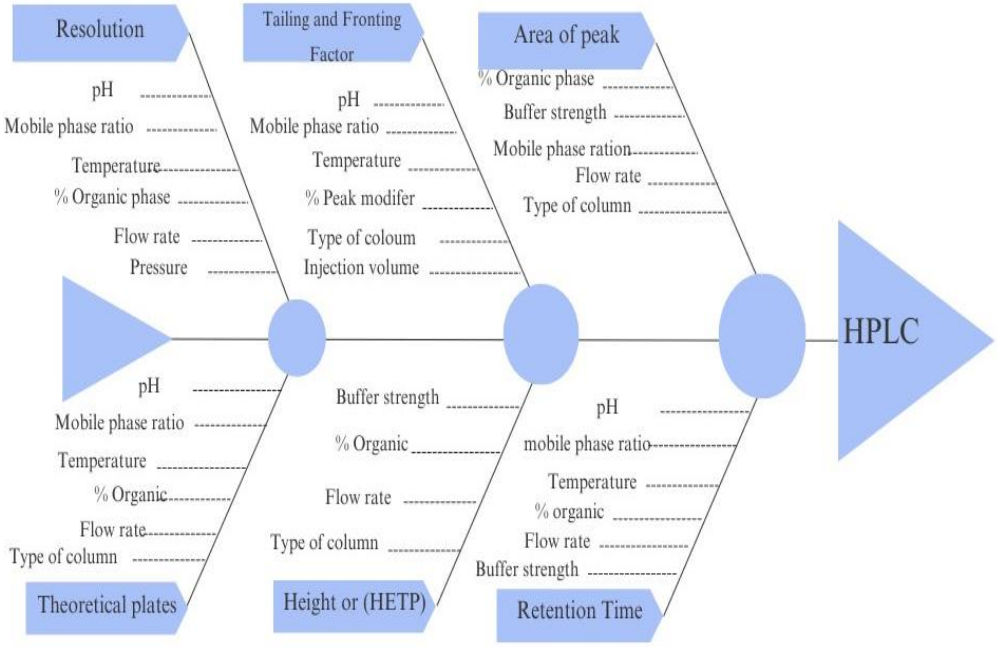

Figure 8: Ishikawa Fishbone diagram for HPLC method

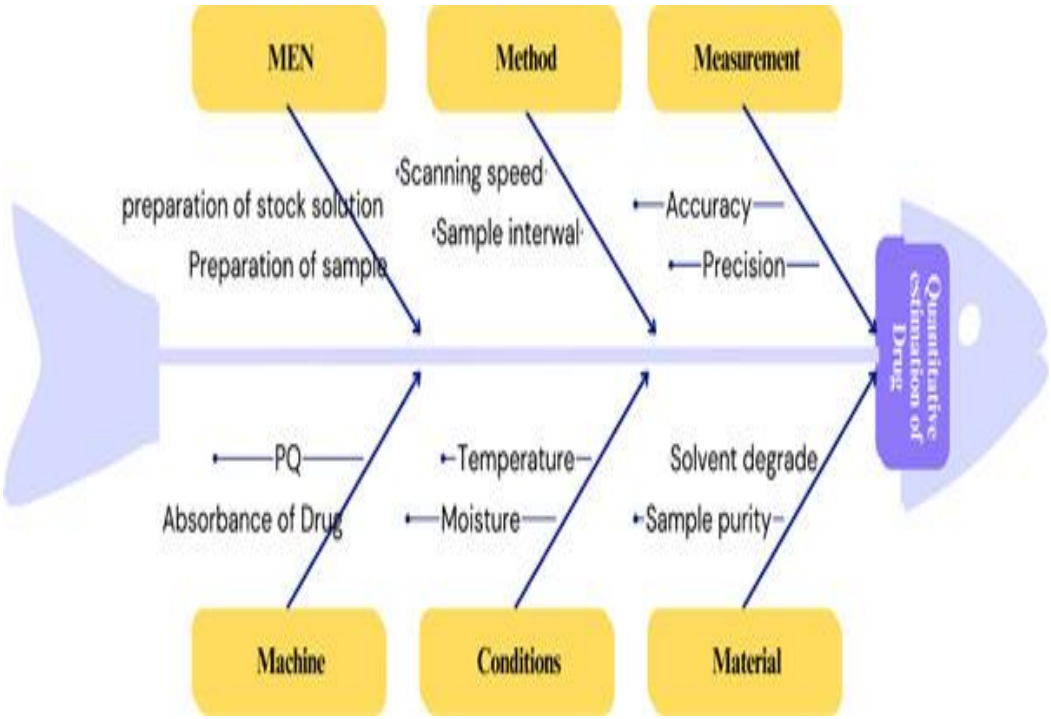

Figure 9: Ishikawa Fishbone diagram for UV-Spectrophotometric method

Step-5: DoE based method 0ptimization

The experimental design (DoE) is a critical phase in AQbD. DoE is a set of statistical methods that include filtering and design optimization, allowing for better results with fewer trials. The screening design identifies the most relevant independent variable (e.g., analytical condition) that influences the dependent variable (e.g., chromatographic response). Furthermore, the optimization design allows for the construction of mathematical equations that define the relation between dependent variable and independent variables. The DOE approach allows for the consideration of a variety of objectives. These include response surface approaches based on empirical models that predict outputs across domains of interest. After defining the potential and CMPs through an, DoE was used to check and rectify the CMPs based on statistical analysis. The term Experimental Design refers to an organized collection of methodologies for discovering the link between factors that influence a process' output. One approach to the research characterization process is to use an experimental design so that data can be used to understand and define the design astronomical. It is then executed, and the results are analyzed to control the importance of the constraints and their role in the strategy space. Can be given for each item operation or as a mixture of numerous technique variables and their connections and reactions. This method offers a fantastic opportunity to filter out numerous circumstances that arise from multiple tests. The data should next be evaluated using statistical tools to find the CMVs and the suitable ideal range for the process variable quantity in order to obtain a vigorous area for critical process attributes. [143-147]

Implementation of control strategy

After method development, it is vital to implement a control strategy. The creation of an ATP was instrumental in steering the optimization of the systematic control way. The analytical controller plan constitutes a deliberate array of controls resulting from an appreciation of various parameters, encompassing suitability for determination, investigative procedure, and risk managing. These criteria guarantee alignment between the process performance and the quality outcomes specified in the ATP. [43]

Continual improvement of life cycle

Achieving effective management of the analytical lifecycle is facilitated through continual improvement. This involves ensuring quality consistency and conducting routine maintenance on HPLC/UV instruments, computers, software, and other laboratory equipment. Additionally, it entails the regular updating of various laboratory instruments and apparatus. [43]

Step-V: Analytical method validation

Validated in terms of numerous parameters as per the ICH Q2 (R1) procedures were planned for proposed methods. The optimized HPLC, UV-Spectrophotometric and Conductometric methods were authenticated in terms of specificity assessment, selectivity analysis, linear response, range, detecting amount, quantifying limits, preciseness, ruggedness, robustness, and accuracy-recovery-analysis. [150]

Step-VI: Assessment of selected agents in marketed preparations

To examine the applicability of the projected analytical procedures, marketed formulations containing chosen anti-diabetic medications were evaluated in triplicate, and drug content and percentage assay values were determined. To extract the analyte from the marketed formulations, an appropriate extraction technique was established for each method. All newly designed, optimized, and validated chromatographic, spectrophotometric, and conductometric approaches were effectively employed for QC investigation of commercial anti-diabetic pharmaceutical preparations. [122, 151-152]

Chapter 4

Results and Discussion

METHOD 1: DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR ESTIMATION OF METFORMIN HCL

MATERIALS AND METHODS

Chromatographic conditions

The suggested investigation utilized an Agilent 1100 (Autosampler) HPLC equipment with Chemstation software. Metformin HCl was chromatographically separated on an Agilent C18 column (internal diameter 5µm, 250 x 4.6 mm). The solvent phase used composed of Methanol and Ortho-Phosphoric Acid (OPA) 0.1% in water (pH of 2.8) in the proportion of 4:96 v/v. Metformin HCl found to eluted at a (Flow rate) FR of 0.7 ml/min and detected by UV detection at 231 nm. The temperature of system was kept at ambient, and the volume of injection was 20 µL.

Standard stock solutions

The Metformin HCl primary stock solution was prepared by dissolving 10 mg of the in 10 mL of Methanol. After sonication, 1 mL of this solution was carefully transferred to a 10 mL volumetric flask (VF) and then diluted to the mark with the mobile phase serving as the diluent. Subsequent concentrations for chromatographic analysis were derived from this stock solution, with each dilution prepared using the mobile phase as the diluting agent. [111]

Method Development

For identifying the best stationary phase, preliminary experimental runs were carried out to identify the stationary phase and mobile phase composition. Initial trials were undertaken out employing a literature review. Methanol was chosen as a trial study to establish cost-effective approach. Aqueous solvents with varying concentrations of OPA were used for efficient separation at various pH levels. Based on the results of the preceding experiments, the C18 stationary phase was chosen for future investigation. 113

Identification of the detecting wavelength

The Metformin HCl standard solution was scanned in the 200-400 nm range to find the Wavelength of maximum absorbance/ Wavelength maxima (λmax) for the analysis. The UV spectra of the standard medication exhibited a maximum absorption at 231nm. As an outcome, it worked as the determining wavelength.[102]

QUALITY BY DESIGN APPROACH

QbD is an organized method to development that commences with specified goals and highlights product and method understanding via solid knowledge and quality-risk-management. In this study, we investigated numerous features of AQbD are applied to a method development and optimization. It started with the finding of an ATP and progressed to the fishbone technique for investigating CAAs and RA. The subsequent technique uses DOE to aid in the factor optimization of liquid chromatographic technology. [41, 123-142]

Outlining and specifying the desired characteristics and parameters for an analytical method

The initial and crucial stage in developing a QbD-based analytical procedure involves defining and establishing the ATP. The ATP can be described as the compilation of data necessary to achieve the desired quality profile for method development. Various elements of the ATP were examined, encompassing aspects such as the target drug, samples, and method application, as well as considerations for the analytical process, machine specifications and necessities, sample extraction stages, and quality attributes of the analytical procedure. Subsequently, an ATP specific to the proposed study was identified. [43]

Identification of critical analytical attributes

To satisfy the requirements of the analytical procedure's target profile, numerous CQAs/CAAs must be chosen, which is a key phase in developing an AQbD-based methodology. In the case of proposed chromatographic development, CQAs typically comprise Tailing Factor (TF), Retention Time (RT), Peak Area (PA), Theoretical Plates (TP), peak spectral purity, and test limit. Among these characteristics, RT, TP, PA and TF had significant effects on the performance of the analytical technique and were chosen for further examination. [41, 43]

Identification of critical method variables and risk assessment

Risk assessment is an important stage in creating QBD-based methods. The RA approach was utilized to predict the procedure's failure risk. To accomplish this, a cause-and-effect model for the connection between CMPS with critical analytical features was developed and using a IFB diagram. [151]

Design of Experiment (DoE)-based method optimization

In the selection of DoE, preference was given to a response surface design as compared to a factorial design. The Box-Behnken Design (BBD) was subsequently opted for in-depth exploration due to its effective performance in three-factor and three-level designs, along with the advantage of requiring fewer runs compared to the Central Composite Design (CCD). The Design Expert program was used to optimize a unique chromatographic method for Metformin HCl. In this particular application, the BBD was employed to optimize CMPs (% organic modifier, flow rate, and wavelength) and explore their interactions with CQAs (RT, PA, TP, and TF). The BBD, known for its efficiency in three-factor, two-level: low (-1) and high (+1) designs with a reduced number of experimental runs, was chosen. After conducting initial factor screening trials, the performance of the method was fine-tuned using the BBD at two levels. In the DoE framework, CMP parameters (% organic solvent, FR, and wavelength) were designated as input variables, while CAAS such as RT, PA, TP, and TF were identified as response variables **Table 3**. Using Design Expert tools, we generated a design matrix with 14 experimental runs. "The screening strategy employed for BBD aimed to assess the various interaction and quadratic effects as outlined in **Table 4.** [143-145]

Table 3: BBD matrix for HPLC analysis of Metformin HCL

Independent Variable	Levels	
	+1	**-1**
FR (ml)	**0.6**	**0.9**
% Methanol	6	2
Wavelength (nm)	233	229

Table 4: Optimization matrix by BBD for HPLC analysis of Metformin HCL

	Factor 1	Factor 2	Factor 3
Run	**A: FR**	**B: Methanol**	**C: Wavelength**
	ml/min	**Percentage**	**nm**
1	0.7	3	230
2	0.8	2	232
3	0.8	3	229
4	0.6	4	231
5	0.7	5	230
6	0.9	3	232
7	0.7	5	232
8	0.7	4	231
9	0.9	3	230
10	0.8	4	231
11	0.9	5	230
12	0.9	5	232
13	0.8	5	231
14	0.8	4	232

Method development based on experimental design

The factor screening method optimized the identification of CMPs influencing procedure performance by employing three factors at two middle levels. Throughout all chromatographic runs, Metformin HCl at a quantity of 10 µg/ml was consistently utilized to assess CAAs.

Data analysis and model validation

The responses generated after performing the proposed trial were entered into software, and various graphs such as 3D response surface plots and graph plots were produced. These charts depict the impact of CMPs on the specified CQAs. The examination of these plots was used to determine which parameters produced satisfactory answers. Based on these findings, the procedure's final CMPs were discovered, and the optimal conditions were chosen. Statistical tools, such as "analysis of variance" (ANOVA) for each response, were employed to investigate the importance of each parameter utilized in the study using the p value.

ANALYTICAL METHOD VALIDATION

The established technique was validated by following the ICH Q2 (R1) guidelines in terms of system suitability test, test for selectivity and specificity, test for linearity and range, sensitivity (Limit of Detection-LOD and Limit of quantification-LOQ), precision study, investigating ruggedness, robustness and recovery analysis. [150]

System suitability: Six replicate analysis of standard Metformin HCl were performed using chromatographic analysis to determine system compatibility. SD and percentage RSD of PA, TF, RT, and TP were calculated.

Selectivity and specificity: The procedure's specificity and selectivity were evaluated using HPLC chromatograms of standard, sample Metformin HCl solutions, and mobile phase. It aids in the identification of the analyte and reduces interference from other peaks.

Linearity and range: The devised method was tested for linearity by preparing the solutions of Metformin HCl between 5 and 25 µg/ml and building a standardization curve of PA versus Metformin HCl concentration. The method's linearity was verified by performing regression statistics.

Sensitivity (Limit of Detection-LOD and Limit of Quantification-LOQ): The sensitivity of proposed method was studied in terms of LOD and LOQ. The detecting and quantification limits values were calculated using the following formulas:

LOD = 3 x σ/S and

LOQ =10 x σ/S

Where, σ is the SD of the y-intercept and S is the slope of the calibration curve.

Precision: The precision of the proposed approach was evaluated by analysing 3 distinct quantities of Metformin HCl (10, 15 and 20 µg/ml) at various time intervals within the same day (intraday precision) and across consecutive days (interday precision). The %RSD was computed for PA of each chromatogram.

Ruggedness: Another analyst analyzed the standard solution of Metformin HCl to assess the ruggedness of the suggested method. The mean and % RSD were computed for PA in the chromatogram.

Robustness: By varying the solvent composition of Methanol: buffer (3:97 to 5:95 v/v), changing the wavelength (233 and 230) and altering the FR (0.6 ml/min to 0.8 ml/min), the robustness was evaluated using working solution of 25 µg/ml. The overall mean and percentage RSD were computed for PA in the chromatogram.

Recovery analysis: The recovery was calculated using the addition method. Metformin HCl (5 µg/ml) sample solution was spiked with reference solution at 100% (5 µg/ml), 120% (6 µg/ml) and 80% (4 µg/ml) concentrations. These solutions were injected into HPLC in triplicates to get chromatograms. Furthermore, amount recovered and % RSD were calculated to ensure accuracy of the data.

ESTIMATION OF METFORMIN HCL IN MARKETED TABLETS

Metformin HCl assay was performed utilizing the devised RP-HPLC approach to determine the applicability of the suggested method. 118, 119

Sample extraction procedure: Twenty Metformin HCl tablets were weighed, crushed, and the resulting powder containing 10 mg of Metformin HCl was introduced into a 10 ml volumetric flask (VF). To this, 2 mL of a solvent system was added, followed by 15 minutes of sonication to ensure dissolution. The volume was then adjusted to the mark with the same solvent. The produced solution subjected to filtration using Whatman filter paper, and 1 mL of this filtrate was further diluted to 10 mL using the mobile phase. 2.5 mL of the solution was transferred to a 10 mL VF, and the volume was adjusted to the mark using the mobile phase as the diluent for having targeted quantity of 25 µg per mL. This solution was injected into a stabilized chromatographic system for elution and subsequent analysis. The process was repeated in triplicate.

RESULTS

METHOD DEVELOPMENT

The initial experiments involved a literature review and trial-and-error approach to establish a straightforward, cost operative, specific, and precise technique for assessing Metformin HCl in pharmaceutical tablet dosage forms. The method development commenced with the selection of a mobile phase from various solvent combinations, including Methanol and OPA. Different concentrations of the buffer phase were tested to optimize the elution of Metformin HCl. In the preliminary trials, it was determined that using 0.1% OPA at a pH of 2.8 yielded faster and more effective drug separation, with Metformin HCl elution occurring at lower RT, low TF and achieving acceptable peak symmetry. **Figure 10** presents the UV spectrum for standard Metformin HCl. **Figure 11** illustrates the optimized chromatogram. The optimized method parameters are displayed in **Table 5**.

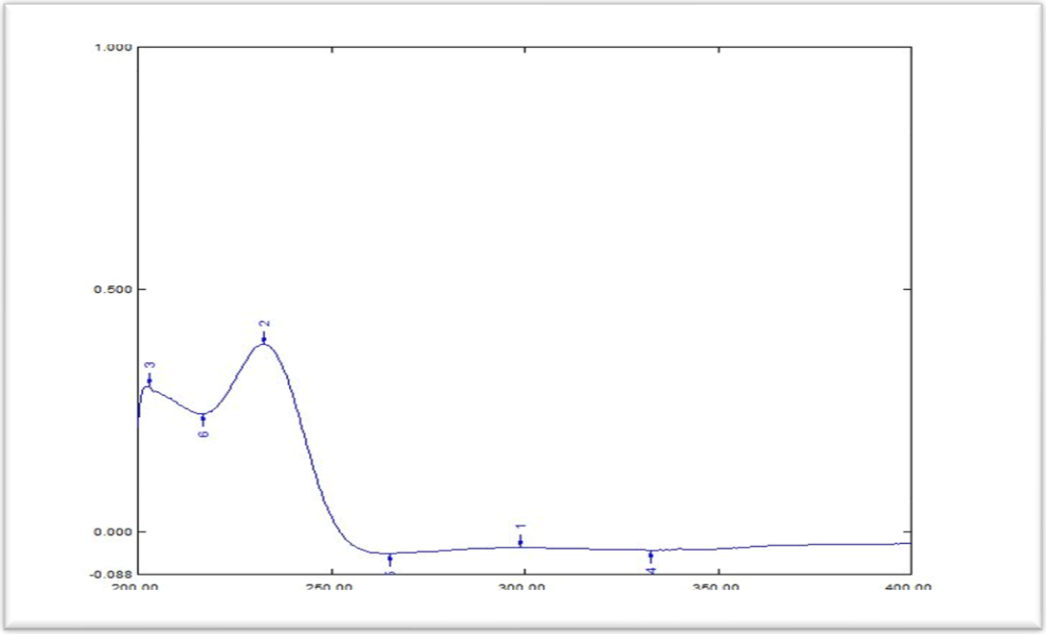

Figure 10: UV Spectrum of standard Metformin HCl

Table 5: Developed HPLC method specifications for Metformin HCl

Sr. No.	Description	Parameters/Specifications
1	Technique	Chromatographic separation
2	Device	HPLC
3	Model	Agilent
4	Brand	Agilent 1100 (Autosampler)
5	Software	Chemstation possessing
6	Column system	Agilent C_{18} column
7	Solvent system	0.1% OPA: Methanol in the ratio of 96:4% v/v
8	FR	0.7 ml
9	Identification	231 nm
10	Volume of injection	20 µL.
11	Analyte	Metformin HCl
12	RT	2.9 min

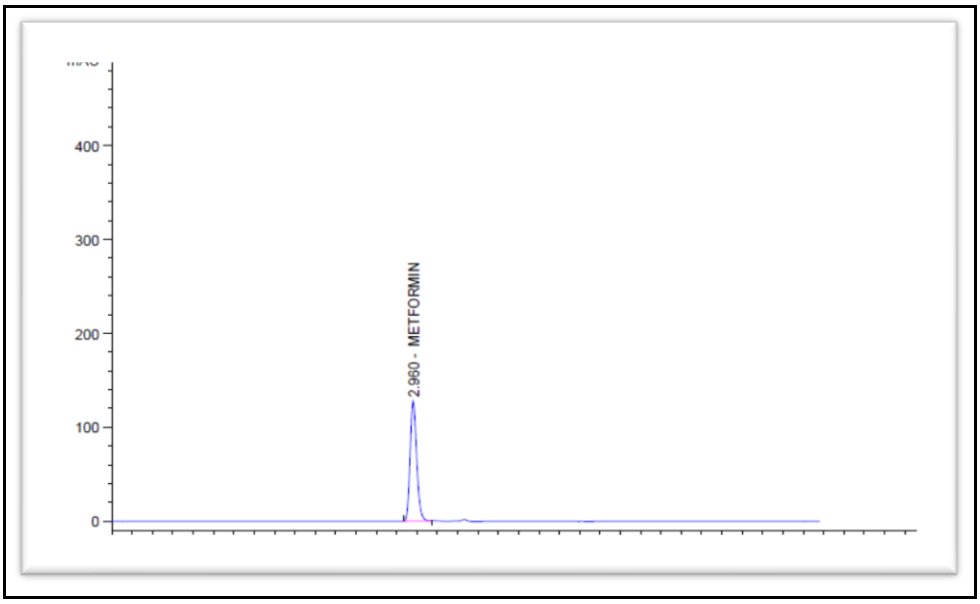

Figure 11: Optimized HPLC chromatogram of standard Metformin HCl

QUALITY BY DESIGN APPROACH

The steps involved in QbD-based method optimization were carried out in a systematic manner. As described in methodology, we completed procedures such as creating an analytical goal profile, identifying critical quality attributes, identifying critical method parameters, risk assessment using the fishbone approach, and method optimization using DoE Software. The results are presented as follows:

Development of analytical target profile

ATP defines the method requirements that are intended to be measured. The analytical target profile is defined using the information and scientific reasoning of the analytical process. Various components of ATP were identified through a thorough review of available literature reports and drug profiles, and we discovered that ATP for method development primarily involved developing a fast, reliable, and profitable analytical process for estimating Metformin in pharmaceutical preparation. As a result, depending on the primary purpose of this study, an HPLC method was used to analyse the Metformin HCl. **Table 6** shows ATP for the suggested HPLC technique for Metformin HCl.

Identification of critical quality characteristics

In the case of chromatographic development, essential quality attributes often include retention time, theoretical plates, peak area, and tailing factor, as well as peak spectral purity. The retention time, theoretical plates, peak area, and tailing factor have a significant effect on method performance.

Table 6: Analytical target profile for HPLC analysis of Metformin HCl

Sl. No.	ATP Parameters	Target
1	Target Analyte	Metformin HCl
2	Target Sample	Metformin HCl Tablets
3	Method category	Chromatographic Method
4	Instrument requirement	HPLC
5	Nature of analyte	Solid (Solution)
6	Standard stock solution	Dilution of main drug in linear manner
7	Application of Method	Estimation of Metformin HCl
8	Validation parameters	System suitability, specificity, selectivity, sensitivity, LOD and LOQ), precision, ruggedness, robustness, and accuracy.

Identification of critical process parameters and analysis of risk

To analyse the CMPs, which are high-risk factors that significantly affect the CAAs, a risk assessment investigation was carried out. An IFB diagram (**Figure 12**) was made for this inquiry in order to pinpoint the main risk factors that might have an impact on the effectiveness of the technique. After being discovered, the high-risk procedure variables were investigated further with the use of a suitable experimental optimization methodology. The FR of mobile phase, the quantity of organic solvent used (Methanol), and the detection wavelength were selected as the high-risk technique factors.

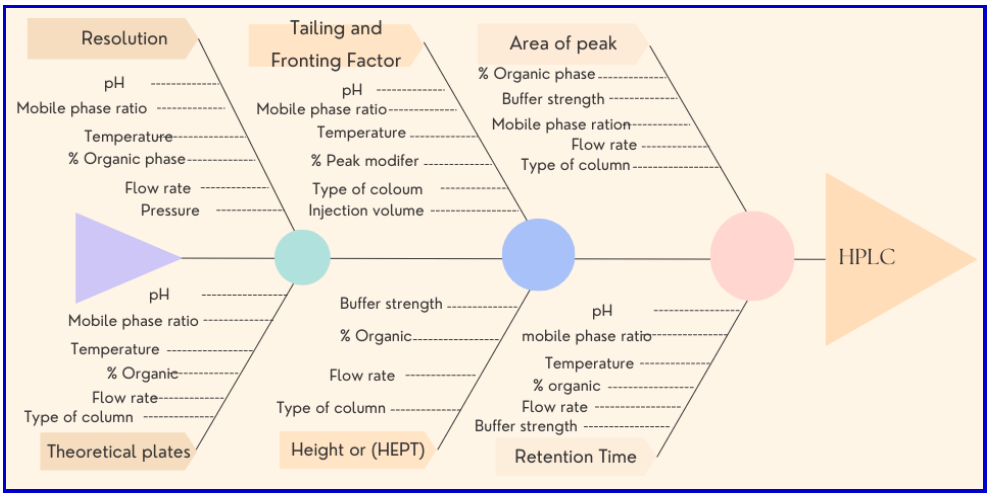

Figure 12: Ishikawa Fishbone diagram for HPLC method of Metformin

DoE optimization

It is a crucial part of the DOE process, and we selected high-risk components such the organic phase percentage in the solvent system, the detection wavelength, and the mobile phase FR in order to optimize the RP-HPLC process. The optimization of RT, PA, TF, and TP involved investigating the primary, interaction, and quadratic effects of these parameters. This optimization was performed using the BBD which was aimed to see the effects of wavelength, mobile phase ratio, FR, interactions on RT, PA, TP, and TF, and to study their primary and quadratic effects. After the 14 runs were completed, the data were statistically examined using software. The wavelength of detection, the proportion of organic phase (X1), and FR (X1) were selected as the independent variables, and the dependent responses were RT, PA, TP, and TF. The chromatographic runs were performed in accordance with **Table 4** recommendations. The effects of multiple independent factors on response values are displayed in **Table 7**. ANOVA and statistical optimization were carried out using the software (**Tables 8** and **9**). Additionally, the 2-D and 3-D graphs were utilized to define the design space and show how independent parameters affected the response variables. It was found that the ANOVA parameters were consistent with these graphs. **Figures 13-16** show a 3-D and 2-D graphs that indicate the effects of FR, organic solvent quantity, and wavelength on selected CAA variables. The BBD was used to further optimize factors in the design space. The analysis of data for both main and interaction effects utilized the quadratic equation. The optimal factors for the model are presented in **Tables 8** and **9**. Every CAA parameter has a quadratic equation produced by the model. Detection at 231 nm, a 4% Methanol composition, and a 0.7 ml/min FR were used for additional validation.

Equation for retention time (for coded factors) = +2.62 - 0.3527 A + 0.0062 B + 0.0011 C + 0.0424 AB +0.1428 A^2 + 0.1242 B^2 + 0.1190 C^2. It was concluded that as β1 negative coefficient (-0.3527) suggests that the flow rate (A) decreases and β2 positive coefficient (0.0062) suggests that as amount of methanol (B) increases, and β3 positive coefficient (0.0011) suggests that the wavelength (C) increases, the value of retention time was increased. In similar way other equations of dependent variables were studied.

Table 7: Box-Behnken optimization design along with responses for HPLC analysis of Metformin HCl

Run	RT	PA	TP	TF
1	3.45	4850.37	11675	0.82
2	2.95	4019.71	10892	0.84
3	3.369	4521.28	11693	0.83
4	3.89	5516.24	12789	0.8
5	3.33	4896.5	11458	0.83
6	2.61	3555.04	9810	0.86
7	3.32	4665.06	11373	0.82
8	2.92	4373.36	10645	0.84
9	2.62	3755.37	9777	0.85
10	2.62	3707.75	9733	0.86
11	2.71	4018.88	9950	0.86
12	2.69	3785.56	9793	0.85
13	3	4326.7	10679	0.84
14	3	4484.22	10716	0.84

Table 8: ANOVA replies of BBD for HPLC analysis of Metformin HCl

Source	Sum of squares	df	Mean square	F-value	p-value	
For Retention Time						
Model	1.95	7	0.2783	211.69	< 0.0001	significant
A-Flow rate	1.10	1	1.10	833.33	< 0.0001	
B-Methanol	0.0005	1	0.0005	0.4083	0.5464	
C-Wavelength	0.0000	1	0.0000	0.0124	0.9149	
For Peak Area						
Model	3.294E+06	1	3.294E+06	57.63	< 0.0001	significant
A-Flow rate	3.294E+06	1	3.294E+06	57.63	< 0.0001	
For Theoretical Plates						
Model	1.120E+07	7	1.600E+06	288.01	< 0.0001	significant
A-Flow rate	6.682E+06	1	6.682E+06	1202.92	< 0.0001	
B-Methanol	40072.40	1	40072.40	7.21	0.0363	
C-Wavelength	358.64	1	358.64	0.0646	0.8079	
For Tailing Factor						
Model	0.0040	7	0.0006	646.40	< 0.0001	significant
A-Flow rate	0.0020	1	0.0020	2334.61	< 0.0001	
B-Methanol	0.0000	1	0.0000	0.0000	1.0000	
C-Wavelength	0.0000	1	0.0000	0.0000	1.0000	

Table 9: Statistical analysis data for adjusted and predicted values for HPLC analysis of Metformin HCl

ANOVA Parameters	Retention Time	Peak Area	Theoretical Plates	Tailing Factor
R-Squared	0.9960	0.8277	0.9970	0.9987
Adjusted *R*-squared	0.9913	0.8133	0.9936	0.9971
Predicted *R*-Squared	0.9583	0.7698	0.8559	0.8529
Standard Deviation	0.0363	239.06	74.53	0.0009
% CV	1.19	5.53	0.69	0.1116
F-value	211.69	57.63	288.01	646.40
p-value	<0.0001	<0.0001	<0.0001	<0.0001

R-squared = Coefficient of determination.

F-value = Value on the F distribution.

p-value = Probability of falsely detecting a significant effect.

C.V.% = Percent Coefficients of variance.

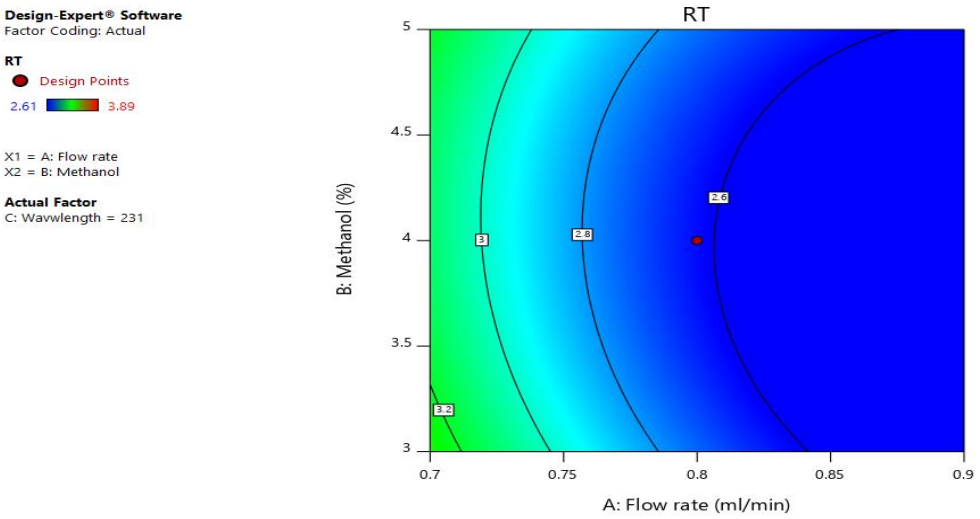

Figure 13a: 2D Contour plot depicting the effect of flow rate and % methanol on retention time of Metformin HCL

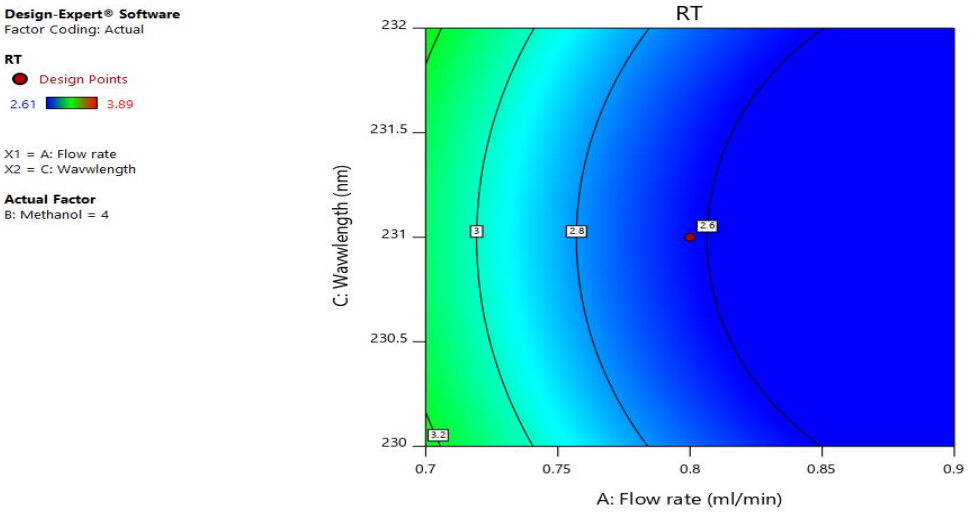

Figure 13b: 2D Contour plot depicting the effect of flow rate and wavelength on retention time of Metformin HCL

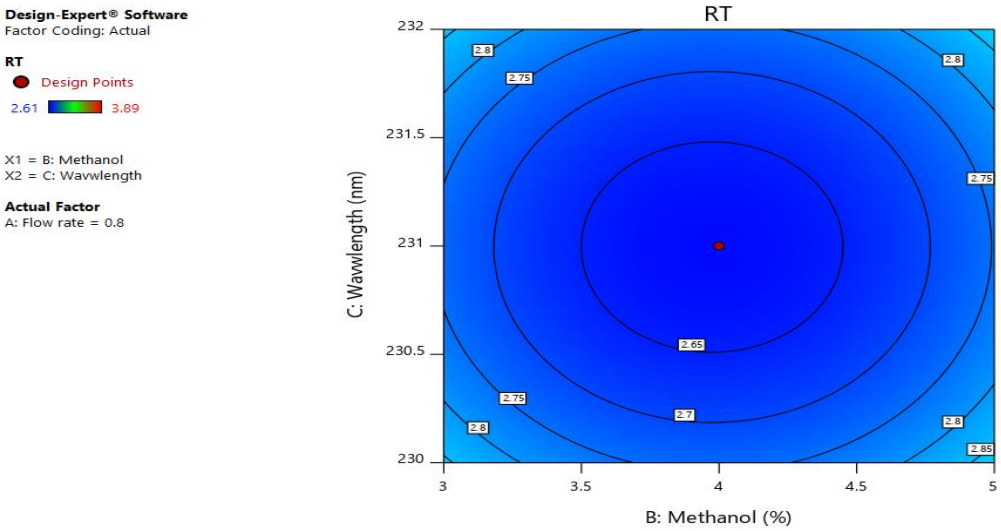

Figure 13c: 2D Contour plot depicting the effect of % methanol and wavelength on retention time of Metformin HCL

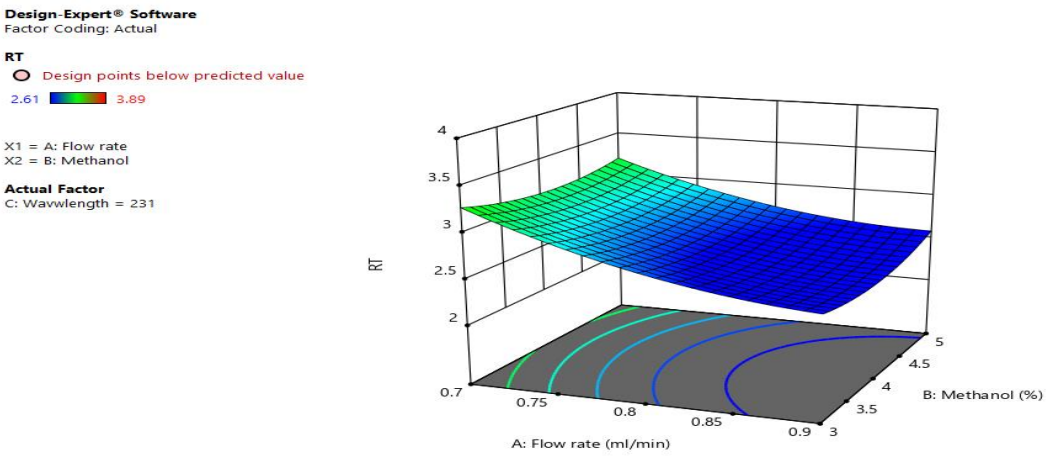

Figure 13d: 3D Contour plot depicting the effect of flow rate and methanol (%) on retention time of Metformin HCL

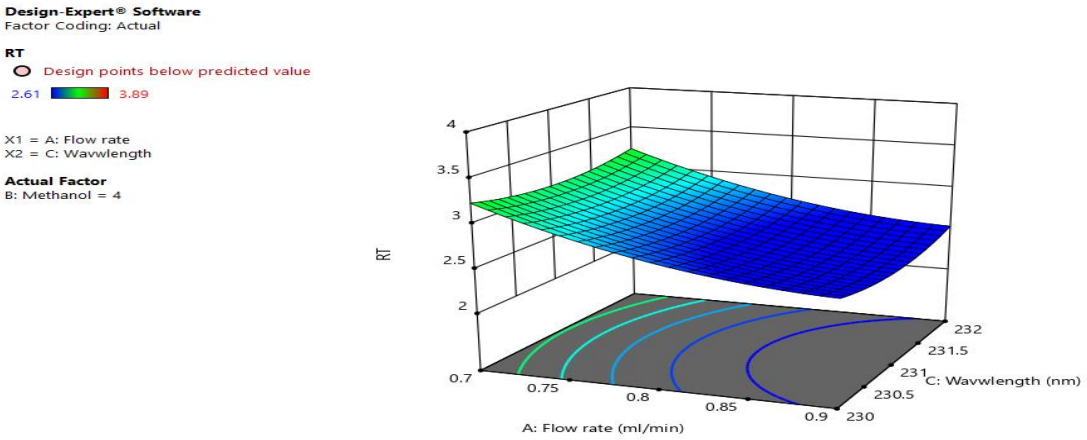

Figure 13e: 3D Contour plot depicting the effect of flow rate and wavelength on retention time of Metformin HCL

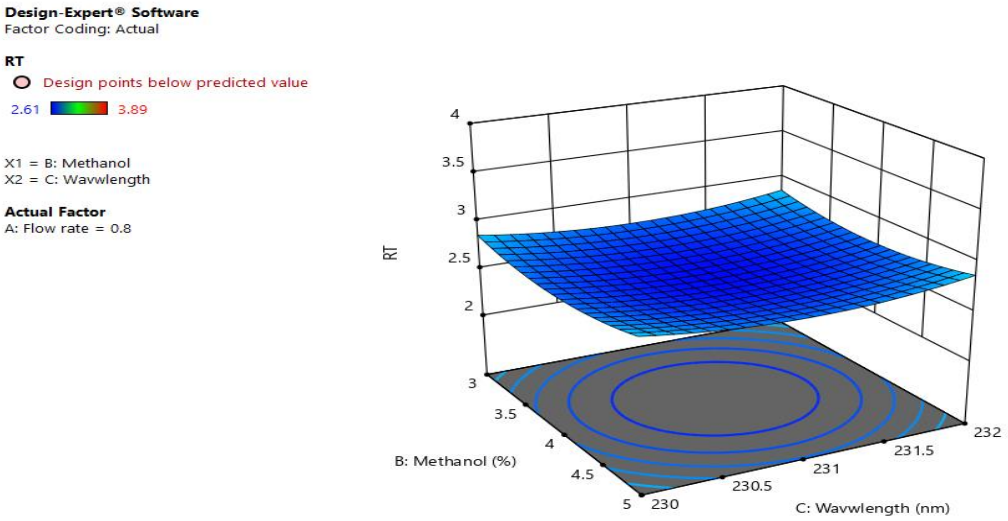

Figure 13f: 3D Contour plot depicting the effect of methanol (%) and wavelength on retention time of Metformin HCL

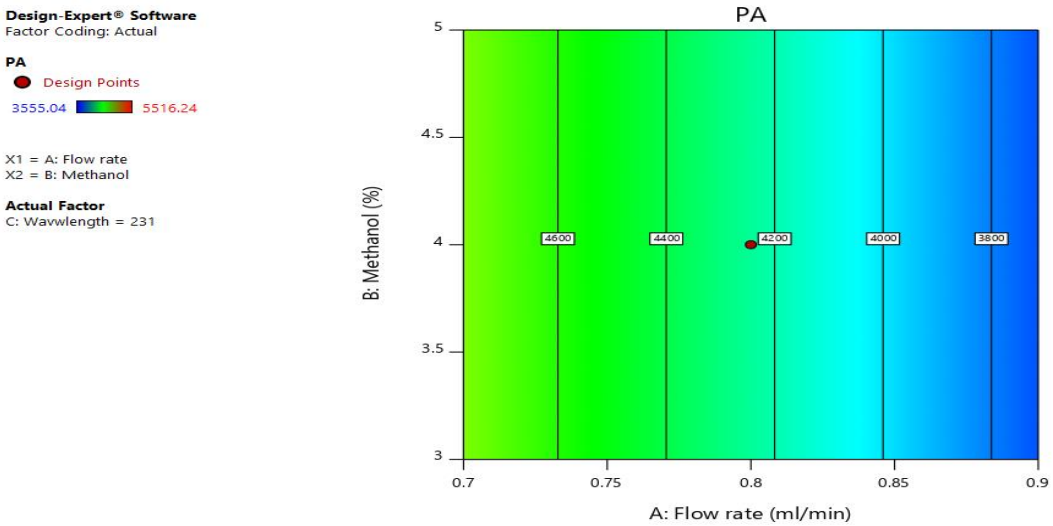

Figure 14a: 2D Contour plot depicting the effect of methanol (%) and flow rate on peak area of Metformin HCl

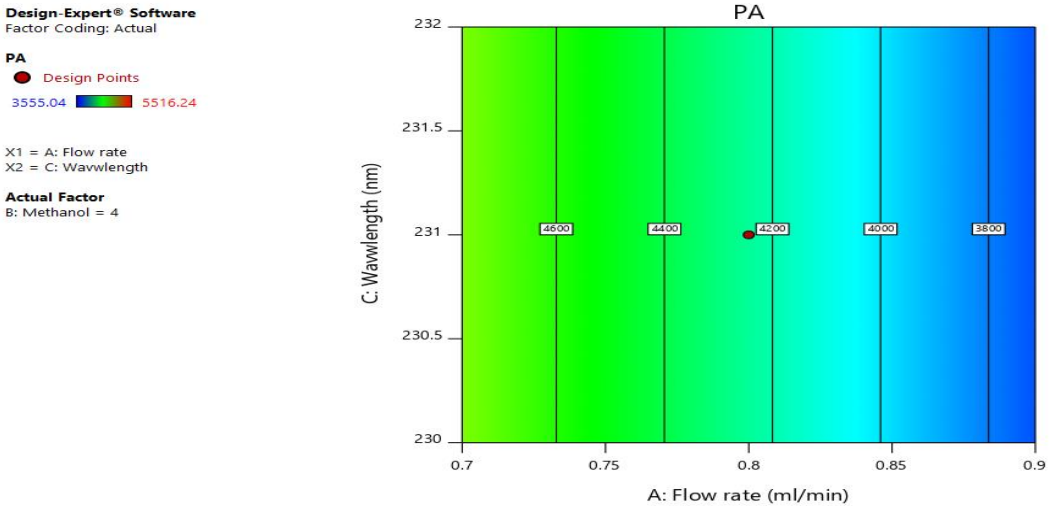

Figure 14b: 2D Contour plot depicting the effect of wavelength and flow rate on peak area of Metformin HCL

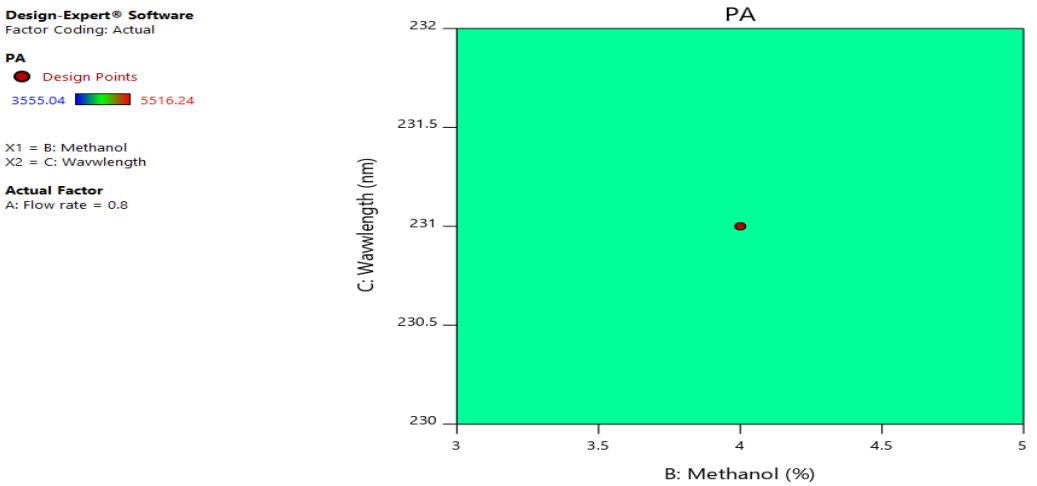

Figure 14c: 2D Contour plot depicting the effect of wavelength and methanol (%) on peak area of Metformin HCL

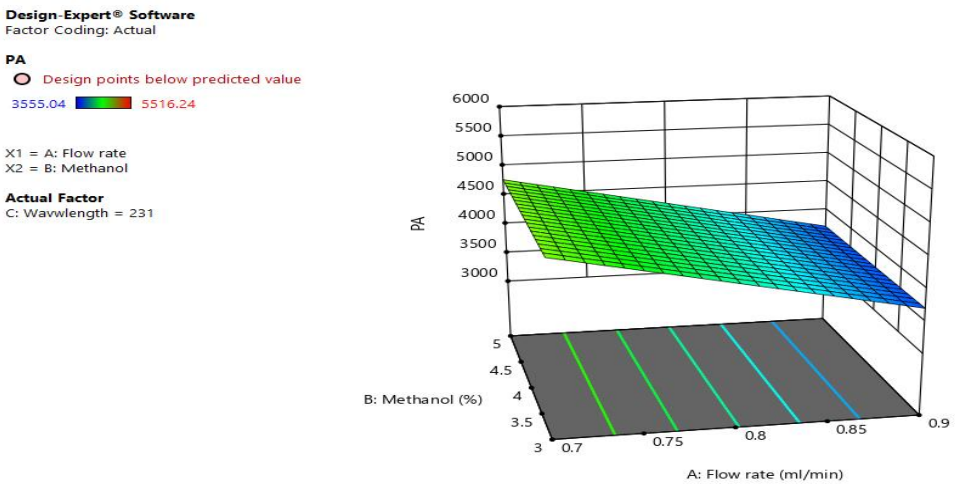

Figure 14d: 3D Contour plot depicting the effect of flow rate and methanol (%) on peak area of Metformin HCL

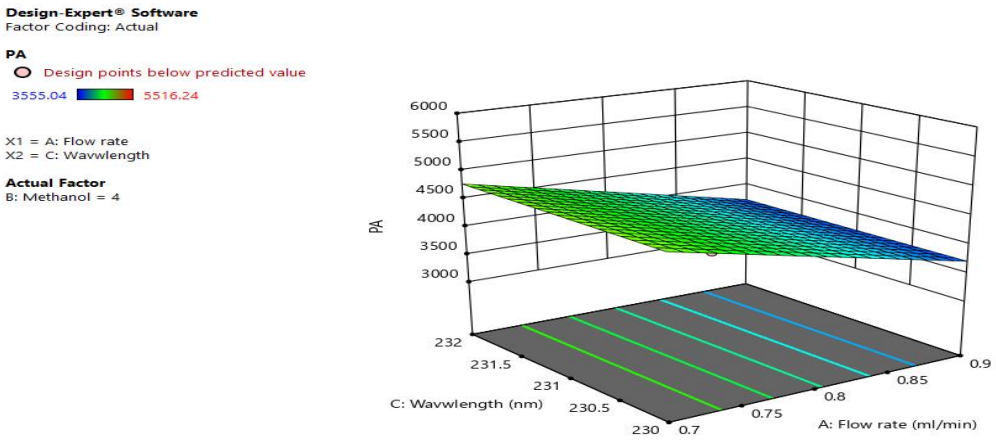

Figure 14e: 3D Contour plot depicting the effect of flow rate and wavelength on peak area of Metformin HCL

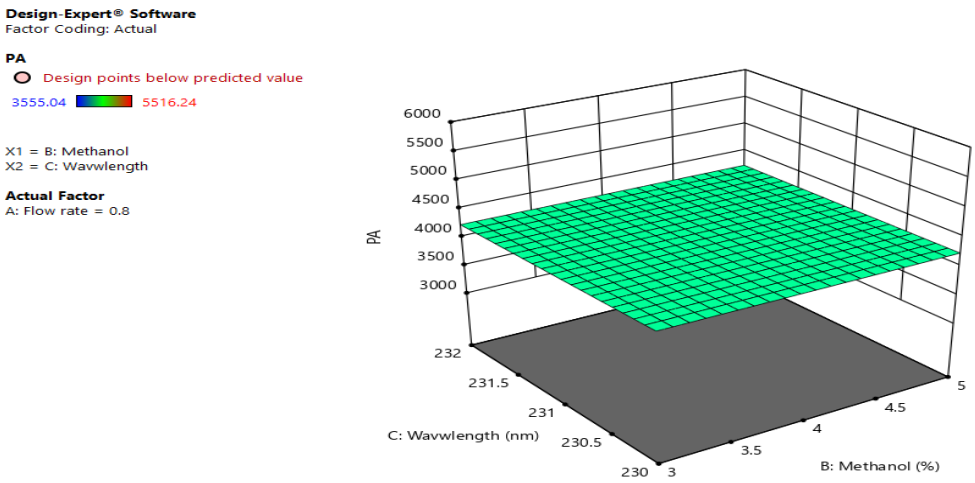

Figure 14f: 3D Contour plot depicting the effect of methanol (%) and wavelength on peak area of Metformin HCL

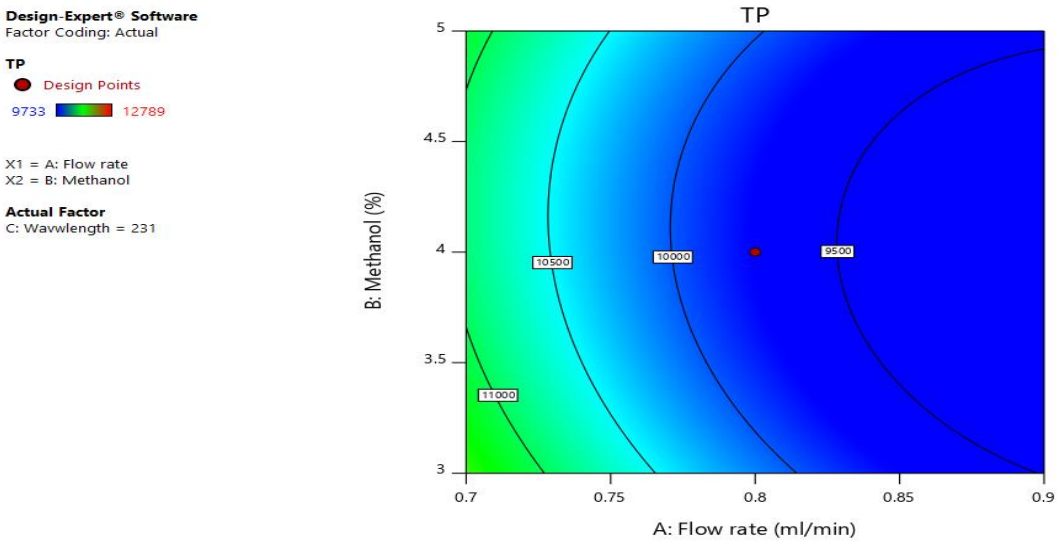

Figure 15a: 2D Contour plot depicting the effect of methanol (%) and flow rate on theoretical plates of Metformin HCl

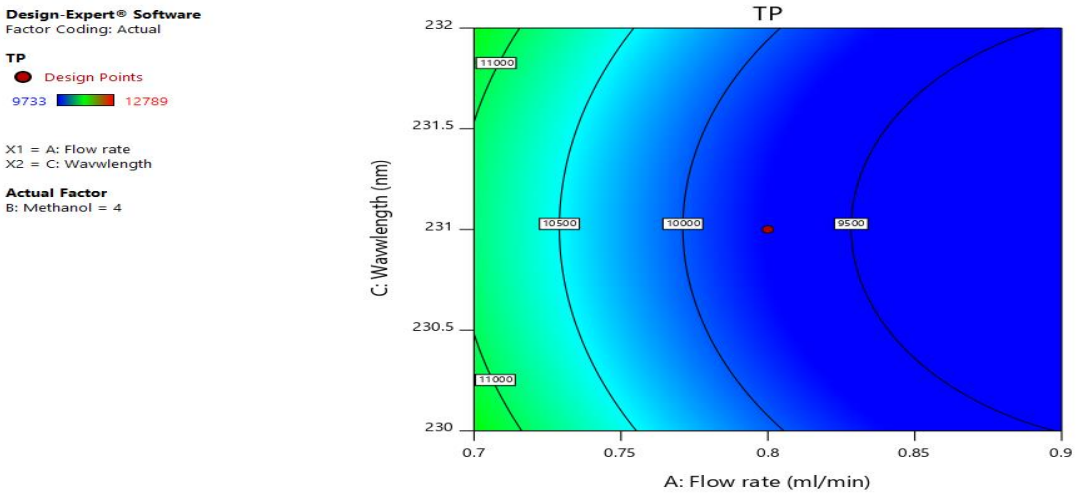

Figure 15b: 2D Contour plot depicting the effect of wavelength and flow rate on theoretical plates of Metformin HCl

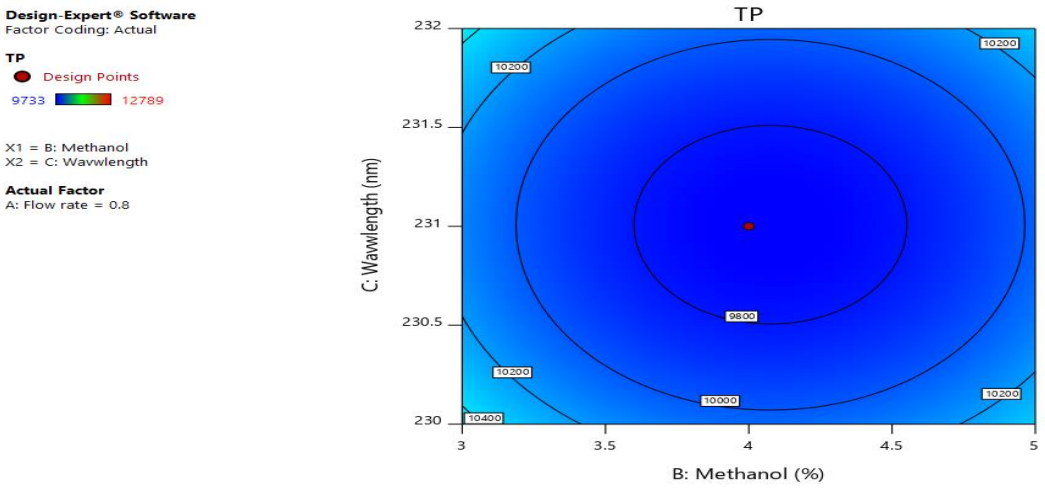

Figure 15c: 2D Contour plot depicting the effect of wavelength and % methanol on theoretical plates of Metformin HCl

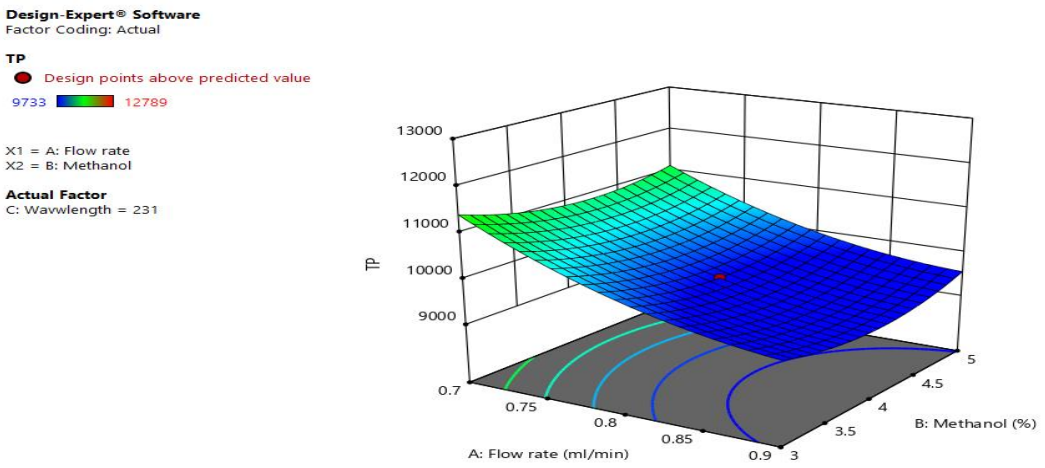

Figure 15d: 3D Contour plot depicting the effect of flow rate and % methanol on theoretical plates of Metformin HCl

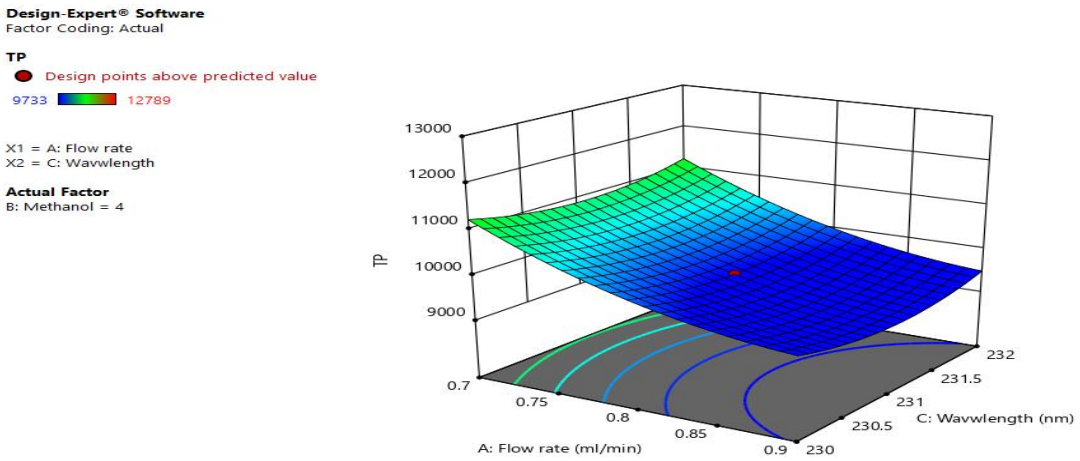

Figure 15e: 3D Contour plot depicting the effect of flow rate and wavelength on theoretical plates of Metformin HCl

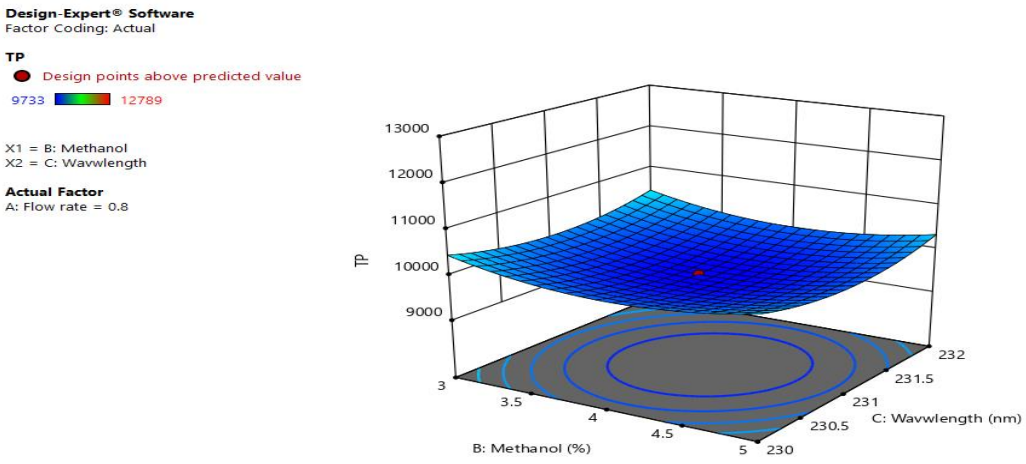

Figure 15f: 3D Contour plot depicting the effect of % methanol and wavelength on theoretical plates of Metformin HCl

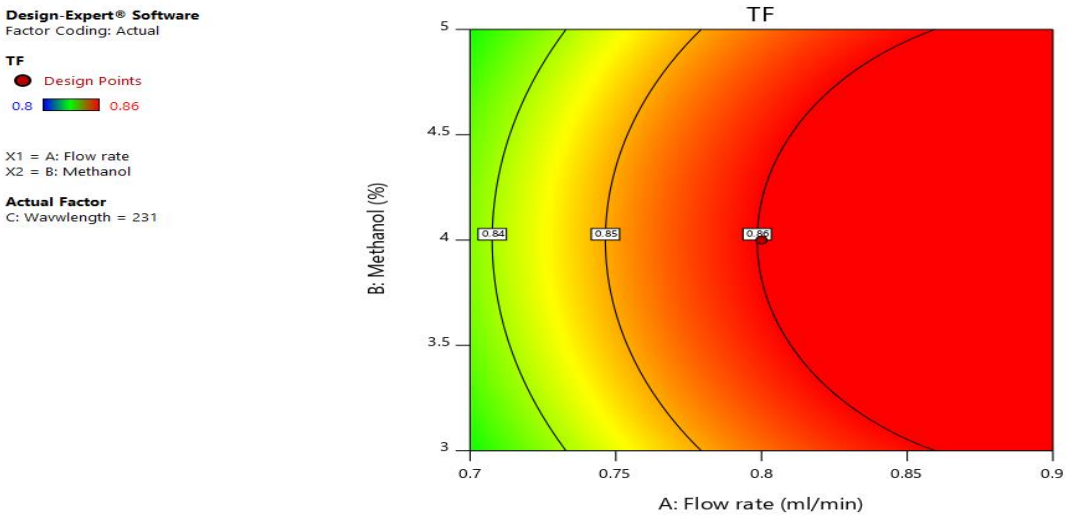

Figure 16a: 2D Contour plot depicting the effect of methanol (%) and flow rate on tailing factor of Metformin HCl

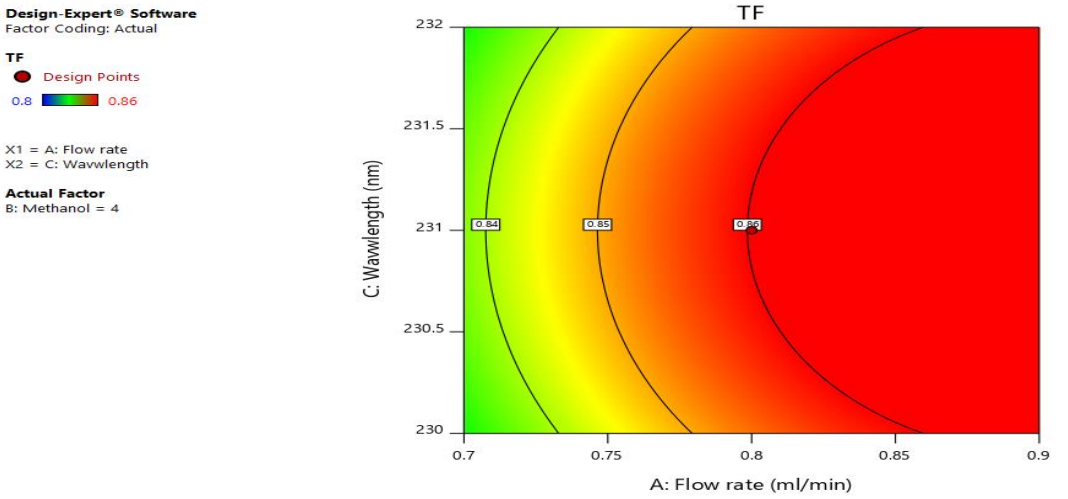

Figure 16b: 2D Contour plot depicting the effect of wavelength and flow rate on tailing factor of Metformin HCl

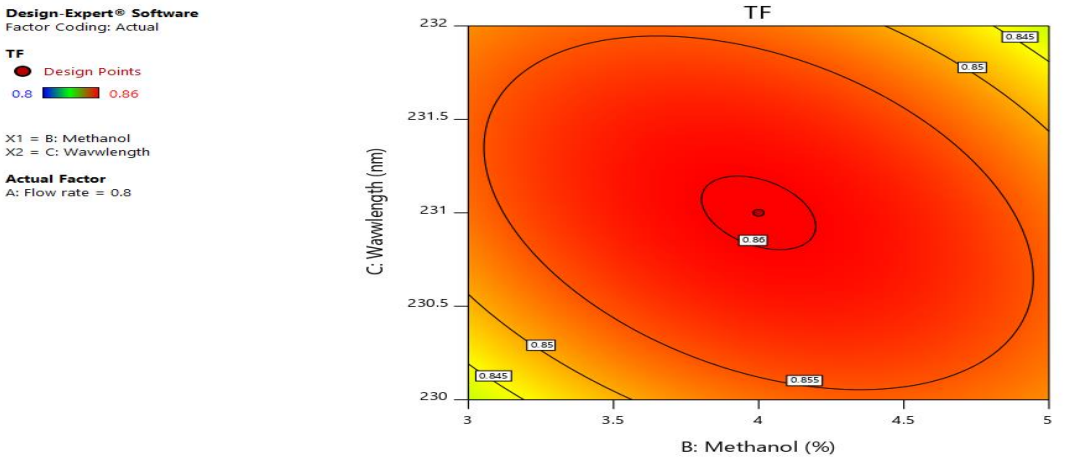

Figure 16c: 2D Contour plot depicting the effect of wavelength and methanol on tailing factor of Metformin HCl

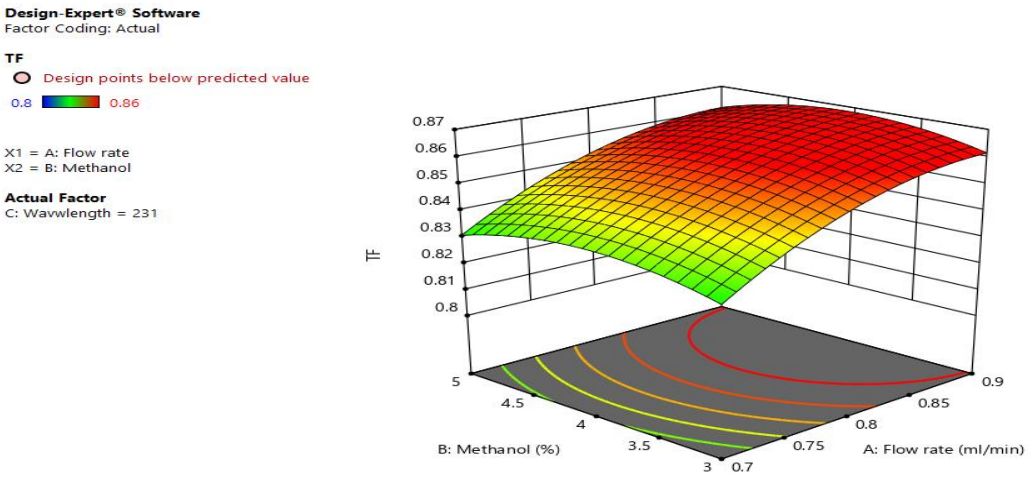

Figure 16d: 3D Contour plot depicting the effect of flow rate and methanol on tailing factor of Metformin HCl

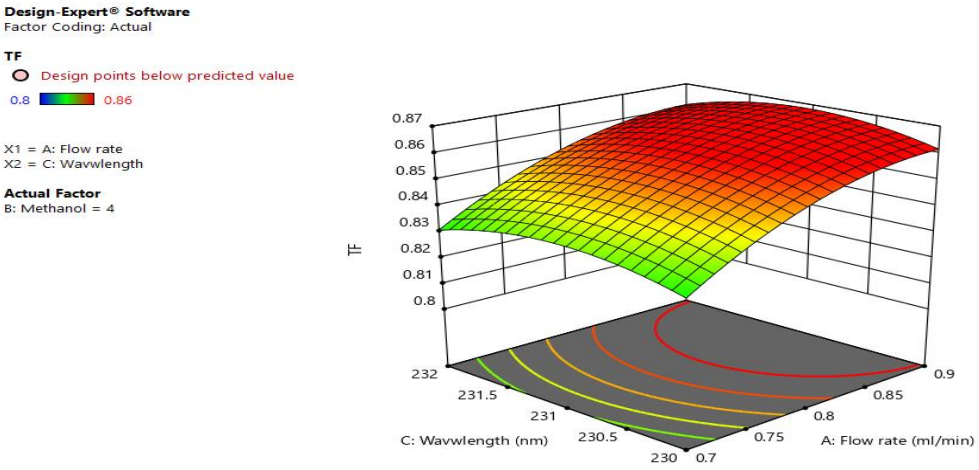

Figure 16e: 3D Contour plot depicting the effect of flow rate and wavelength on tailing factor of Metformin HCl

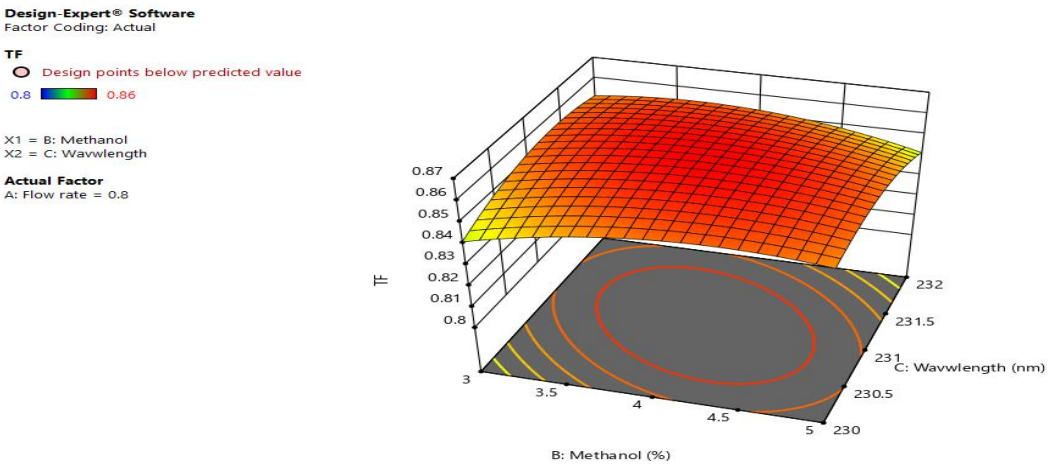

Figure 16f 3D Contour plot depicting the effect of methanol and wavelength on tailing factor of Metformin HCl

ANALYTICAL METHOD VALIDATION

System suitability: The system suitability data suggest that there is no noteworthy alteration in RT, PA, TF and TP of Metformin HCl across six replicates. During system testing, factors such as RT, PA, TP and TF were evaluated, and %RSD was computed before doing the analysis. The percentage RSD for all system suitability parameters was less than 2% (**Table 10, Figure 17**).

Table 10: System suitability data of Metformin HCl by RP-HPLC method

Parameter	RT	PA	TP	TF
Mean	2.93	576.29	10669.11	0.81
SD	0.03	3.98	39.77	0.01
%RSD	1.09	0.69	0.37	0.93

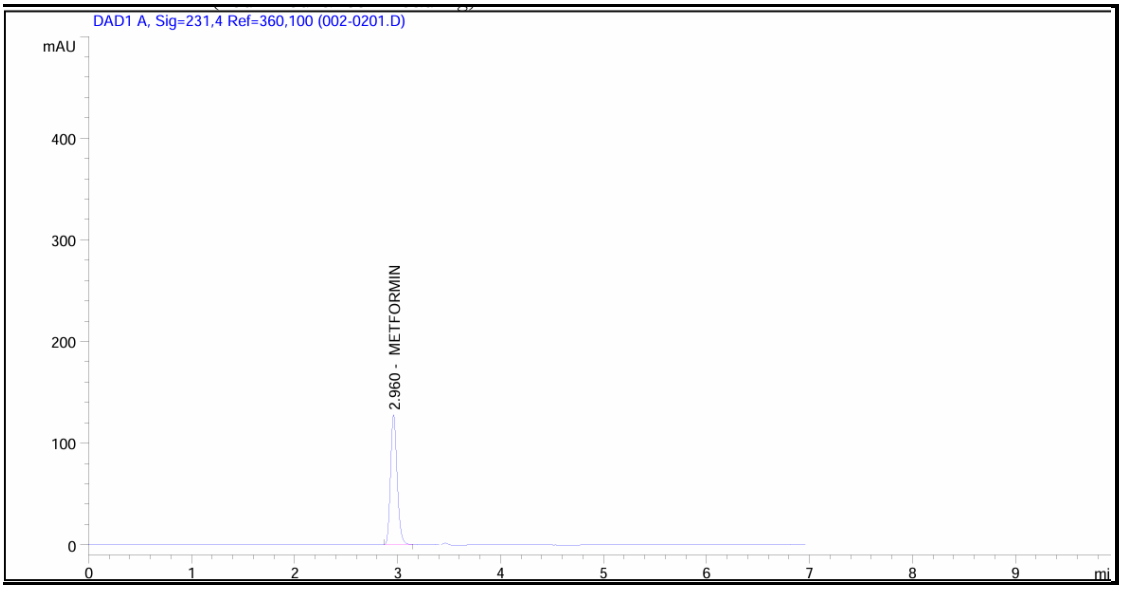

Figure 17: System suitability chromatogram of Metformin HCl

Specificity and selectivity: The HPLC chromatogram of the standard and sample indicated a retention duration of 2.9 minutes (**Figures 18 a-b**). The blank chromatogram revealed no peak at RT of Metformin HCl. No interference was seen during the RT of the Metformin HCl peak in the API and sample chromatograms, indicating that the technique is specific and selective for Metformin HCl.

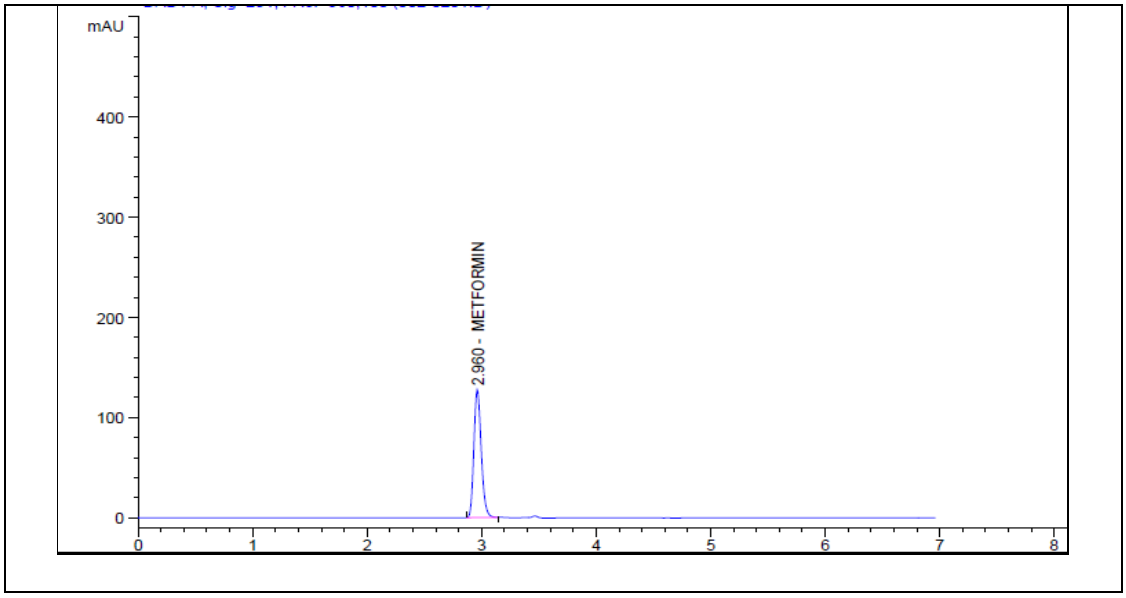

Figure 18a: Chromatogram of standard Metformin HCl

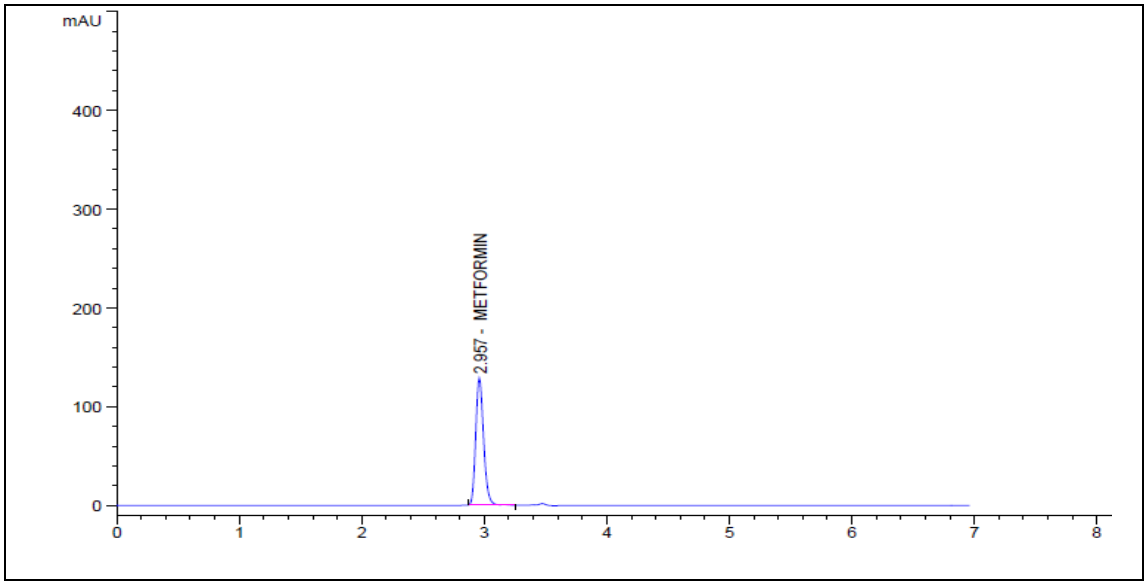

Figure 18b: Chromatogram of sample Metformin HCl

Linearity and range: The linear standardization curve for Metformin HCl in concentrations ranging from 5- 25 µg/ml demonstrated good linearity with a r^2 0.999 (**Table 11, Figure 19-20a-f**).

Table 11: Linearity and range data of Metformin HCl by RP-HPLC method

Sl. No.	Description	Results
1	Range	5 to 25 µg/ml
2	r^2	0.999
3	Calibration Equation	y = 58.03x + 1.639
4	Slope	58.03
5	Intercept	1.639
6	Detecting concentration	0.30 µg per ml
7	Quantification quantity	0.93 µg per ml

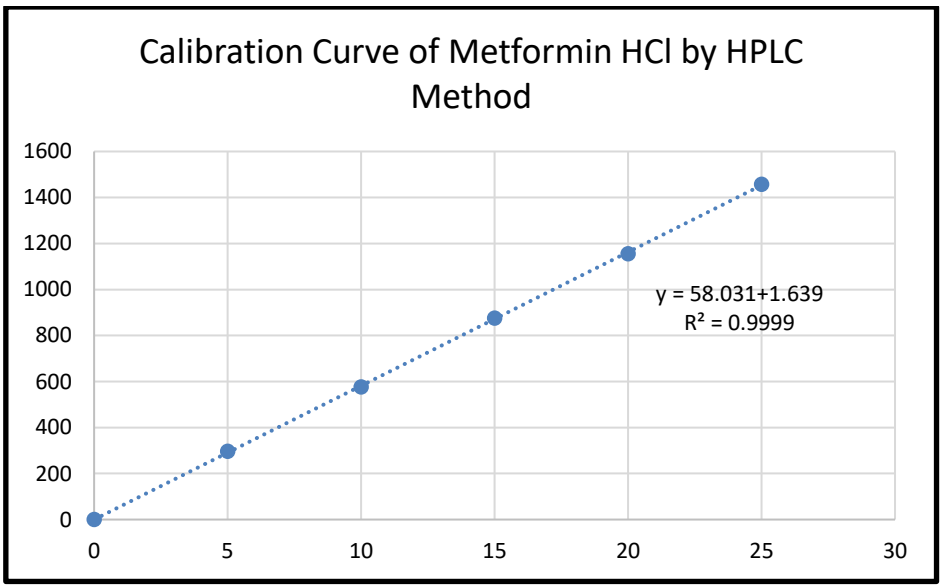

Figure 19: Calibration curve of Metformin HCl by RP-HPLC method

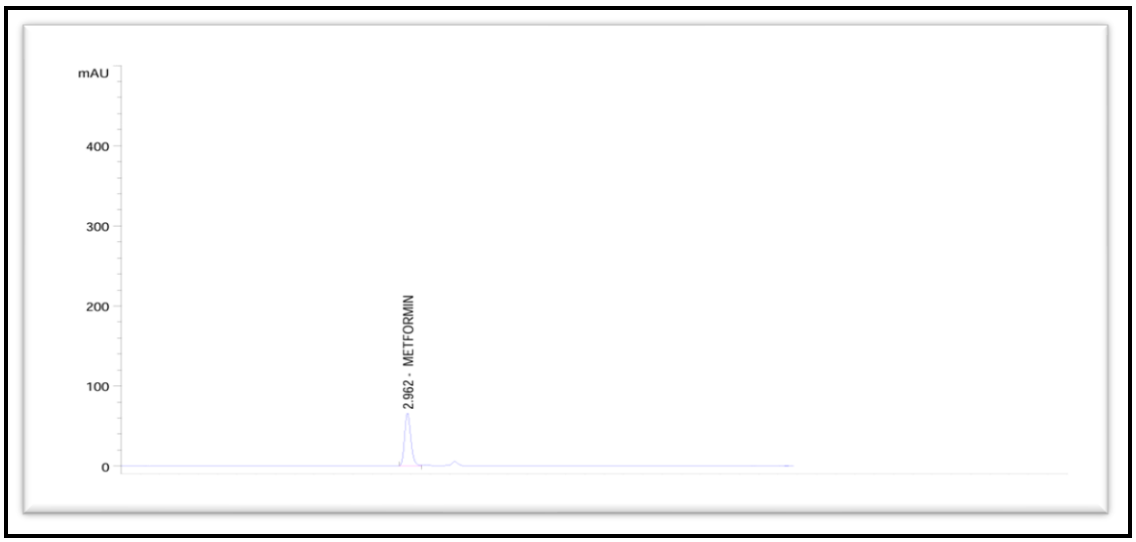

Figure 20a: Chromatogram of Metformin HCl (5 µg/ml)

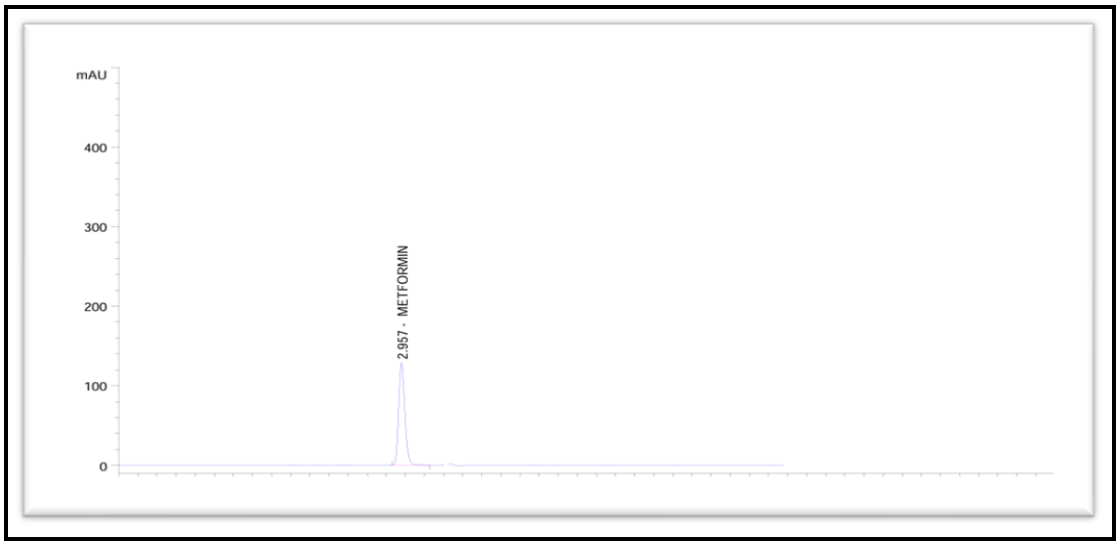

Figure 20b: Chromatogram of Metformin HCl (10 µg/ml)

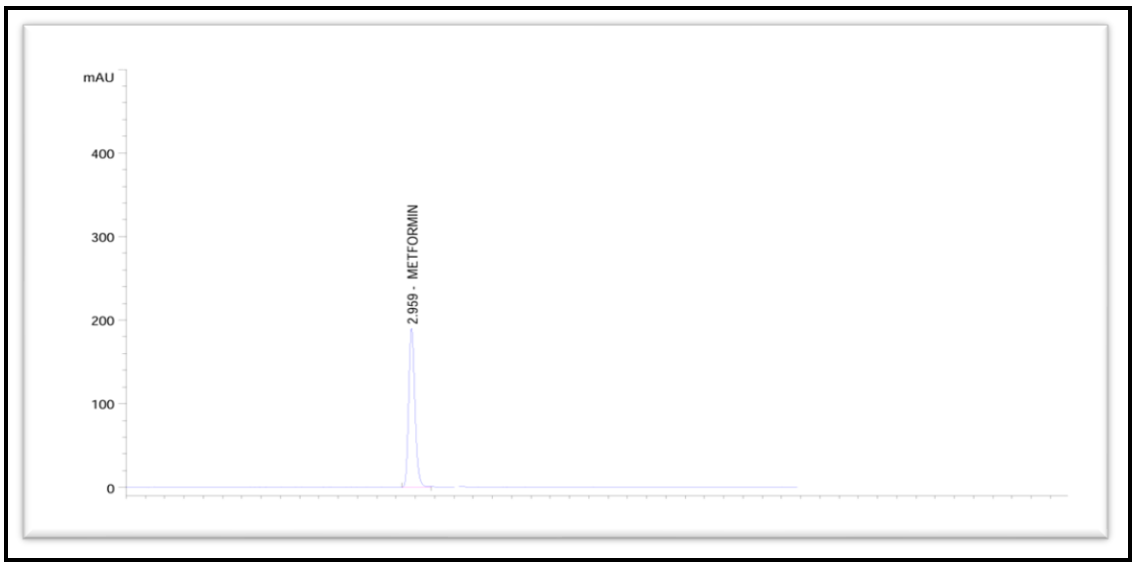

Figure 20c: Chromatogram of Metformin HCl (15 µg/ml)

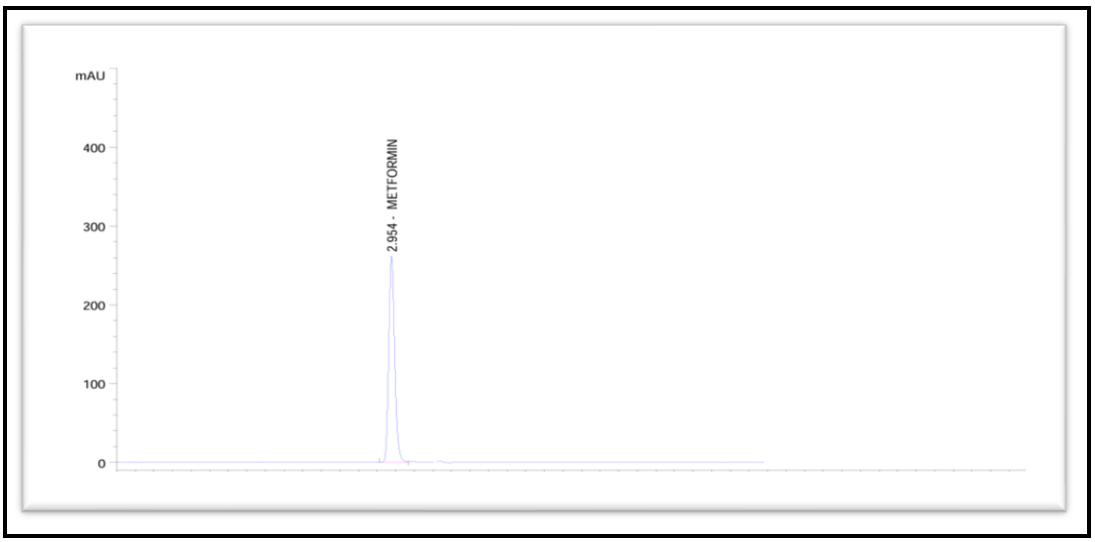

Figure 20d: Chromatogram of Metformin HCl (20 µg/ml)

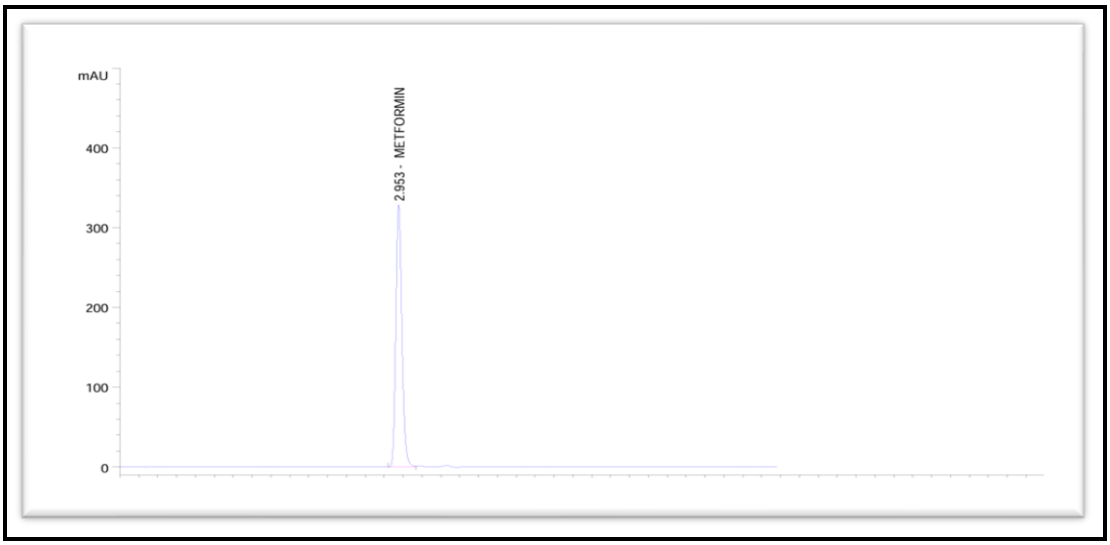

Figure 20e: Chromatogram of Metformin HCl (25 µg/ml)

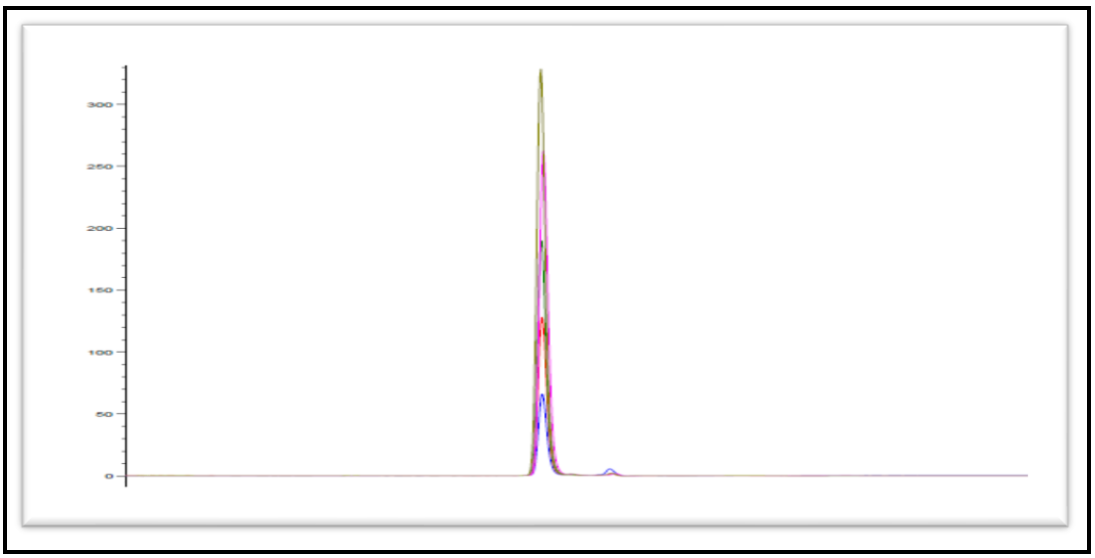

Figure 20f: Linearity overlay chromatogram of Metformin HCl

Detecting limit and quantifying limit: The values of detecting limit and quantifying limit for Metformin HCl were 0.30 µg per ml and 0.93 µg per ml, respectively. The acquired values demonstrate the excellent sensitivity of the HPLC approach.

Precision: The RSD for intra-inter-day-precision of Metformin HCl was determined to be below 2% following three measurements. These results supported the developed method's high level of precision (**Figure 21**). **Table 12** shows the precision data for the approach.

Ruggedness: Different analysts tested the procedure's robustness, and the results were judged to be acceptable (**Figure 22**). **Table 13** demonstrates the robustness of the proposed technique (%RSD <2%).

Robustness: After deliberately introducing slight variations in the developed method parameters, the obtained %RSD values were deemed acceptable. This observation indicates that the process exhibits robustness for manufacturing analysis. Specifically, the %RSD of PA was found to be within the acceptable limit (<2%).” (**Table 14, Figure 23a-f)**.

Accuracy: The accuracy data demonstrated favorable percentage recovery ranging from 98 percentage to 101 percentage. The percentage RSD, consistently below 2 %, signifies a high level of recovery for the established approach, as illustrated in **Table 15** and **Figure 24a-c**.

Table 12: Precision analysis of Metformin HCl by RP-HPLC technique

Precision Parameter	Concentration µg/ml	Mean PA	Standard Deviation	%RSD
Intraday (Morning)	10 µg/ml	579.45	0.68	0.12
	15 µg/ml	873.15	3.24	0.37
	20 µg/ml	1162.57	1.76	0.15
Intraday-2 (Afternoon)	10 µg/ml	582.81	6.23	1.07
	15 µg/ml	877.15	10.23	1.17
	20 µg/ml	1171.50	16.54	1.41
Intraday-3 (Evening)	10 µg/ml	579.45	0.68	0.12
	15 µg/ml	873.15	3.24	0.37
	20 µg/ml	1162.57	1.76	0.15
Interday-1	10 µg/ml	584.55	1.69	0.29
	15 µg/ml	877.94	2.72	0.31
	20 µg/ml	1175.03	3.36	0.29
Interday-2	10 µg/ml	576.84	3.76	0.65
	15 µg/ml	875.72	0.99	0.11
	20 µg/ml	1175.96	9.35	0.80
Interday-3	10 µg/ml	580.66	4.11	0.71
	15 µg/ml	876.18	1.16	0.13
	20 µg/ml	1159.22	2.79	0.24

Table 13: Ruggedness data of Metformin HCl by RP-HPLC method

Ruggedness Type	Concentration	Mean PA	SD	%RSD
Change in Analyst	5 µg/ml	296.67	1.16	0.39
	15 µg/ml	875.74	1.03	0.12
	25 µg/ml	1456.98	3.10	0.21

Table 14: Robustness data of Metformin HCl by RP-HPLC method

Parameters	Range	Mean PA	SD	%RSD
Change in Organic Phase (Methanol: aqueous phase)	3:97 v/v	1434.6	0.21	0.01
	5:95 v/v	1500.40	0.11	0.01
Change in FR	0.6 ml	1637.23	3.17	0.19
	0.8 ml	1286.40	0.09	0.01
Change in wavelength	230	1484.9	1.62	0.11
	232	1405.30	3.34	0.24

Table 15: Recovery data of Metformin HCl by RP-HPLC method

Levels	Quantity of sample (µg/ml)	Quantity of standard (µg/ml)	Total quantity (µg/ml)	Mean % Recovery (µg/ml) ± SD	SD
80%	5	4	9	98.98	0.69
100%	5	5	10	99.79	0.48
120%	5	6	11	99.20	0.70

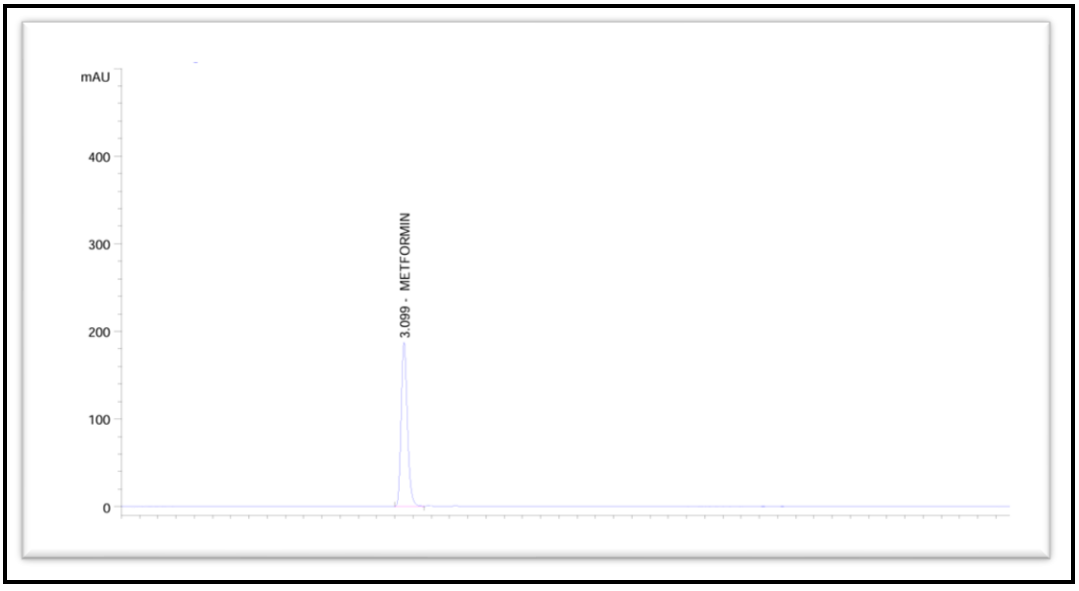

Figure 21: Precision chromatogram of Metformin HCl (15 µg/ml)

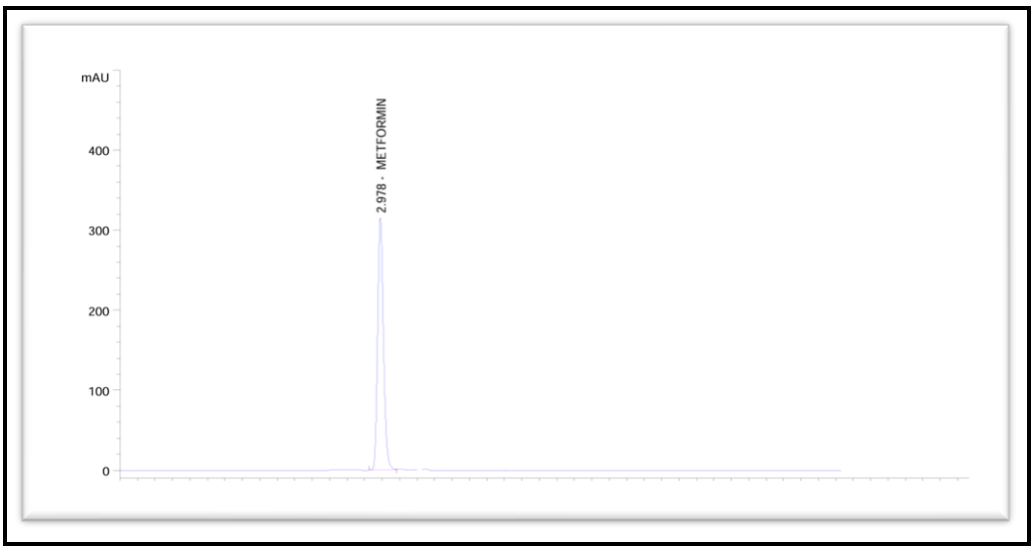

Figure 22: Ruggedness chromatogram of Metformin HCl (15 µg/ml)

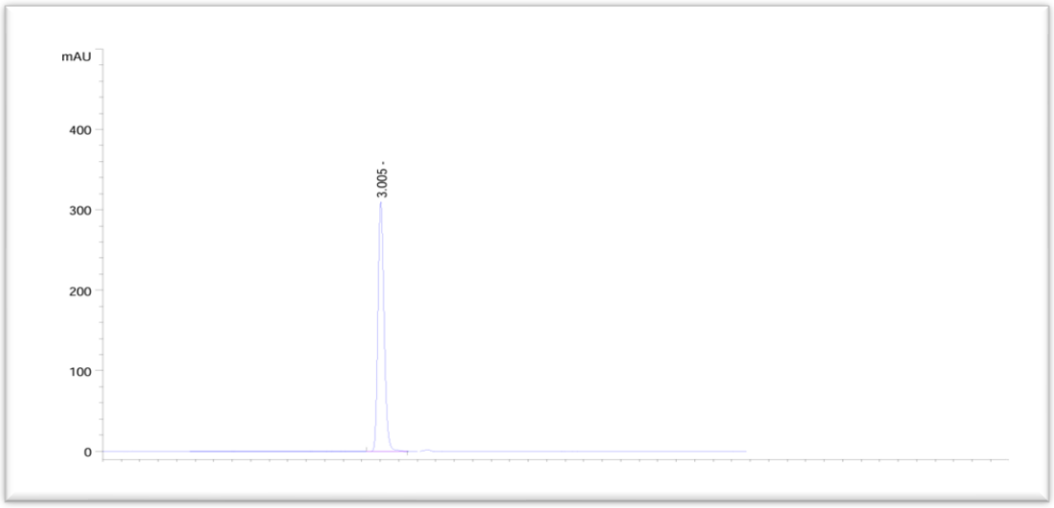

Figure 23a: Chromatogram of Metformin HCl by change in mobile phase concentration Methanol: aqueous phase 3:97 v/v

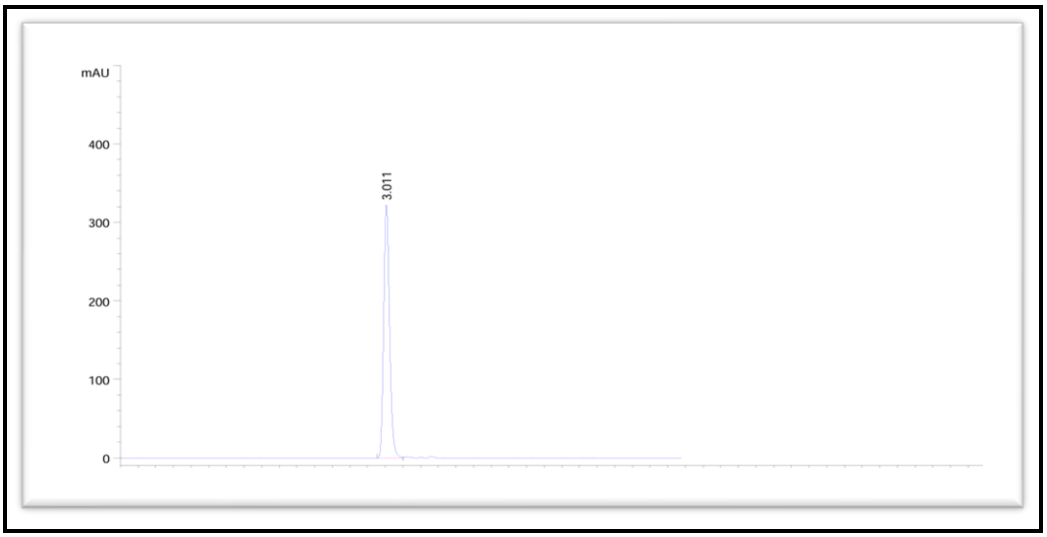

Figure 23b: Chromatogram of Metformin HCl by change in mobile phase concentration Methanol: aqueous phase 5:95 v/v

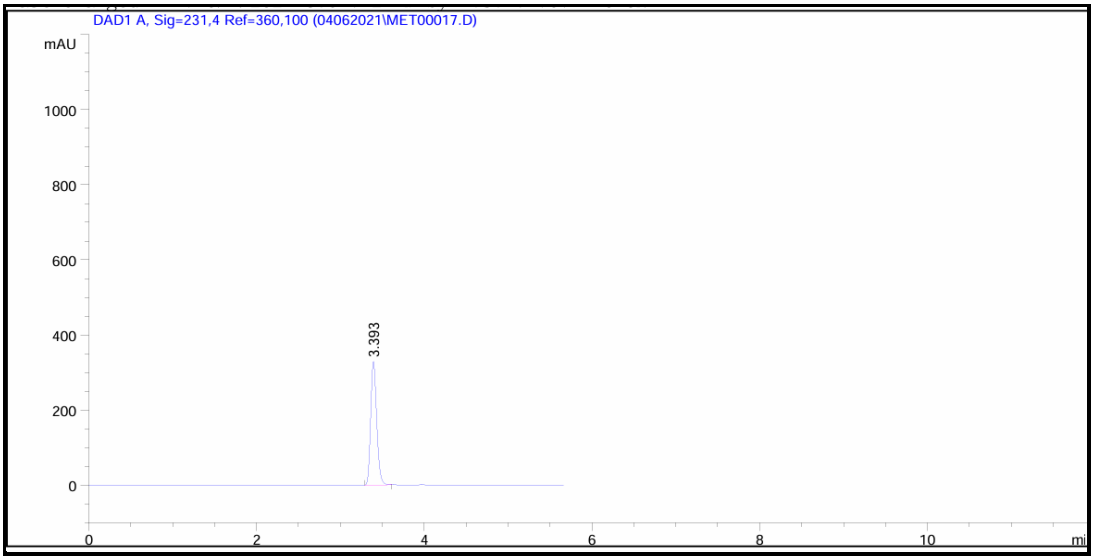

Figure 23c: Chromatogram of Metformin HCl by change in FR rate (0.6 ml)

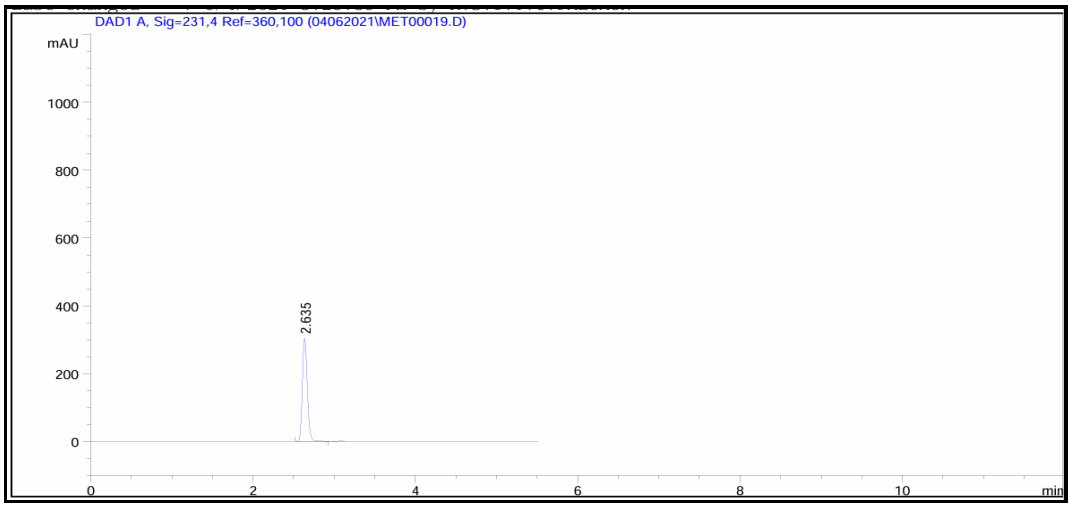

Figure 23-D: Chromatogram of Metformin HCl by change in FR (0.8 ml)

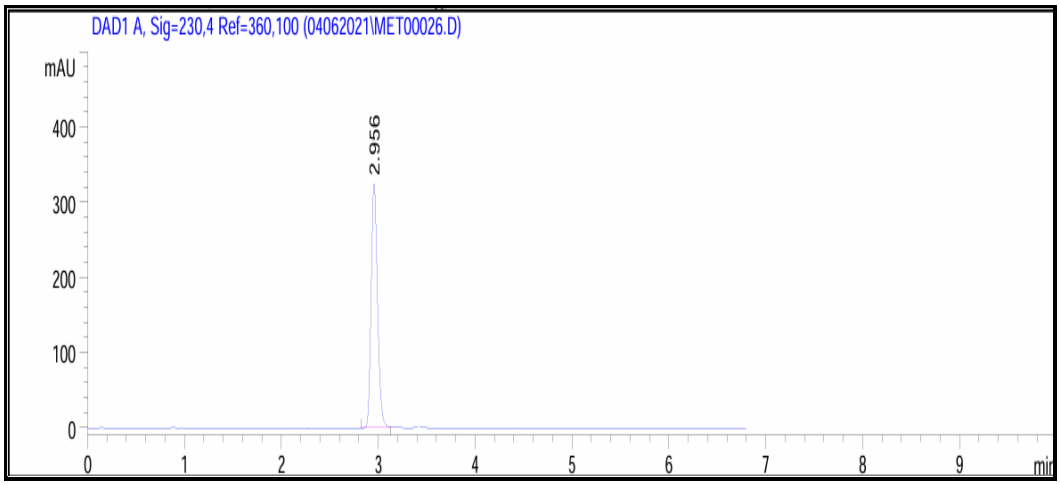

Figure 23e: Chromatogram of Metformin HCl by change in detection wavelength (230nm)

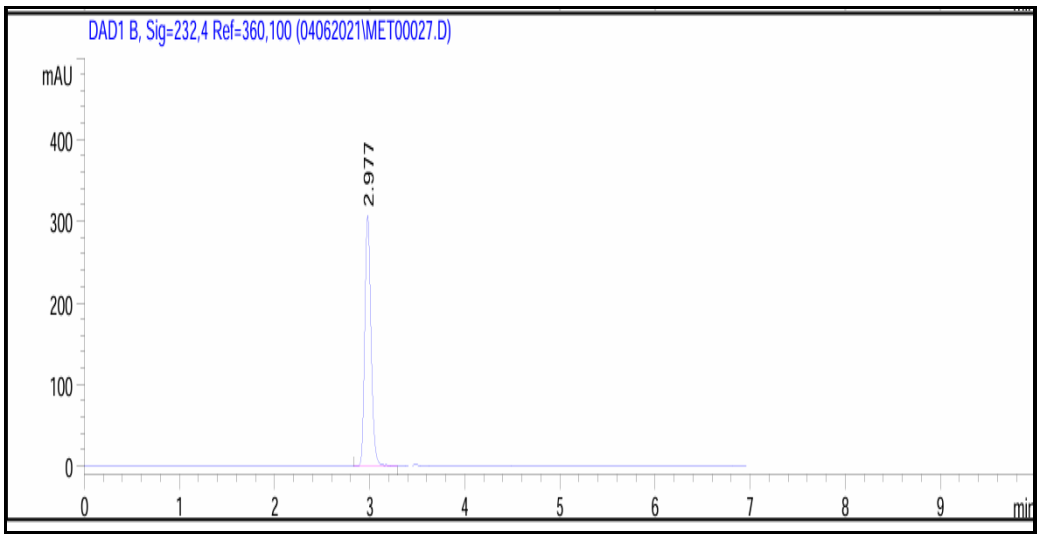

Figure 23f: Chromatogram of Metformin HCl by change in detection wavelength (232nm)

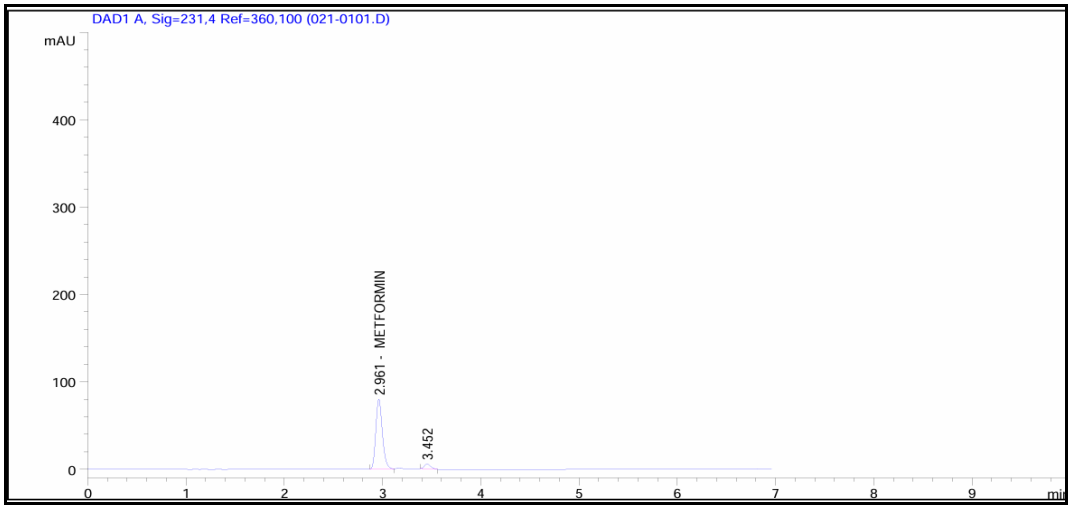

Figure 24a: Chromatogram of Metformin HCl (Accuracy 80%)

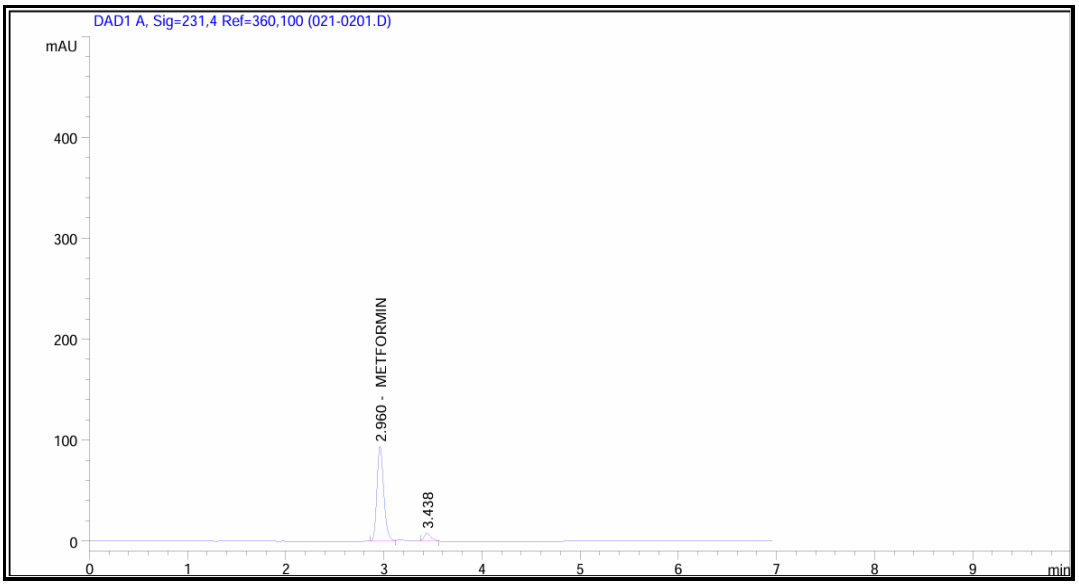

Figure 24b: Chromatogram of Metformin HCl (Accuracy 100%)

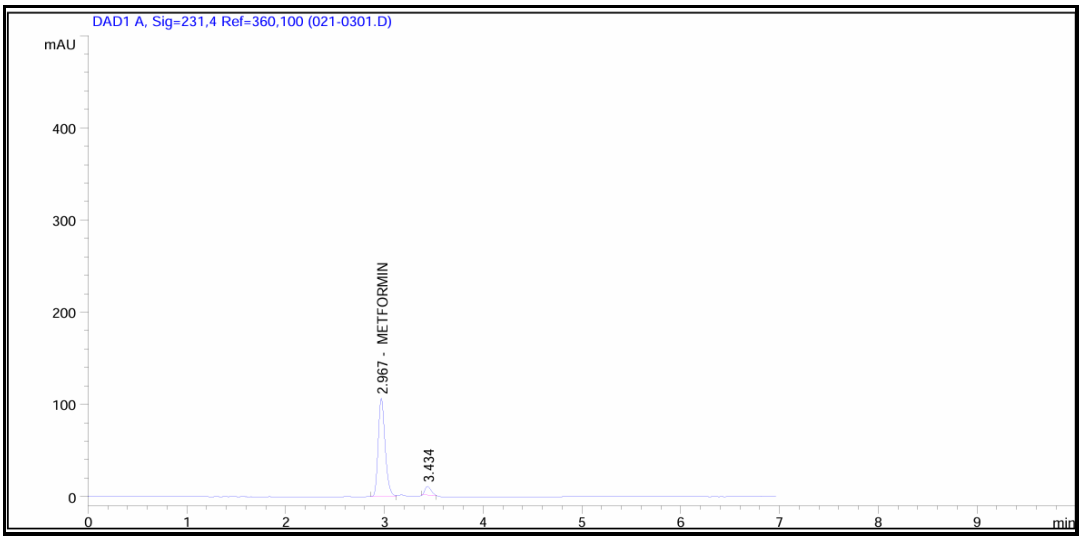

Figure 24c: Chromatogram of Metformin HCl (Accuracy 120%)

ESTIMATION OF METFORMIN HCL BY HPLC METHOD

The investigation using BBD determined RP-HPLC for promoted Metformin HCl revealed commendable recovery, with a determined percent recovery of 99.89% as outlined in **Table 16**. The retention duration of Metformin HCL in the dosage remained consistent when compared to the Metformin HCL API. The TF and TP values were observed to be within acceptable levels. Notably, no additional peaks were observed in the chromatograms for any of the sample solutions, indicating the absence of added Metformin HCL in the dosage form. This confirms the suitability of the planned RP-HPLC process for routine QC of Metformin HCL in API and tablet formulation." The assay chromatogram is represented in the **Figure 25**.

Table 16: Assay data of Metformin HCl by RP-HPLC method

Sl. No.	Marketed Formulation	Label Claim (mg)	% Assay	SD	%RSD
Brand 1	Tablet	500 mg	99.89%	0.21	0.32
Barnd 2	Tablet	500 mg	98.03%	0.49	0.50

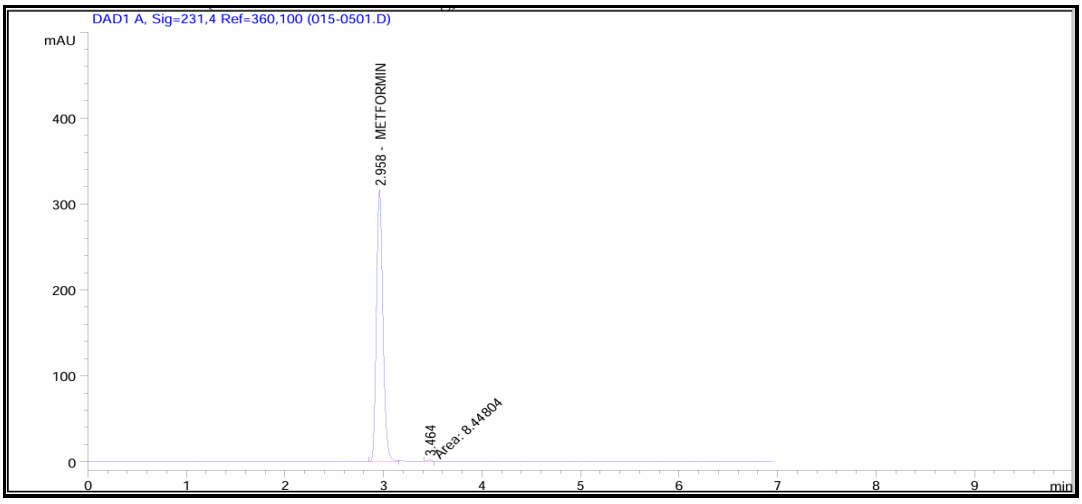

Figure 25: Assay chromatogram of Metformin HCl

DISCUSSION

This study presents a straightforward, precise, consistent, and effective application of QbD tools in developing a chromatographical technique for the assessment of Metformin HCl, aiming for enhanced robustness. The BBD was employed for 3 factors at 3 levels using the DoE. Each factor underwent analysis through experimental data, including Analysis of Variance, indicative graphs, and classical graphs. The impact of each component on the response outcome was thoroughly examined in the findings section. The established RP-HPLC technology underwent validation following ICH Q2 R1 guidelines, covering system suitability, specificity, selectivity, linearity, accuracy, precision, repeatability, and sensitivity studies, The absence of any undesired peaks and the consistent drug RT indicated the method's suitability for measuring Metformin HCl in pharmaceutical preparations, affirming that the methodology meets stringent requirements for high standards and selectivity.

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CONCLUSION

The present study effectively employed the AQbD approach to enhance the performance of HPLC procedure for the effective assessment of Metformin HCl. “This resulted in a more profound understanding of the critical factor-response relationship and an overall improvement in process performance. The BBD-optimized RP-HPLC method confirmed the robustness of the investigative process even before the validation inquiry. This innovative methodology aids researchers and analysts in designing control methods to mitigate the adverse effects of critical method variables on method performance. Validation reports provided assurance of the proposed method's specificity, outstanding linearity, sensitivity, recovery, preciseness, and repeatability. The newly designed HPLC technique, based on the QbD approach, was positively functional to the analysis of marketed tablets, yielding satisfactory results. Consequently, this method proves to be appropriate for routine QC of Metformin HCl in the pharmaceutical industry.

METHOD 2: DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR ESTIMATION OF DAPAGLIFLOZIN

MATERIALS AND METHODS

Chromatographic Conditions

In the suggested investigation, an Agilent 1100 (Autosampler) HPLC system with Chemstation software was used. Dapagliflozin was separated chromatographically using an Agilent C18 stationary phase (250 x 4.6 mm internal diameter and 5 µm)." The solvent system was a solvent system consisting of Methanol and 0.05% OPA in water in proportions of 74:26 v/v. Dapagliflozin was eluted at 0.95 millilitre/min and identified by UV at 224 nm. The temperature was kept at ambient, and the injection volume was 20 µL. [151]

Preparation of standard stock solution

The Dapagliflozin stock was made by dissolving 10 mg of Dapagliflozin in 10 ml of mobile phase. The resulting stock was sonicated to solubilize the Dapagliflozin, and then 1 ml of this solution was transferred into 10 ml of VF, and it was diluted 10 ml with solvent system as diluent. This stock was utilized for optimization and validation of experimental parameters. The further working solutions of Dapagliflozin were obtained by serial dilutions. The series of concentrations were obtained by using solvent system as diluent. The final solutions were filtered using a 0.45µm syringe filter. The final dilutions were then transferred to vials before chromatographic runs. [119]

METHOD DEVELOPMENT

Chromatographic trials

Preliminary experimental runs were conducted to explore potential combinations and to determine the most suitable column and solvent system. Initial trials were initiated based on insights gained from a literature review. Methanol was chosen as a trial study to establish cost-effective approach. Aqueous solvents with varying concentrations of OPA were used for efficient separation at various pH levels. Based on the results of the preceding experiments, the C18 stationary phase was chosen for future investigation. [151]

Identification of the detecting wavelength

The standard dapagliflozin was scanned between 200-400 nm to determine its λ max for analysis. The UV spectra of the standard drug exhibited a maximum absorption at 224nm. As a result, it served as the detection wavelength for HPLC analysis. [119]

QUALITY BY DESIGN APPROACH

The steps involved in quality by design approach have been followed in similar way as mentioned in the **Method 1**.

Optimization study by BBD

Chromatographic optimization of parameters was carried out using a BBD with three factors and two levels to assess the main, interaction, and quadratic effects of critical factors on the detected responses (**Table 17**). The BBD comprised 17 experimental runs, and the screening phase involved two steps. Initially, CMPs were chosen based

on FR, % organic phase, and detection wavelength. The second step focused on the selection of CQAs, which in this study included RT, PA, TP and TF These responses were evaluated during practical testing, utilizing Design Expert tools to construct a design matrix of 17 experimental runs. The Box-Behnken screening strategy was then applied to explore numerous interaction effects and quadratic effects (**Table 18**). [151]

Development of experiments as per BBD

The factor screening approach efficiently refined the selection of CMPs influencing procedure performance, employing three factors at two equidistant levels. Throughout all chromatographic runs, Dapagliflozin at a concentration of 5 µg/ml was consistently utilized to assess CAAs (RT, PA, TP, and TF). [41]

Data analysis and model validation

The responses generated after performing the proposed trial were entered into software, and various graphs such as 3D response surface plots and graph plots were produced. These graphs depict the impact of CMPs on the specified CQAs. The examination of these plots was used to determine which parameters produced satisfactory answers. Based on these findings, the procedure's final CMPs were discovered, and the optimal conditions were chosen. Statistical tools, such as ANOVA for each response, were employed to investigate the importance of each parameter utilized in the study using the p value. [151]

Table 17: BBD matrix for HPLC analysis of Dapagliflozin

Independent Variables	Units	Low (-1)	High (+1)
Organic Phase (A)	%	73	75
FR (B)	ml/min	0.9	1
Wavelength (C)	nm	223	225

Table 18: Optimization parameters by BBD for HPLC analysis of Dapagliflozin

Run	A Methanol	B: FR	C: Wavelength
	%	ml/min	nm
1	73	0.95	223
2	73	0.95	225
3	73	0.9	224
4	73	1	224
5	74	0.9	225
6	74	0.95	224
7	74	0.95	224
8	74	0.95	224
9	74	0.95	224
10	74	1	225
11	74	0.95	224
12	74	1	223
13	74	0.9	223
14	75	0.95	223
15	75	0.95	225
16	75	0.9	224
17	75	1	224

ANALYTICAL METHOD VALIDATION [150, 151]

System suitability: Six replicate analysis of standard Dapagliflozin (10 µg/ml) were used to assess system fit, including estimating SD and % RSD of PA, TF, RT and TP.

Specificity and selectivity: The procedure's specificity and selectivity were evaluated using HPLC chromatography of Dapagliflozin (15 µg/ml) solution, standard, sample, and blank solvent chromatograms. It aids in the identification of the analyte and reduces interference from other peaks.

Linearity and range: The linearity of the developed method was established by conducting a serial dilution of the Dapagliflozin stock solution within the range of 5 to 25 µg/ml. The resulting calibration curve of PA versus the concentration of Dapagliflozin was graphed to evaluate linearity.

Sensitivity (Limit of Detection-LOD and Limit of Quantification-LOQ): The sensitivity of proposed method was studied in terms of LOD and LOQ. The detecting and quantification limits values were calculated using the following formulas:

LOD = 3 x σ/S and

LOQ =10 x σ/S

Where, σ is the SD of the y-intercept and S is the slope of the calibration curve.

Precision: The precision of the developed approach was evaluated by analysing three diverse concentrations of Dapagliflozin at various time intervals on the same day and consecutive days. The percentage Relative Standard Deviation for PA in each chromatogram was calculated to assess precision.

Ruggedness: The suggested approach was tested for ruggedness by evaluating a reference solution of Dapagliflozin (15 µg/ml) by another analyst. The overall mean and percentage RSD for PA were computed from the chromatograms of each peak.

Robustness: The procedure's robustness was evaluated by altering the solvent composition from Methanol to aqueous phase (73:27 to 75:25 v/v), changing the wavelength from 223 to 225, and adjusting the FR from 0.9 to 1.0 ml/min. All robustness parameter experiments employed a working solution of 20 µg/ml.

Accuracy: Recovery was assessed through the addition method. A sample solution of Dapagliflozin (10 µg/ml) was spiked with 80%, 100%, and 120% of the standard concentration. Subsequently, the recovery and %RSD were determined to ensure the accuracy of the data.

ESTIMATION OF DAPAGLIFLOZIN IN MARKETED TABLETS

To demonstrate the applicability of the proposed HPLC approach, marketed tablets containing Dapagliflozin were analyzed and a percentage assay was calculated (n=3). The assay was done in triplicate, and the analyte recovery from the formulation was reported. [151]

RESULTS

Preliminary experiments using a literature search and trial and error were conducted to optimize a accurate approach for assessing and QC of Dapagliflozin in bulk and tablets. Initially, development began by selecting a mobile phase from a variety of solvent combinations, including Methanol and o-phosphoric acid. To improve Dapagliflozin separation, several concentrations of the buffer phase are tested. Initial runs revealed that using OPA at a concentration of 0.05% results in successful analyte separation with Dapagliflozin elution at reduced RT and low peak tailing. The optimized chromatogram was showed in **Figure 26.** The Optimized method parameters are presented in **Table 19**.

Table 19: Developed HPLC method specifications for Dapagliflozin

Sr. No.	Description	Specifications
1	Technique	Chromatographic separation
2	Device	HPLC
3	Model	Agilent
4	Brand	Agilent 1100 (Autosampler)
5	Software	Chemstation possessing
6	Column system	Agilent C_{18} column
7	Solvent system	Methanol: 0.05% OPA in water, pH 2.8 74:26 v/v
8	FR	0.95 ml
9	Identification	224 nm
10	Injection volume	20 µL.
11	Analyte	Dapagliflozin
12	RT	4.8 min

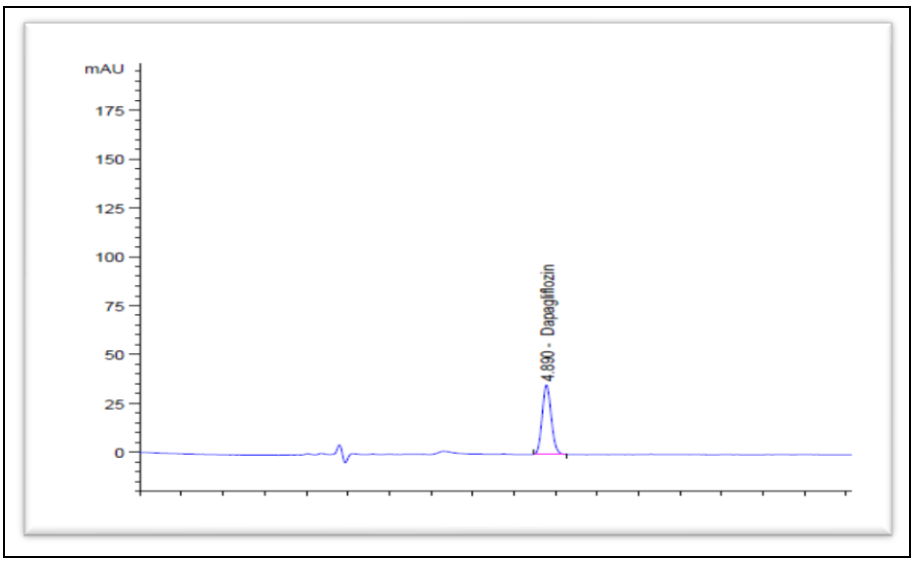


Figure 26: Optimized HPLC Chromatogram of standard Dapagliflozin

QUALITY BY DESIGN APPROACH

The steps involved in QbD-based method optimization were carried out in a systematic manner. The results are presented as follows.

Development of analytical target profile

ATP defines the method requirements that are intended to be measured. The analytical target profile is defined using the information and scientific reasoning of the analytical process. **Table 20** shows ATP for the suggested HPLC technique for Dapagliflozin.

Identification of CAAs

In the case of chromatographic development, important quality parameters often involve RT, TP, PA, and TF, as well as peak spectral purity. RT, TP, PA, and TF all have significant impacts on the performance of technique.

Identification of CMPs

A risk assessment study was conducted to scrutinize CMPs, specifically high-risk factors with a significant impact on CAAs. In this investigation, an IFB diagram (**Figure 8**) was constructed to pinpoint crucial risk variables that could potentially affect the method's performance. The identified high-risk procedural variables were then subjected to a suitable experimental optimization approach for further examination.

Table 20: Analytical target profile for HPLC analysis of Dapagliflozin

Sl. No.	ATP Parameters	Target
1	Target Analyte	Dapagliflozin
2	Target Sample	Dapagliflozin Tablets
3	Method category	Chromatographic Method
4	Instrument requirement	HPLC
5	Nature of analyte	Solid (Solution)
6	Standard stock solution	Dilution of main drug in linear manner
7	Application of Method	Estimation of Dapagliflozin
8	Validation parameters	Specificity, system suitability, linearity, range, precision, accuracy, ruggedness, robustness, selectivity and sensitivity.

DoE optimization

It constitutes a crucial phase within the DoE process, and we selected high-risk factors for the RP-HPLC method, namely the mobile phase FR, the percentage of the organic phase in the mobile phase, and the detection wavelength. These factors underwent optimization using the BBD to elucidate the primary, interaction, and quadratic effects on RT, PA, TF and TP. This study aimed to discern the effects of mobile phase ratio, FR, and wavelength on RT, PA, TP, and TF through the completion of 17 runs, with subsequent statistical analysis of the data using software.

Chromatographic runs were executed under the conditions outlined in **Table 18**, and **Table 21** illustrates the effects of various independent variables on response values. ANOVA and statistical optimization were performed using software (**Tables 22**). Additionally, 3-D graphs and 2-D graphs were utilized to delineate the design space and showcase how independent parameters influence the response variables. These visual representations were found to align with the ANOVA parameters.

Figures 27a-d present 3-D surface graphs illustrating the effects of FR, organic phase composition, and wavelength on RT, PA, TP, and TF. The BBD was employed for optimizing various design characteristics, with the quadratic model utilized to scrutinize data for both main and interaction effects.

Table 21: BBD optimization along with responses by HPLC analysis of Dapagliflozin

Run	RT	PA	TP	TF
1	5.621	574.774	10352	0.9
2	5.619	575.134	10799	0.89
3	5.976	599.769	8415	0.89
4	5.732	585.165	9989	0.85
5	5.155	570.948	12828	0.9
6	4.812	839.565	10340	0.88
7	4.862	822.565	10440	0.88
8	4.818	824.565	10540	0.86
9	4.818	821.565	10640	0.85
10	4.646	829.565	10240	0.89
11	4.861	839.565	10440	0.88
12	4.894	514.78	9860	0.88
13	5.151	574.322	10634	0.91
14	4.185	625.937	10561	0.86
15	4.115	568.71	10848	0.88
16	4.384	572.348	10888	0.89
17	3.935	516.414	10012	0.88

Table 22: ANOVA responses of BBD design for HPLC analysis of Dapagliflozin

Source	Sum of squares	df	Mean square	F-value	p-value	
For Retention Time						
Model	5.39	9	0.5991	130.75	< 0.0001	significant
A-% Organic modifier	5.01	1	5.01	1092.79	< 0.0001	
B-Flow rate	0.2661	1	0.2661	58.07	0.0001	
C-Wavelength	0.0125	1	0.0125	2.72	0.1428	
For Peak Area						
Model	2.418E+05	9	26862.67	6.52	0.0109	significant
A-% Organic modifier	330.68	1	330.68	0.0802	0.7852	
B-Flow rate	2065.20	1	2065.20	0.5009	0.5020	
C-Wavelength	8099.11	1	8099.11	1.96	0.2038	
For Theoretical Plates						
Model	3.179E+07	6	5.298E+06	142.32	< 0.0001	significant
A-% Organic modifier	3.102E+07	1	3.102E+07	833.37	< 0.0001	
B-Flow rate	55112.00	1	55112.00	1.48	0.2516	
C-Wavelength	11858.00	1	11858.00	0.3185	0.5849	
For Tailing Factor						
Model	0.0500	3	0.0167	32.17	< 0.0001	significant
A-% Organic modifier	0.0496	1	0.0496	95.82	< 0.0001	
B-Flow rate	0.0003	1	0.0003	0.6036	0.4511	
C-Wavelength	0.0001	1	0.0001	0.0966	0.7609	

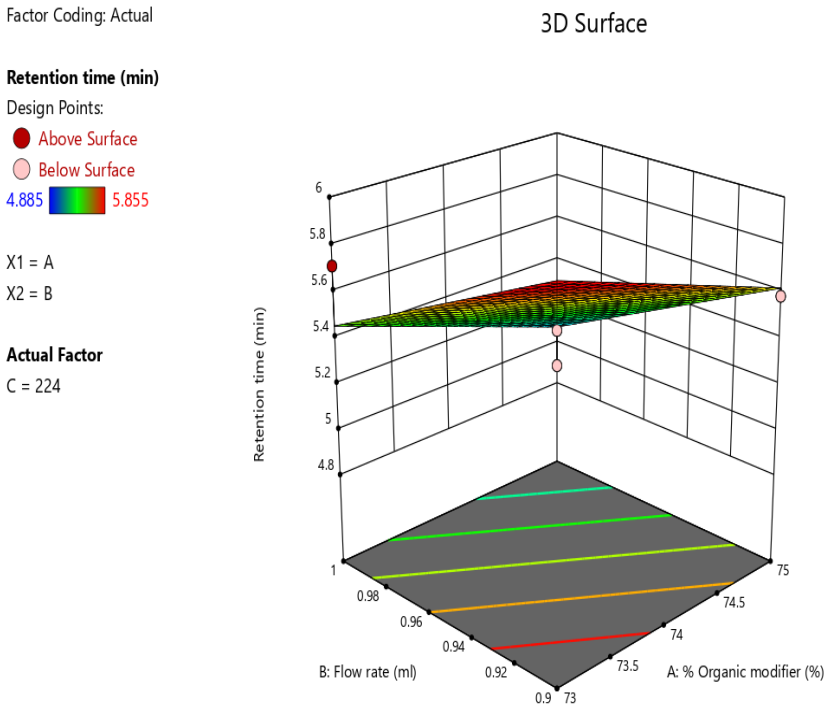


Figure 27a: 3D Contour plot depicting the effect of flow rate and methanol (%) on retention time of Dapagliflozin

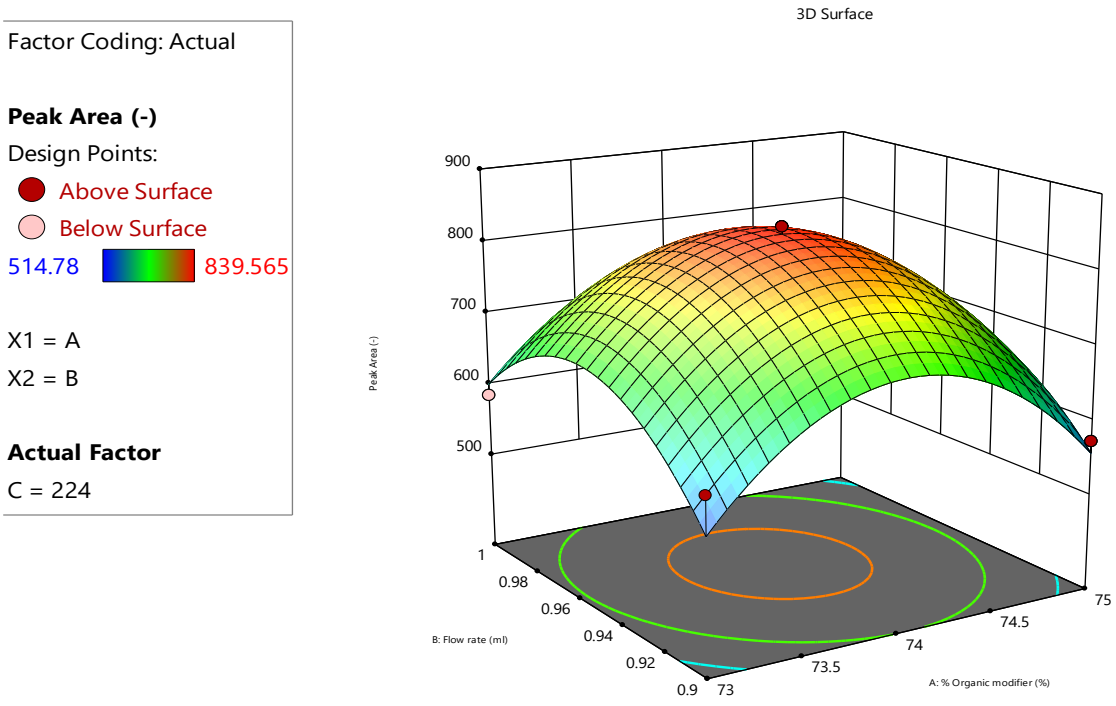


Figure 27b: 3D Contour plot depicting the effect of flow rate and methanol (%) on peak area of Dapagliflozin

Factor Coding: Actual

Theoretical Plates (-)

Design Points:

Above Surface

Below Surface

10352 14888

X1 = A

X2 = B

Actual Factor

C = 224

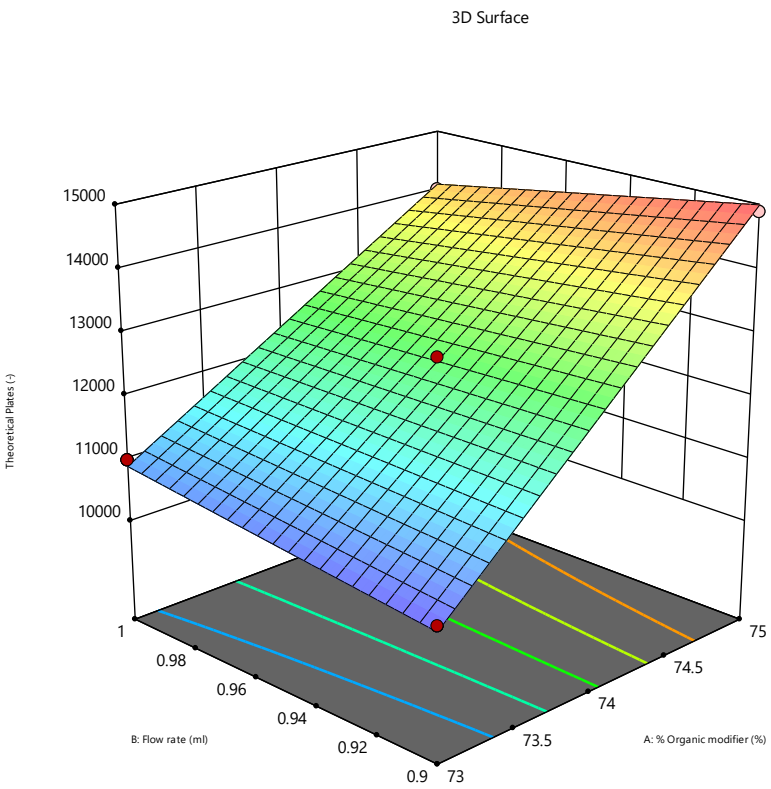


Figure 27c: 3D Contour plot depicting the effect of flow rate and % methanol on theoretical plates of Dapagliflozin

Factor Coding: Actual

Tailing Factor (-)

Design Points:

Above Surface

Below Surface

0.71 0.91

X1 = A

X2 = B

Actual Factor

C = 224

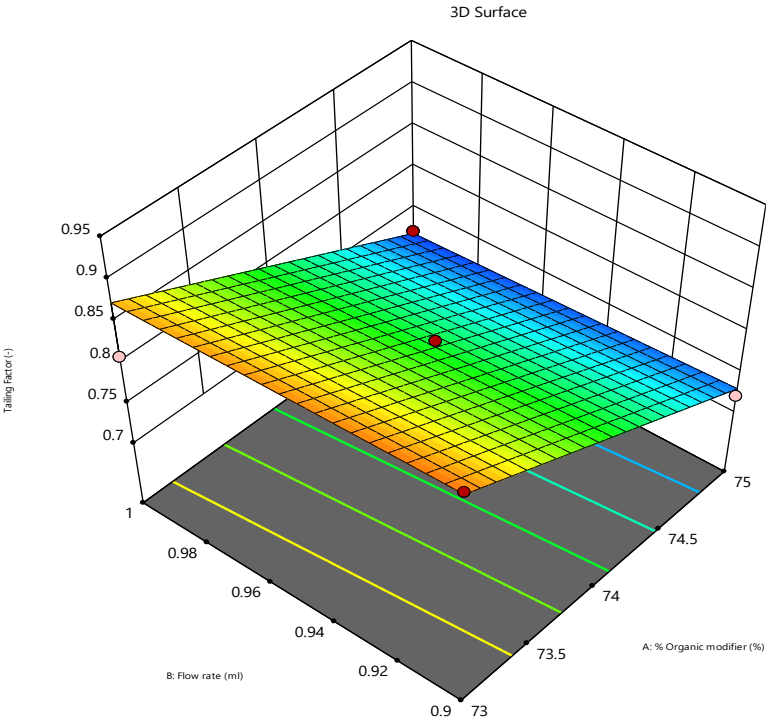


Figure 27d: 3D Contour plot depicting the effect of flow rate and methanol on tailing factor of Dapagliflozin

METHOD VALIDATION

System suitability: System suitability results demonstrate that there is no significant variation in Dapagliflozin's PA, RT, theoretical plate, or TF across six replicates. During system testing, factors such as retention duration, PA, TP, and TF were evaluated, and %RSD was computed before doing the analysis. The percentage RSD for all system suitability parameters was less than 2% (**Table 23, Figure 28**).

Specificity and selectivity: Figure 29a shows the HPLC chromatogram of standard Dapagliflozin at RT 4.8 mins. **Figure 29b** depicts the chromatogram of Dapagliflozin in a sample at 4.7 min. The blank chromatogram exhibited no peak at RT for Dapagliflozin. There was no interference observed during the RT of the Dapagliflozin peak in the standard and sample chromatograms, indicating that the technique was specific and selective for Dapagliflozin.

Linearity and range: The standard calibration curve for Dapagliflozin was graphed in the concentration range of 5-25 µg/ml. **Figure 30** depicts the standard calibration curve, while Figures **31a–e** depict chromatograms. The process was linear in the concentration range of 5-25 µg/ml, with a r2 value of 0.999 (**Table 24**).

Sensitivity: The limits of detection and quantification were found to be 1.26 and 3.38 µg/ml, respectively. The acquired values demonstrate the excellent sensitivity of the HPLC approach.

Precision: The percentage RSD of intraday and interday precision for Dapagliflozin was less than 2% after three measurements. These findings supported the high level of precision of the developed approach. **Table 25** shows the precision data for the approach.

Ruggedness: Different analysts tested the procedure's robustness, and the results were judged to be acceptable. **Table 26** shows the robustness of the suggested technique (%RSD <2%).

Robustness: Acceptable % RSD values were obtained by making tiny purposeful adjustments in the developed technique parameters. This shows that the approach is suitable for industrial analysis. **Table 27** shows that the percentage RSD of PA was less than 2%, which is within the acceptable range.

Recovery

Table 28 shows that the developed approach is highly accurate, with percentage recovery ranging from 98.67 to 102.33% at all three levels and an RSD value of less than 2%.

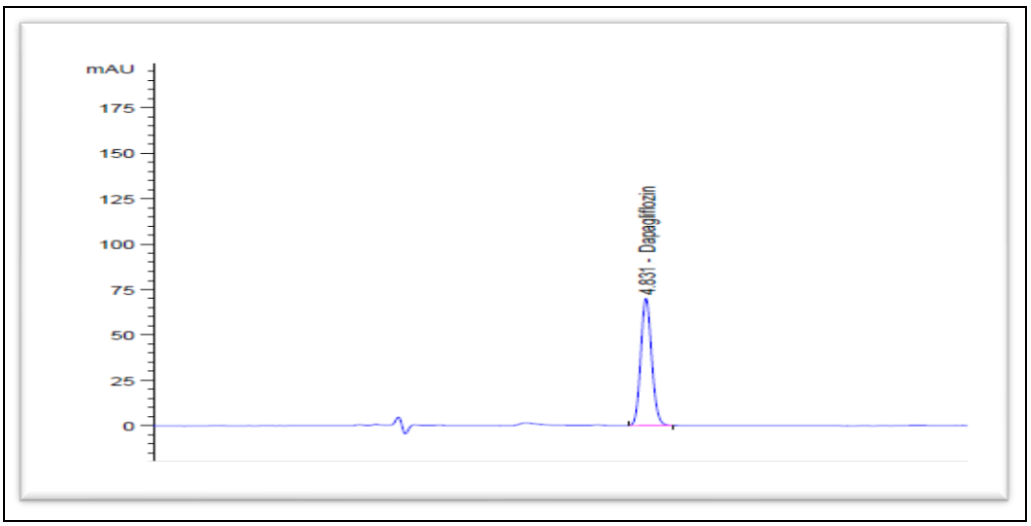


Figure 28: System suitability chromatogram of Dapagliflozin

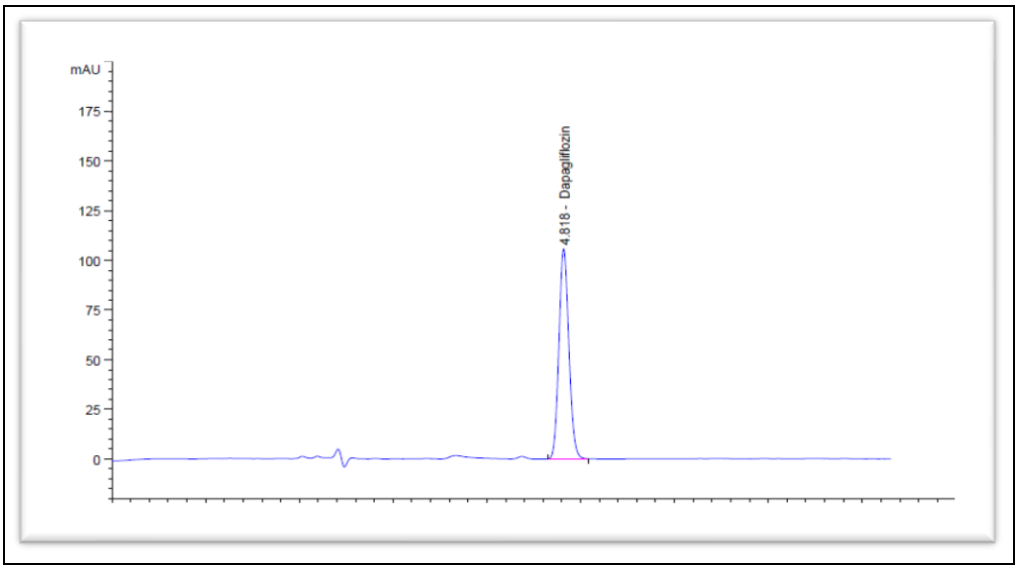


Figure 29a: Chromatogram of standard Dapagliflozin

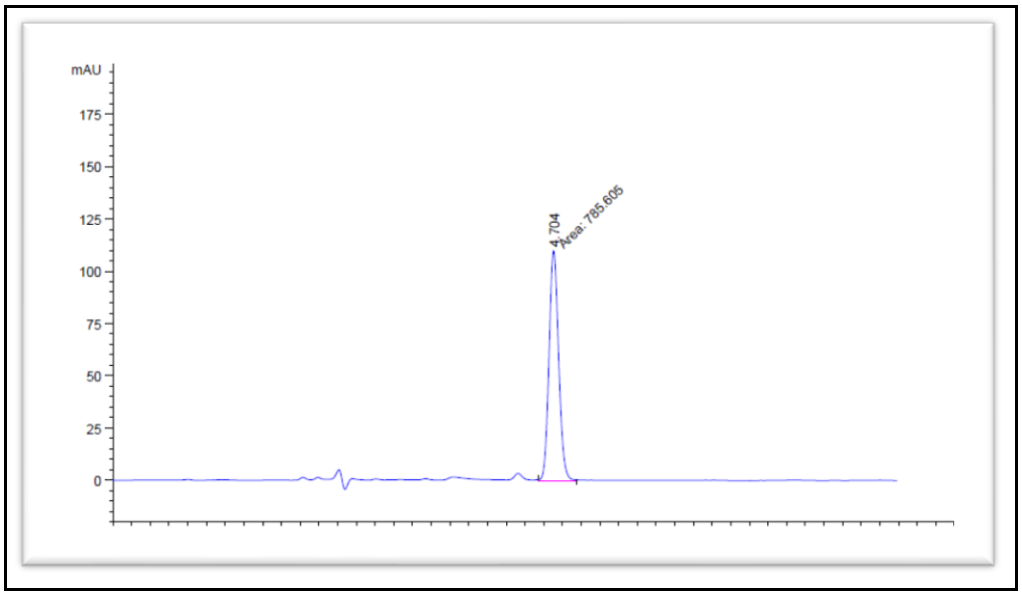


Figure 29b: Chromatogram of Dapagliflozin sample

Table 23: System suitability data of Dapagliflozin by RP-HPLC method

Parameter	RT	PA	TP	TF
Mean	4.85	1433.29	11602.39	0.88
SD	0.04	12.23	55.81	0.01
%RSD	0.87	0.85	0.48	0.86

Table 24: Linearity and range data of Dapagliflozin by HPLC method

Sl. No.	Parameters	Results
1	Linearity Range	5 to 25 µg/ml
2	Correlation coefficient (r^2)	0.9994
3	Calibration Equation	y = 141.46x + 2.532
4	Slope	141.46
5	Intercept	2.532
6	Limit of Detection	1.26 µg/ml
7	Limit of Quantification	3.83 µg/ml

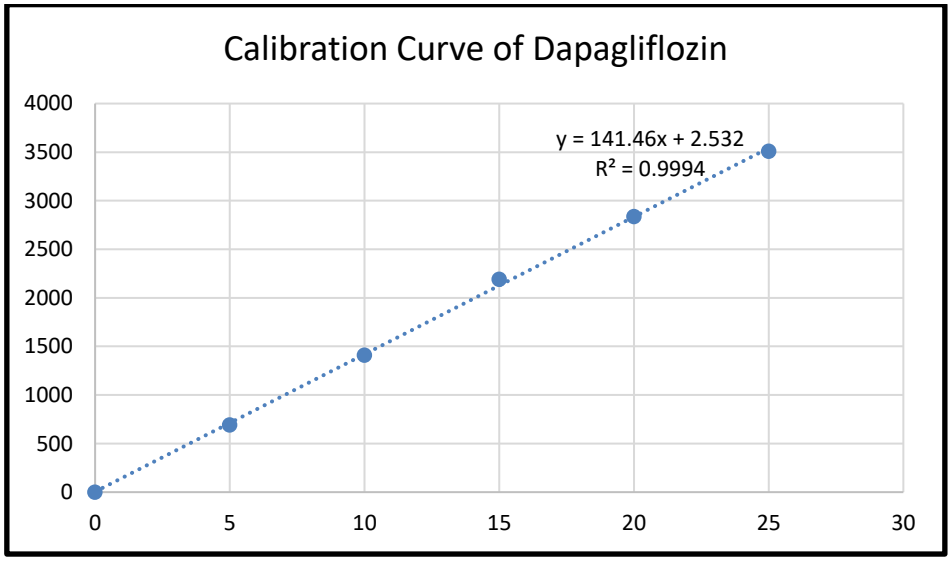


Figure 30: Calibration curve of Dapagliflozin by HPLC method

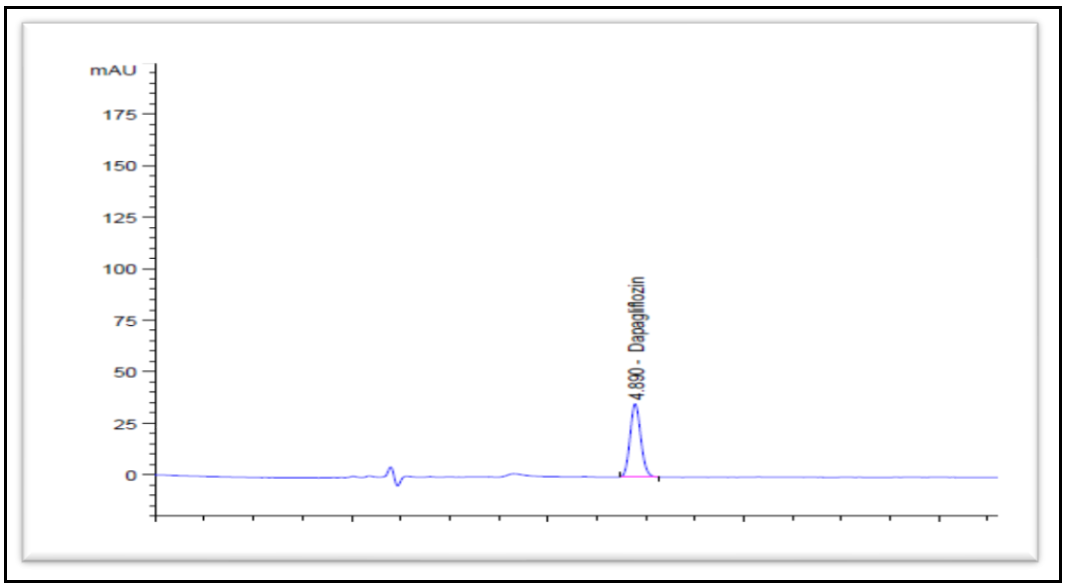


Figure 31a: Chromatogram of Dapagliflozin (5 µg/ml)

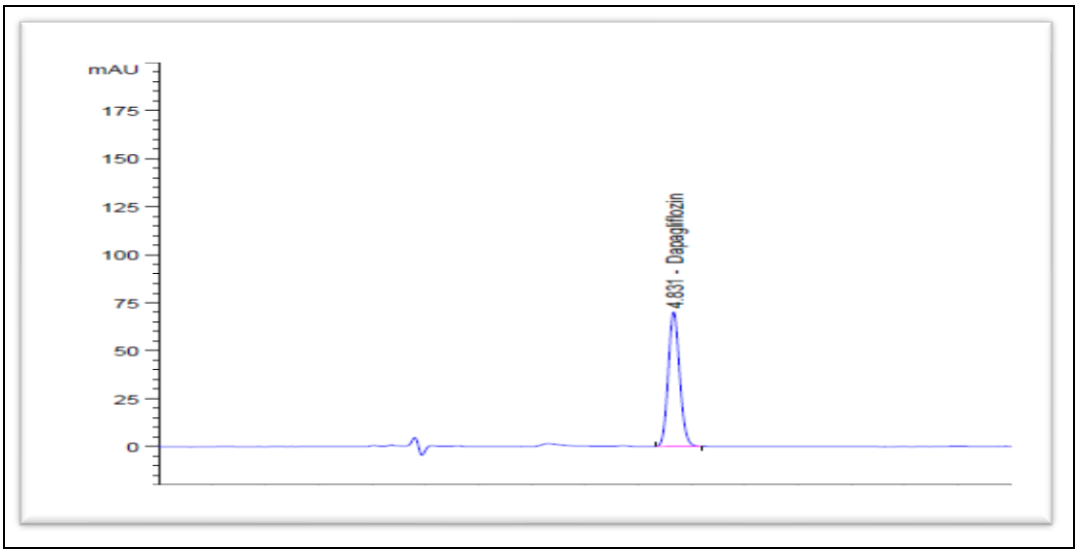


Figure 31b: Chromatogram of Dapagliflozin (10 µg/ml)

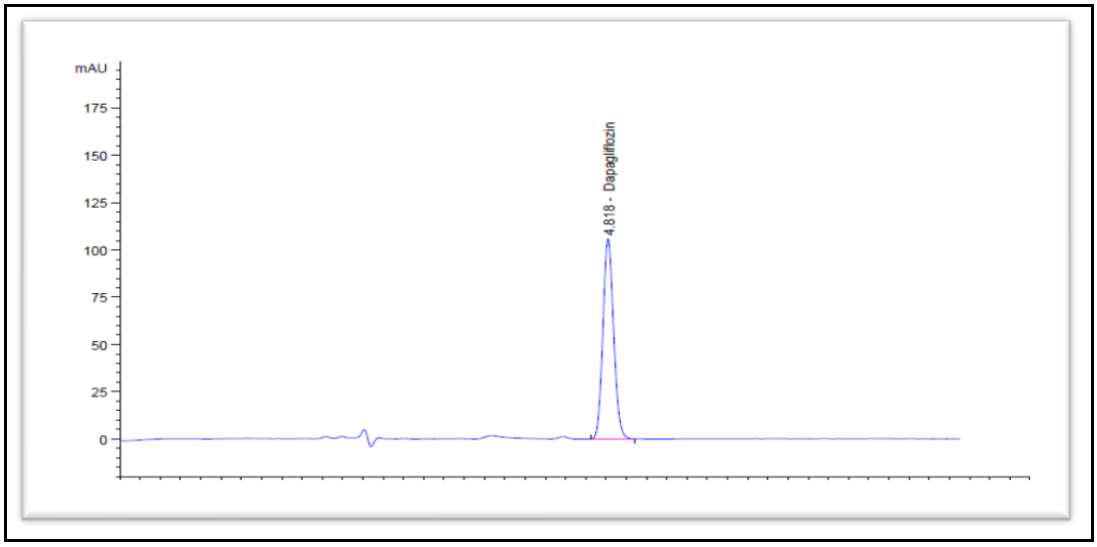


Figure 31c: Chromatogram of standard Dapagliflozin (15 µg/ml)

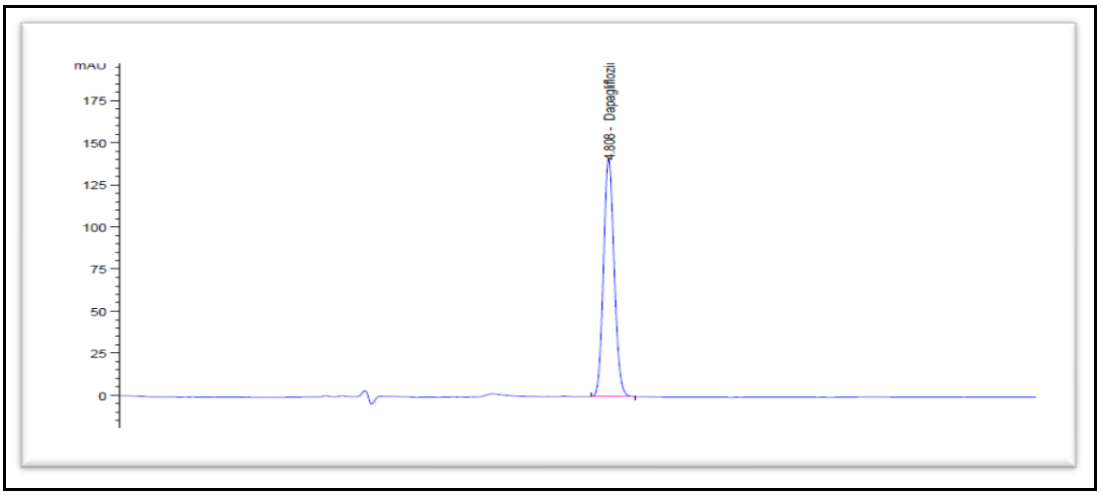


Figure 31d: Chromatogram of standard Dapagliflozin (20 µg/ml)

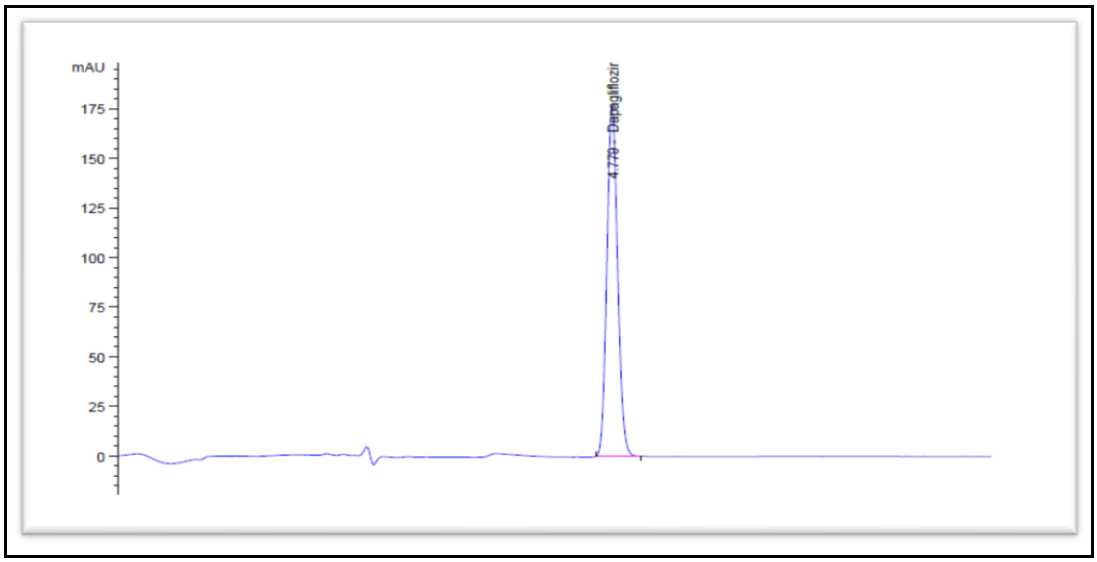


Figure 31e: Chromatogram of standard Dapagliflozin (25 µg/ml)

Table 25: Precision data of Dapagliflozin by HPLC method

Precision Parameter	Concentration µg/ml	Mean PA	Standard Deviation	%RSD
Intraday	5 µg/ml	692.67	3.59	0.52
	15 µg/ml	2130.66	2.67	0.13
	25 µg/ml	3512.12	9.56	0.27
Interday	5 µg/ml	387.37	4.09	1.06
	15 µg/ml	1121.38	5.72	0.51
	25 µg/ml	1822.61	3.17	0.17

Table 26: Ruggedness data of Dapagliflozin by HPLC method

Ruggedness Type	Concentration	Mean PA	SD	%RSD
Change in Analyst	25 µg/ml	3510.13	3.15	0.09

Table 27: Robustness data of dapagliflozin by HPLC method

Parameters	Range	Mean PA	SD	%RSD
Change in Organic Phase (Methanol: aqueous phase)	73:27 v/v	3014.67	3.51	0.12
	75:25 v/v	2653.80	3.34	0.73
Change in FR	0.9 ml	3263.51	3.40	0.10
	1.0 ml	2493.84	3.51	0.14
Change in wavelength	223	3077.89	3.52	0.11
	225	2642.84	3.51	0.13

Table 28: Recovery data of Dapagliflozin by HPLC method

Levels	Sample	Standard	Area found	Amt found	Amt recovered	% Recovery
80%	10	8	**Mean**	17.94	7.94	99.31
			SD	0.018	0.018	0.22
			%RSD	0.100	0.226	0.23
100%	10	10	**Mean**	20.23	10.23	102.33
			SD	0.020	0.020	0.20
			%RSD	0.100	0.198	0.20
120%	10	12	**Mean**	21.84	11.84	98.67
			SD	0.021	0.021	0.18
			%RSD	0.098	0.181	0.18

ESTIMATION OF DAPAGLIFLOZIN USING HPLC METHOD

The AQbD based RP-HPLC analysis of Dapagliflozin's commercial formulation exhibited excellent recovery. The recovery rate was determined to be 101.07±0.116%, with a standard deviation of 0.11. Notably, the RT of Dapagliflozin in the dosage remained consistent compared to the Dapagliflozin standard. The TF and TP factors were within acceptable ranges. Chromatograms of all sample solutions displayed no additional peaks, indicating that additions in the dosage form did not impact Dapagliflozin levels. This underscores the viability of the proposed tool for routine QC of Dapagliflozin in tablet forms. The assay chromatogram is depicted in **Figure 32**.

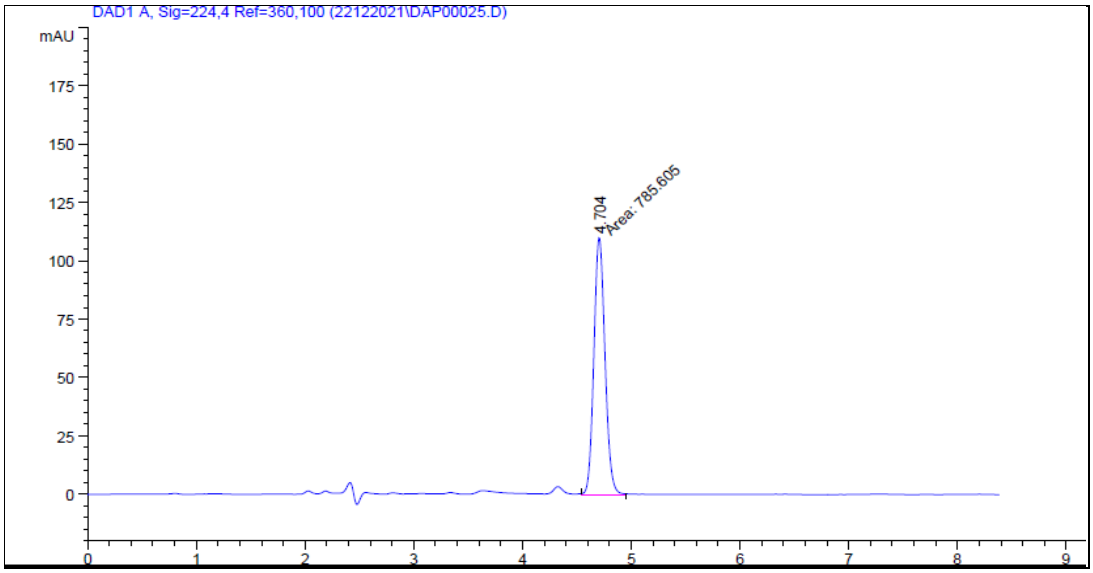


Figure 32: Assay chromatogram of Dapagliflozin

DISCUSSION

The current study focuses on the design of the method for estimating Dapagliflozin in bulk and formulation. We decided to optimize the approach utilizing the notion of DoE, as the pharmaceutical industry and different regulatory guidelines are currently advocating that the analytical method be developed using the QbD principle. The use of the DoE technique enabled systemic and easy screening of method variables and their optimization, as well as a better understanding of the CMVs in relation to analytical answers. Response surface methodology-based BBD was utilized to optimize chromatographic variables, assisting in the determination of the optimal Methanol proportion in mobile phase, detection wavelength, and FR for the analytical procedure, as well as the establishment of a mathematical link between CMVs and analytical results. [41] Under these three conditions, an optimum RT (4.8 min) with a large number of TP resulted in minimal tailing, indicating that a time-saving and specific RP-HPLC method was created. The developed RP-HPLC technique is accurate and precise, aligning with regulatory criteria, as evidenced by consistent and acceptable% RSD (<2%) results across validation parameters. Furthermore, the validated method yielded low detecting limit and quantifying limit values, indicating that the method is very sensitive. The proposed approach was found to be specific for the measurement of Dapagliflozin in the presence of formulation excipients, and it was then used to estimate drug concentration in commercialized Dapagliflozin formulations.

CONCLUSION

This paper describes the invention and validation of a simple, sensitive, quick, and accurate RP-HPLC analytical method for estimating the anti-diabetic medication Dapagliflozin. The use of the QbD technique and DoE software aids in optimizing the precise conditions needed to build the final analytical method for Dapagliflozin. Dapagliflozin was accurately quantified using Methanol and o-phosphoric acid buffer as the mobile phase, with a FR of 0.95mL/min and a RT of 4.8 mins. The method can detect and quantify Dapagliflozin concentrations as low as 1.26 µg/ml and 3.83 µg/ml, respectively. The devised approach was demonstrated to be effective for accurately quantifying Dapagliflozin in bulk and in marketed formulations.

METHOD 3: DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR ESTIMATION OF EMPAGLIFLOZIN

MATERIALS AND METHODS

Chromatographic conditions

The proposed experiment utilized an Agilent 1220 (Autosampler) HPLC equipment with Chemstation software. Empagliflozin was separated chromatographically using an Agilent C-18 column (250x4.6 mm i.d.,5 µm). The solvent system was composed of Methanol and MW in proportions of 70:30 v/v. Empagliflozin was eluted at 1.0 mL per min and detected using UV at 233 nm. The column temperature was set to ambient, and the injection volume was 10 µL. [151]

Standard preparation

A stock solution of Empagliflozin was prepared by dissolving 10 mg of Empagliflozin in 10 mL of the designated mobile phase. The resulting solution underwent sonication to ensure complete dissolution of Empagliflozin. Subsequently, 1 mL of this stock solution was withdrawn and transferred into a 10 mL volumetric flask, followed by dilution with 10 mL of the mobile phase as the diluent. This prepared sample was utilized for calibrating and validating the experimental parameters. Further Empagliflozin solutions were prepared by successive dilutions, maintaining the solvent system as the diluent. Concentration series were generated using these solutions. Prior to chromatography, the final solution underwent filtration through a 0.45µm syringe filter to remove any particulate matter. The resulting diluted solutions were then transferred into vials for subsequent chromatographic analysis. [151]

METHOD DEVELOPMENT

Initial experimentation involved a thorough review of existing literature and iterative testing to refine a linear, economical, and reliable approach for assessing and managing the quality of Empagliflozin in both bulk and tablet formulations. The process commenced with the exploration of different solvent combinations such as acetonitrile, Methanol, and MW to establish an optimal mobile phase. [127]

Identification of the detecting wavelength

The Empagliflozin solution was scanned in the 200-400 nm range to find the λ max for the study. The UV spectra of the standard medication exhibited a maximum absorption at 233nm. As a result, it served as the detecting wavelength. [119]

QUALITY BY DESIGN APPROACH

In this study, we focused on several aspects of AQbD and applied them to a practical technique. It was implemented by following the methods outlined in Methods 1 and 2. [123-130]

DOE based optimization

Based on preliminary factor screening trials, the method's performance was enhanced using the BBD design with three levels: low (-1), intermediate (0), and high (+1). The DOE incorporated CMPs such as the percentage of organic solvent, FR, and wavelength as input variables, while CQAs including RT and TF were designated as response variables **(refer to Table 29**). Utilizing DoE tools, a design matrix comprising 17 experimental runs was generated. The BBD screening approach facilitated the examination of various interaction effects and quadratic impacts of the organic solvent percentage, wavelength, and FR on RT and TF **(Table 30).** [41]

Table 29: BBD matrix for HPLC analysis of Empagliflozin

Independent Variable	Levels		
	+1	**0**	**-1**
Organic solvent (%)	**68**	**70**	**72**
FR (ml)	0.9	1.0	1.1
Wavelength (nm)	231	233	235

Table 30: Optimization parameters using BBD for Empagliflozin HPLC analysis

		Factor 1	Factor 2	Factor 3
Std	**Run**	**A: Organic solvent**	**B: FR**	**C: Wavelength**
		%	ml	nm
8	1	72	1	235
6	2	72	1	231
4	3	72	1.1	233
15	4	70	1	233
16	5	70	1	233
9	6	70	0.9	231
3	7	68	1.1	233
10	8	70	1.1	231
7	9	68	1	235
2	10	72	0.9	233
11	11	70	0.9	235
1	12	68	0.9	233
12	13	70	1.1	235
14	14	70	1	233
17	15	70	1	233
5	16	68	1	231
13	17	70	1	233

METHOD VALIDATION [150, 151]

System suitability: The 6 replicate analyses of standard Empagliflozin (10µg/ml) were conducted to ensure system applicability. Measurements included SD and % RSD of PA, TF, RT, and TP.

Specificity and selectivity: It was evaluated by analyzing the HPLC peak of a 10µg/ml Empagliflozin solution, as well as sample and blank chromatograms within the solvent system. This examination aimed to distinguish the analyte accurately and minimize interference from any other peaks present.

Linearity and range: The method's linearity was tested by serially diluting the stock solution of Empagliflozin from 5 to 25µg/ml and graphing the standardization curve of PA vs Empagliflozin concentration. The regression analysis confirmed the method's linearity.

Sensitivity (Limit of Detection-LOD and Limit of Quantification-LOQ): The sensitivity of proposed method was studied in terms of LOD and LOQ. The detecting and quantification limits values were calculated using the following formulas:

LOD = 3 x σ/S and LOQ =10 x σ/S

Where, σ is the SD of the y-intercept and S is the slope of the calibration curve.

Precision: The precision of the newly proposed method was assessed by analyzing three different concentrations of Empagliflozin (5µg/ml, 15µg/ml, and 25µg/ml) at various time intervals within the same day (intraday precision) and across consecutive days. % RSD was calculated for all datasets to quantify the variation.

Ruggedness: Another analyst examined the suggested approach for ruggedness by evaluating six duplicates of a reference solution (15µg/ml) of Empagliflozin. The chromatogram was utilized to calculate the overall mean and percentage Relative Standard Deviation (RSD) for each peak's area.

Robustness: The robustness of the procedure was assessed by varying several parameters. These variations included changing the solvent composition from a Methanol to an aqueous phase (68:32 to 72:28 v/v), adjusting the wavelength from 232 to 234 nm, and increasing the FR from 0.9 to 1.1 mL/min. All robustness experiments were conducted using a working solution of 15µg/ml.

Accuracy: Accuracy was determined using the addition method. The sample solution of Empagliflozin (5 µg/ml) was spiked with 50%, 100%, and 150% of the usual dose. Furthermore, recovery and percent RSD were determined to ensure data correctness.

ESTIMATION OF EMPAGLIFLOZIN IN MARKETED TABLETS

The described method was used to quantify Empagliflozin in marketed tablets. The analysis was done in triplicate and the percentage assay value was provided. The sample was prepared for the assay using the following technique: Empagliflozin weighing 10 mg was precisely placed in a 50 mL volumetric flask. Subsequently, 20 mL of Methanol was added to the flask, followed by vigorous shaking and sonication for 15 minutes. The flask was then agitated and filled to its brim with diluent. The resultant solution was filtered through a Whatman filter paper with a pore size of 0.45 micrometre. The filtration process produced a solution containing Empagliflozin at a concentration of 10 µg/ml, which was then subjected to further evaluation as per the indicated procedure. [151]

RESULTS

METHOD DEVELOPMENT

Preliminary testing found that Methanol and MW made promising mobile phase mixtures, with adequate peak symmetry and reduced peak tailing. **Figure 33** depicts an optimized chromatogram. **Table 31** shows the optimal method parameters.

OPTIMIZATION OF HPLC METHOD WITH QBD APPROACH

The steps involved in QbD-based method optimization were carried out in a systematic manner. The results are presented as follows.

Development of ATP

ATP defines the method requirements that are intended to be measured. **Table 32** shows the ATP for the suggested HPLC technique for empagliflozin.

Identification of CMPs and RA

Research was conducted to assess the risks associated with CMPs, which are influential factors with significant implications for CAAs. In this investigation, an IFB diagram was constructed to pinpoint key risk variables that could potentially impact the efficacy of the method (refer to Figure 8). Through this analysis, several high-risk procedure variables were identified and subsequently subjected to thorough examination using an appropriate DoE. The selected method variables included the FR of the mobile phase, the percentage composition of the organic phase (specifically Methanol in the mobile phase), and the choice of detection wavelength.

Table 31: Developed HPLC method specifications for Empagliflozin

Sr. No.	Description	Specifications
1	Technique	Chromatographic separation
2	Machine	HPLC
3	Model	Agilent
4	Brand	Agilent 1220
5	Processing Software	Chemstation possessing
6	Stationary Phase	C_{18} column
7	Solvent ratio	Methanol: MW (70:30 v/v)
8	FR	1.0 ml
9	λmax	233 nm
10	Volume of injection	10 µL.
11	API	Empagliflozin
12	RT	4.0 min

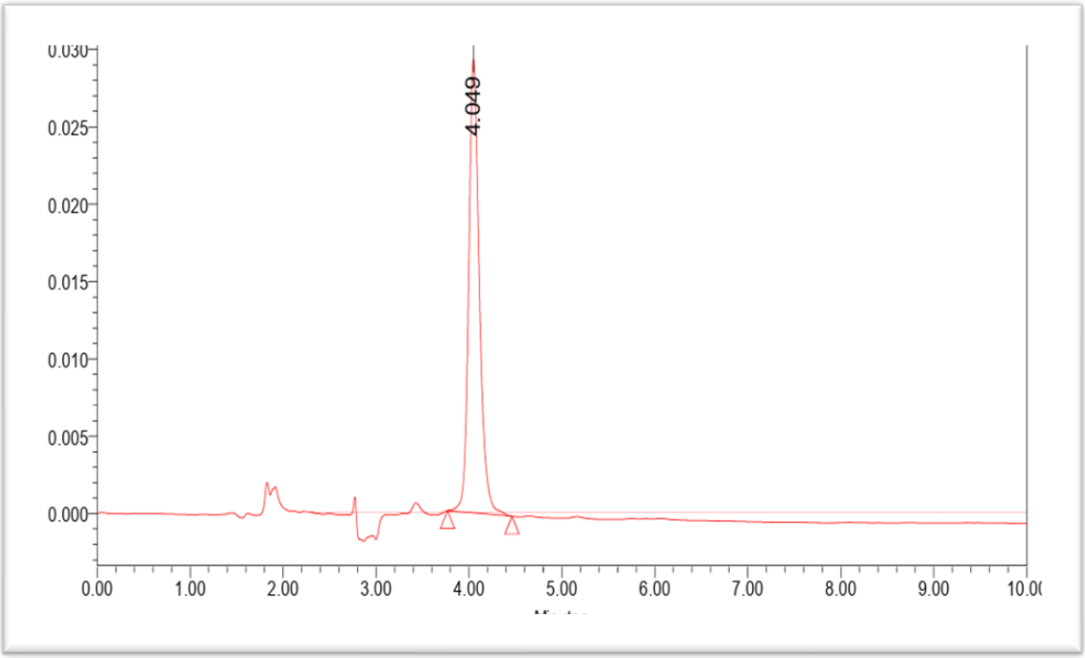


Figure 33: Optimized Chromatogram of standard Empagliflozin

Table 32: Analytical target profile for HPLC analysis of Empagliflozin

Sl. No.	ATP Parameters	Target
1	Target Analyte	Empagliflozin
2	Target Sample	Empagliflozin Tablets
3	Method category	Chromatographic
4	Instrument requirement	HPLC
5	Nature of analyte	Solid (Solution)
6	Standard stock solution	Dilution of main drug in linear manner
7	Application of Method	Estimation of Empagliflozin
8	Validation parameters	Specificity, System suitability, Linearity, Precision, Accuracy, Ruggedness, Robustness, Selectivity and Sensitivity.

DoE optimization

The BBD was utilized to optimize a variety of design characteristics. The quadratic model was used to examine the data for both main and interaction effects. **Table 33** displays the optimal model parameters. The model generates a quadratic equation for each CAA parameter.

The RT equation (for actual value) is 4.02 - 0.50 A - 0.17 B + 0.00 C + 0.00 AB + 0.00 AC + 0.00 BC + 0.16 A^2 + 0.01 B^2 + 0.01 C2. In **Figure 34a**, RT increases as β1 negative coefficient (-0.50) indicates a decrease in mobile phase quantity (A), β2 negative coefficient (-0.17) suggests a decrease in mobile phase FR (B), and β3 positive coefficient (+0.00) indicates an increase in wavelength. Similar way actual values for evaluated for peak area and tailing factor and effects were showed in the **Figure 34b-c.**

Table 33: ANOVA responses of BBD for HPLC method of Empagliflozin

Source	Sum of squares	df	Mean square	F-value	p-value	
For Retention Time						
Model	2.36	9	0.2627	141.47	< 0.0001	significant
A-Organic modifier	2.00	1	2.00	1076.92	< 0.0001	
B-Flow rate	0.2450	1	0.2450	131.92	< 0.0001	
C-Wavelength	0.0000	1	0.0000	0.0000	1.0000	
For Peak Area						
Model	6.164E+06	9	6.849E+05	1358.56	< 0.0001	significant
A-Organic modifier	5.018E+06	1	5.018E+06	9954.31	< 0.0001	
B-Flow rate	8.00	1	8.00	0.0159	0.9033	
C-Wavelength	1250.00	1	1250.00	2.48	0.1593	
For Tailing Factor						
Model	0.5000	3	0.1667	204.63	< 0.0001	significant
A-Organic modifier	0.5000	1	0.5000	613.89	< 0.0001	
B-Flow rate	0.0000	1	0.0000	0.0000	1.0000	
C-Wavelength	0.0000	1	0.0000	0.0000	1.0000	

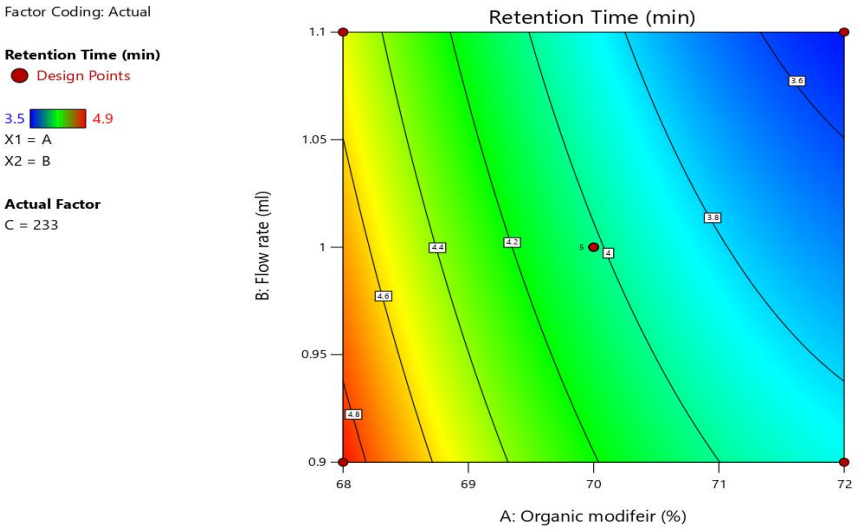


Figure 34a: 2D surface plot for effect of Organic modifier and Flow rate on RT

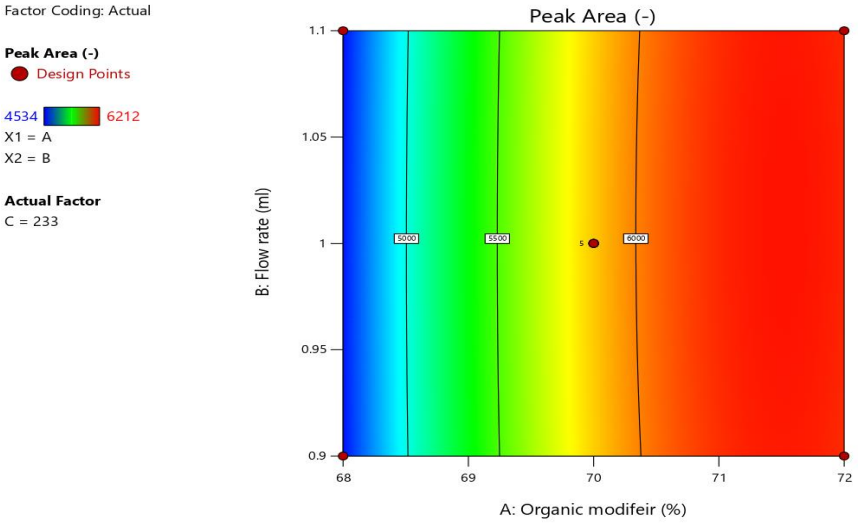


Figure 34b: 2D surface plot for effect of Organic modifier and Flow rate on PA

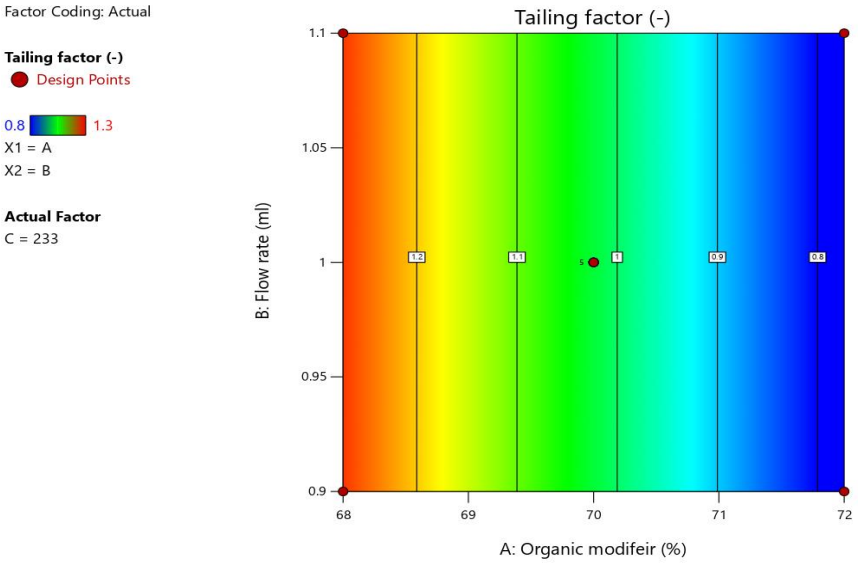


Figure 34c: 2D surface plot for effect of Organic modifier and Flow rate on TF

The optimized solution revealed that a mobile phase composition of 70:30% v/v Methanol: MW and a FR of 1 ml/min produced good results, with all CAAs falling within the intended range (**Table 34**), and **Figure 35** depicts the design space for optimum parameters.

Table 34: BBD optimized solution for HPLC analysis of Empagliflozin

% Organic solvent	FR	Wavelength	RT	PA	TF
70%	1 ml	233	4.0	8881	1.0

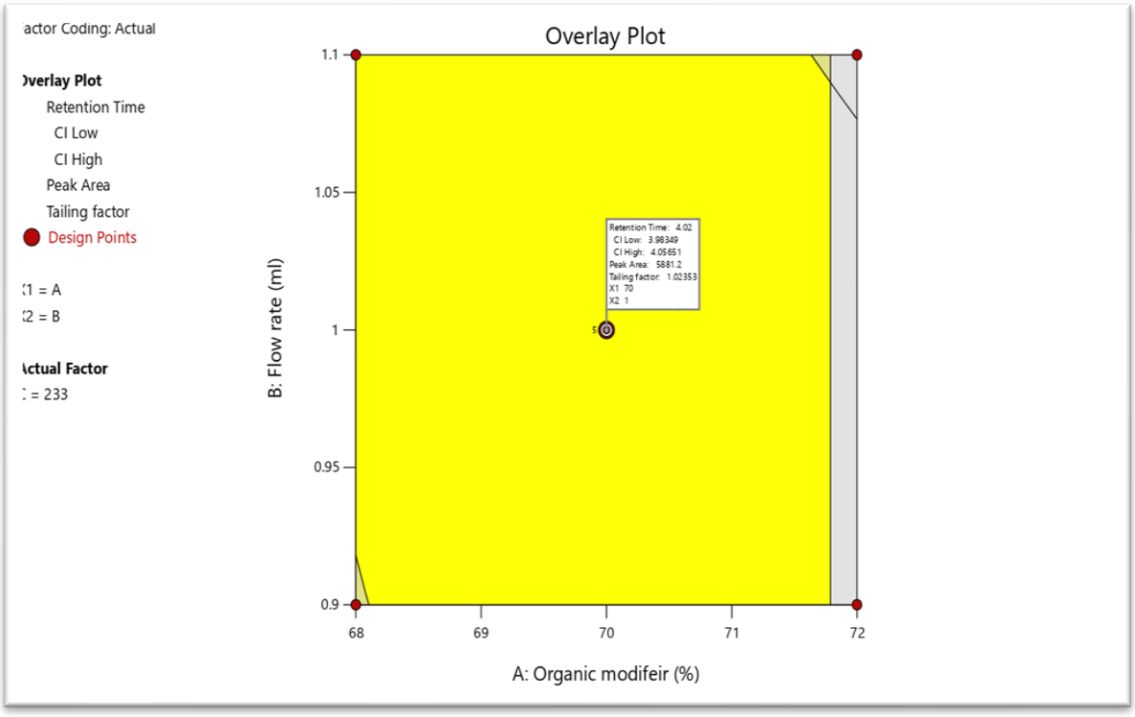


Figure 35: Design space of optimized parameters for Empagliflozin by HPLC

METHOD VALIDATION

System suitability: The results from the system suitability analysis indicate that there were negligible fluctuations observed in the parameters of PA, TF, TP, and RT for Empagliflozin. The percent relative standard deviation (%RSD) was calculated for each of these system suitability measures. Across the board, the %RSD for all parameters remained below 2%, as detailed in **Table 35** and illustrated in **Figure 36**.

Table 35: System suitability data of Empagliflozin by HPLC method

Parameter	RT	PA	TP	TF
Mean	4.01	175402.43	5830.44	1.07
SD	0.04	2801.25	55.75	0.01
%RSD	1.01	1.60	0.96	0.78

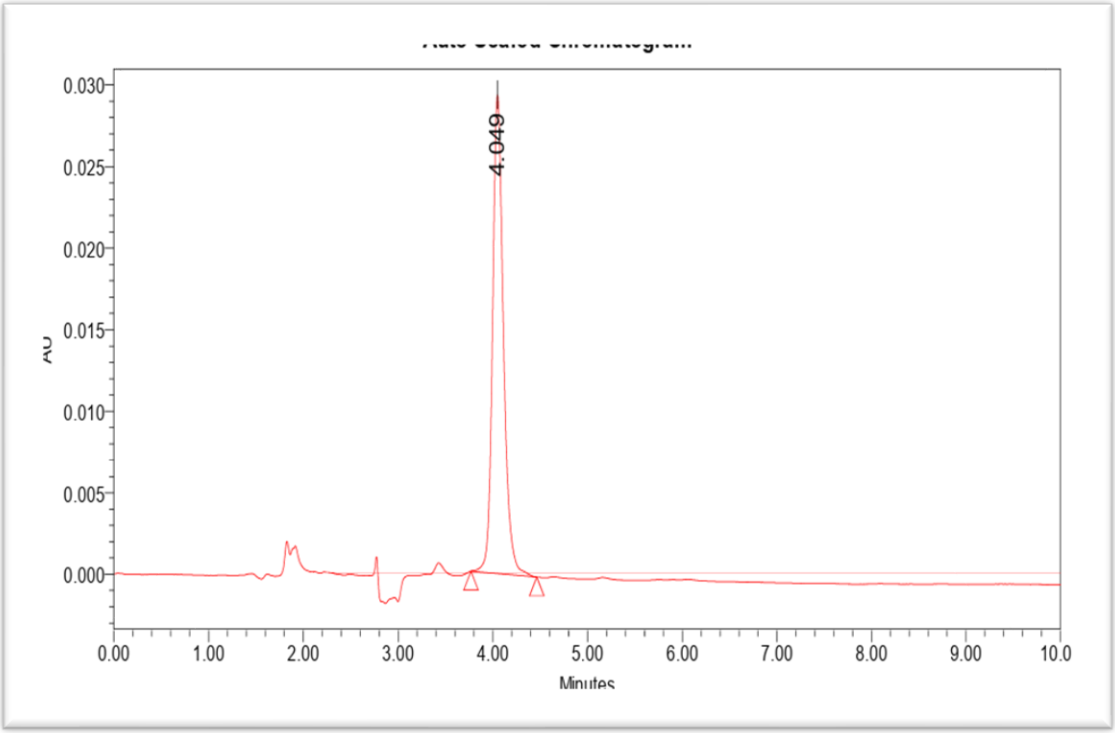


Figure 36: System suitability HPLC chromatogram of Empagliflozin

Specificity and Selectivity: The standard and sample HPLC chromatograms had a retention duration of 4.0 min (**Figures 37a-b**). The blank chromatogram revealed no peak at Empagliflozin RT. No interference was seen at the RT of the Empagliflozin peak in the standard and sample chromatograms, indicating that the technique was specific and selective for Empagliflozin.

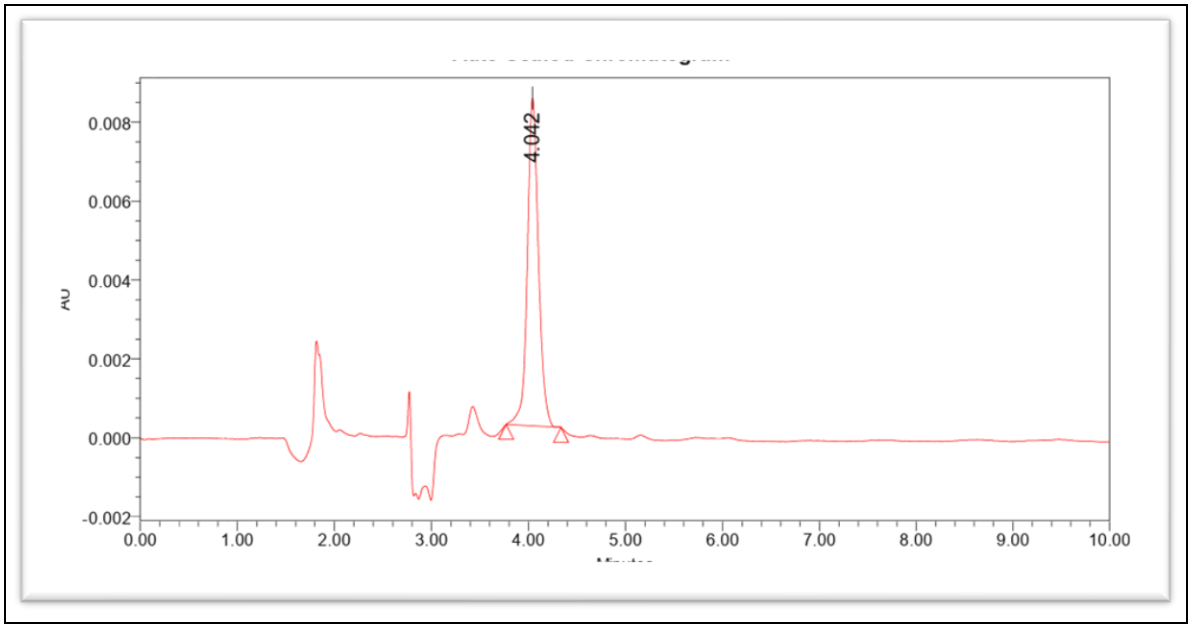


Figure 37a: HPLC Chromatogram of Standard Empagliflozin

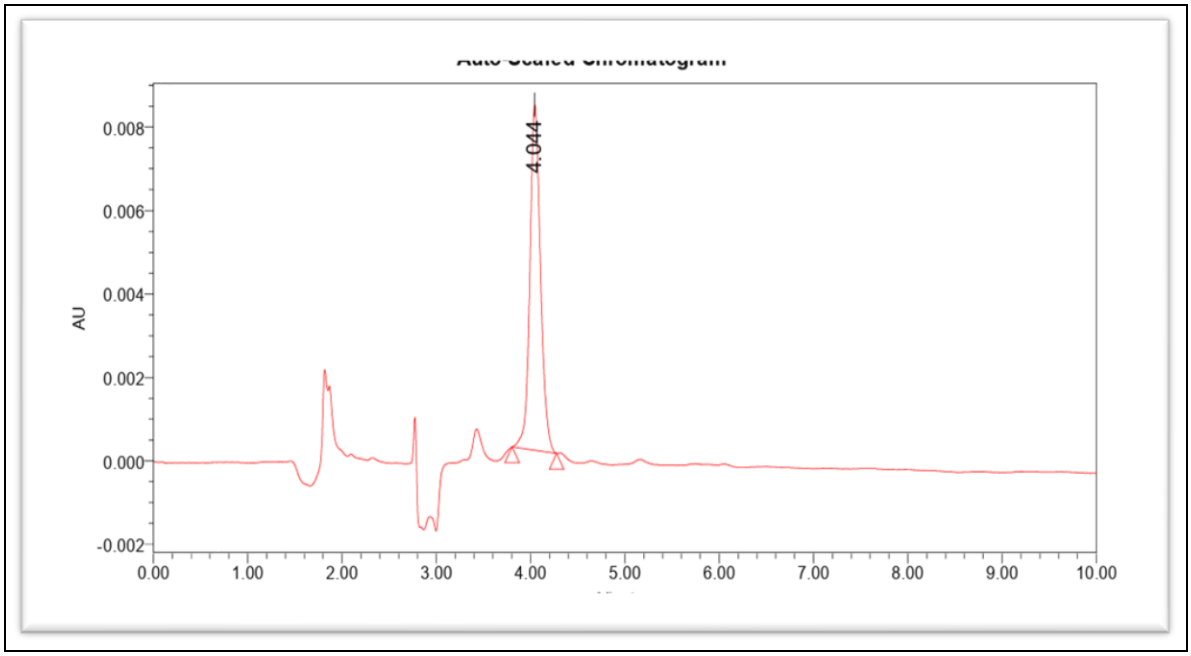


Figure 37b: HPLC Chromatogram of Sample Empagliflozin

Linearity and range: The linear calibration curve constructed for Empagliflozin spanning concentrations from 5 to 25µg/ml exhibited excellent linearity, as evidenced by a correlation coefficient of 0.9991. Detailed results are provided in **Table 36**, while the graphical representation is depicted in **Figure 8**.

Table 36: Linearity and range data of Empagliflozin by RP-HPLC method

Sl. No.	Description	Results
1	Range	5 to 25µg/ml
2	r^2	0.9991
3	Calibration Equation	y = 16522x + 1693.2
4	Slope	16522
5	Intercept	1693.2
6	Detecting limit	1.18µg/ml
7	Quantifying limit	3.57µg/ml

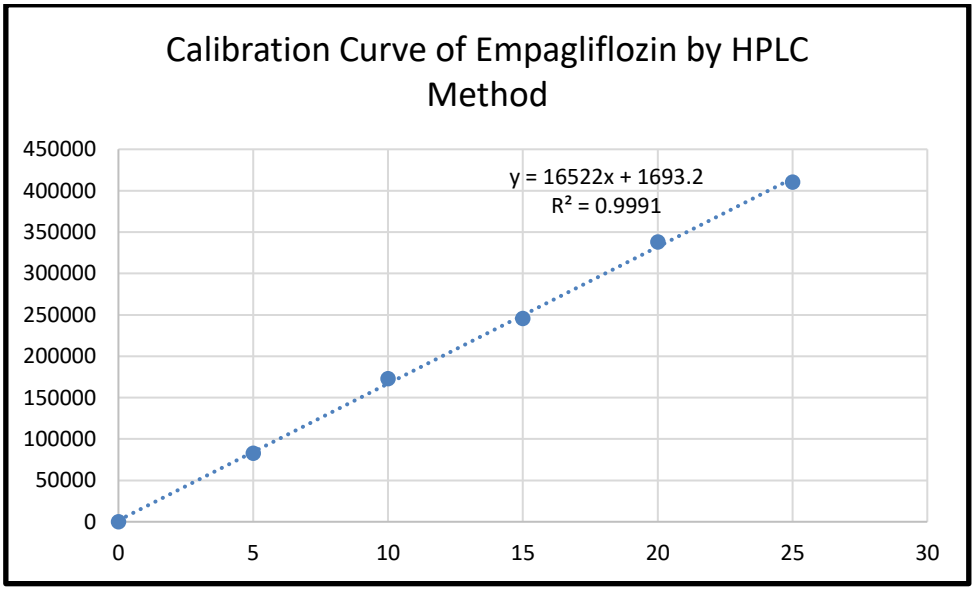


Figure 38: Calibration curve of Empagliflozin by RP-HPLC method

Sensitivity: The detecting limit and quantifying limit values for empagliflozin were 1.18 and 3.57µg/ml, respectively. The results show that the HPLC technique has great sensitivity.

Precision: The percentage RSD of intraday and interday precision for Empagliflozin was less than 2% after three measurements. These findings provided evidence for the developed approach's high level of precision. **Table 37** displays the precision data for the process.

Ruggedness: The ruggedness of the technique was examined by many analyzers, and the results were deemed satisfactory. **Table 38** indicates that the proposed approach is resilient (%RSD < 2%).

Robustness: By implementing slight, purposeful adjustments to the established procedure parameters, the resulting %RSD values were deemed acceptable, indicating the suitability of the approach for industrial analysis. **Table 39** illustrates that all parameters-maintained a %RSD below 2%, aligning well within the acceptable threshold.

Accuracy: The devised approach demonstrated remarkable accuracy, with percentage recovery ranging from 98.80 to 101.05% across all three levels and a percentage RSD value of less than 2% (**Table 40**).

Table 37: Precision of Empagliflozin by HPLC method

Precision Parameter	Concentration µg/ml	Mean PA	Standard Deviation	%RSD
Intraday	5µg/ml	82449.85	1535.75	0.86
	15µg/ml	255083.23	577.35	0.23
	25µg/ml	414439.01	5318.90	1.28
Interday	5µg/ml	72696.44	221.62	0.30
	15µg/ml	246081.86	1154.70	0.47
	25µg/ml	412168.95	2886.75	0.70

Table 38: Ruggedness of Empagliflozin by HPLC method

Ruggedness Type	Concentration	Mean PA	SD	%RSD
Change in Analyst	15µg/ml	275290.36	4026.78	1.46

Table 39: Robustness data of Empagliflozin by HPLC method

Parameter	Statistics	Change in conc. of MeOH (%)		Detection (nm)		Change in FR (ml)	
		68	**72**	**232**	**234**	**0.9**	**1.1**
PA	**Mean**	188783	188783	172637	172711	174259	174092
	SD	1842	952	330	1764	1764	2538
	%RSD	0.98	0.50	0.19	1.02	1.01	1.46

Table 40: Recovery data of Empagliflozin by RP-HPLC method

Levels %	Sample quantity	Standard Quantity	Total quantity	Mean Quantity Recovered (µg/ml) ± SD	Mean % Recovery (µg/ml) ± SD
50	5	5	10	9.88 ±0.07	98.80 ± 0.69
100	5	10	15	15.17 ±0.08	101.15 ± 0.52
150	5	15	20	19.85 ±0.09	99.23 ±0.43

ESTIMATION OF EMPAGLIFLOZIN IN MARKETD TABLES

For the Empagliflozin label claim, the percentage of drug content was 100.42±0.34% (n = 3). The assay results show that the devised HPLC technique is selective for estimating Empagliflozin in pharmaceutical dose form **(Figure 39**).

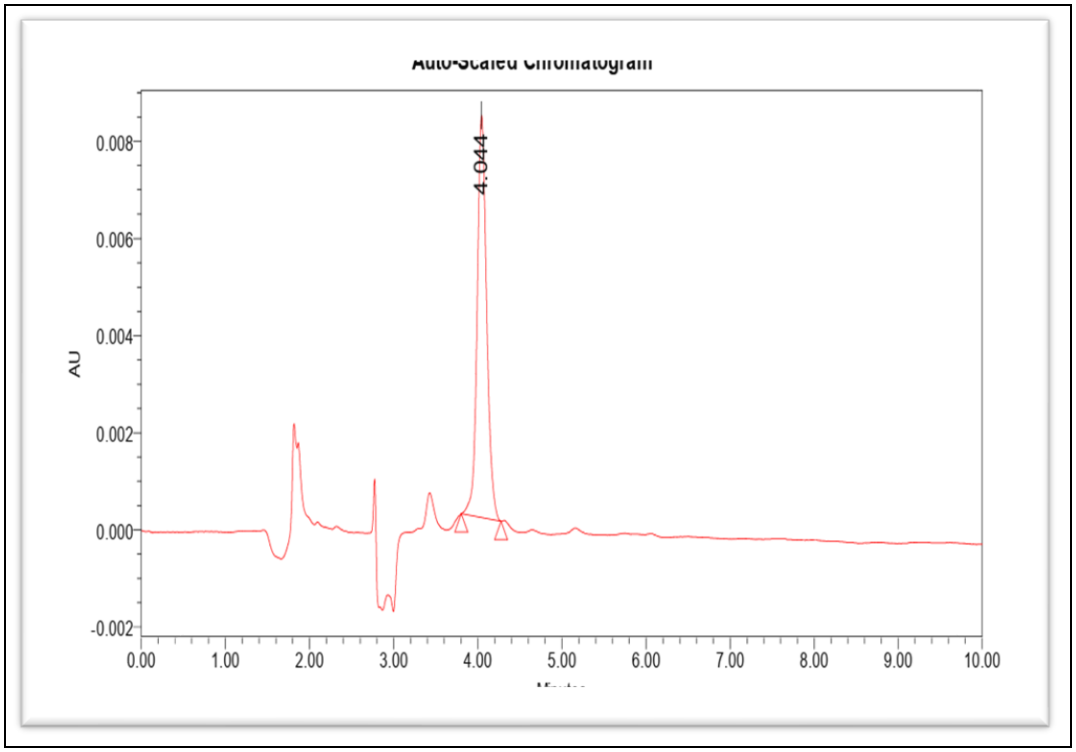


Figure 39: Assay chromatogram of Empagliflozin

DISCUSSION

The primary objective of this study was to develop a straightforward, precise, dependable, and effective application of QBD principles in designing a technique for quantifying Empagliflozin, with enhanced robustness and performance. To achieve this, a BBD was employed, encompassing three factors at 3 different levels, utilizing DoE software. Each factor was meticulously analyzed using DoE data, incorporating techniques such as ANOVA, diagnostic and model graphs to ascertain their influence on the response outcome. Furthermore, the developed method underwent thorough validation in accordance with the ICH Q2 R1 guidelines, assessing various parameters. The absence of any undesirable peaks and the consistent RT of the drug affirmed the method's suitability for quantifying Empagliflozin in pharmaceutical preparations, attesting to its high specificity and selectivity.

CONCLUSION

The QbD-based methodology was successfully applied to establish a simple, rapid, sensitive, and cost-effective analytical method for calculating Empagliflozin in bulk and pharmaceutical dose forms. DOE is used to provide a comprehensive statistical analysis and design spaces for the new approach, and the method is shown to be robust over a wider range of scenarios. It can be utilized effectively for routine QC in research institutes.

METHOD 4: DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR ESTIMATION OF LINAGLIPTIN

MATERIALS AND METHODS

Chromatographic conditions

The Shimadzu Prominence i series LC-2030c 3-D plus (Autosampler) HPLC system, Model: Shimadzu Prominence, with software, Lab solutions Version 5.97 SP1, was utilized in the suggested research project. A Shim-pack GIST C18 column (250 x 4.5 mm i.d., 5µm) and a solvent system comprising Acetonitrile (ACN) and 0.2% OPA (31.7: 68.3 v/v) were used to elute linagliptin using RP-HPLC at a FR of 0.96 ml/min. The column's temperature was kept at 30°C while the UV analysis was conducted at 226 nm. After being extracted from the milli-Q® system, the HPLC grade water was filtered via a membrane filter with a 0.22-µM pore size before being utilized to create the mobile phase composition.

Preparation of Linagliptin standard solutions

A calibrated micro weighing scale was used to weigh 10 mg of Linagliptin standard, which was then quantitatively transferred into a 10 mL VF. A tiny quantity of Methanol was then added, and the mixture was shaken and sonicated for approximately five minutes to dissolve the Linagliptin. Using the same solvent, the volume was adjusted to provide a standard stock solution with a concentration of 1000 µg/ml of linagliptin. One ml was quantitatively transferred from this stock into a ten ml VF, and the volume was adjusted with Methanol to obtain a secondary stock solution (100 µg/ml). To obtain a concentration of 10 µg/ml of linagliptin, 1 mL of the secondary stock solution was diluted with the solvent system to create a working standard solution. [151]

METHOD DEVELOPMENT

Preliminary studies based on a literature search and trial-and-error methodology were conducted in order to optimize a straightforward, economical, accurate, and exact approach for evaluating and quantifying the Linagliptin in its pharmaceutical tablet dosage form. The first step in the development process involved choosing the mobile phase using numerous solvent combinations, including ACN, Methanol, and MW with varying quantities of orthophosphoric acid. [151]

Identification of the detecting wavelength

The 200–400 nm range of the Linagliptin reference solution was examined to find the λmax for the analysis. The standard drug's UV spectrum displayed maximum absorption at 226 nm. As a result, it served as the detecting wavelength. [102]

METHOD OPTIMIZATION BY QBD APPROACH

To optimize the developed method QbD approach, such as identifying an analytical target profile, selecting critical analytical features, defining critical method parameters, and identifying risks, were carried out as previously mentioned in methods followed by DoE optimization. [151]

DoE Optimization

Based on a thorough review of the existing literature, we have pinpointed two Critical Method Attributes, namely the solvent concentration in the Mobile Phase (X1) and the FR of the method (X2), as the focal points for subsequent optimization efforts. The optimization of the method was studied by using 3^2 factorial designs.

The optimization was studied with respect to three dependent variables mainly TP, RT of analyte and TF. The optimization of the method was carried out by using 10 µg/ml of LIN solution. The factorial design was used for a total of 13 analytical runs. We also analyzed study data using statistical analysis derived from the factor screening procedure. Design-Expert® software version 11 was used to perform the optimization. The design-generated model was tested using ANOVA and compared for several metrics such as adjusted correlation coefficient (adjusted R2), correlation coefficient (R2), projected correlation coefficient (projected R2), and appropriate precision. Response surface analysis was conducted through the generation of 2-D-contour graphs and 3-D-response surface graphs. A numerical optimization approach was employed to predict the optimal analytical conditions. Subsequently, these optimal conditions were mapped within the analytical design space region using an overlay graph, which included a flag incorporating all pertinent parameters. [151]

ANALYTICAL METHOD VALIDATION [150]

The newly developed and QbD based optimized RP-HPLC method was validated by following regulatory guidelines of ICH by evaluating validation parameters.

System suitability: To determine the appropriate approach for drug analysis, a system suitability analysis of the chromatographic system was conducted. Analyte solution (6µg/ml) injections were given six times a day, as part of a daily routine to assess sample appropriateness for the system. The percentage RSD of the PA and the analyte's RT were taken into account while evaluating the testing's appropriateness. The PA, RT, TP, TF, and other metrics were used to express the system appropriateness results.

Specificity and selectivity: To evaluate the specificity and selectivity of the procedure, chromatograms of Linagliptin (5 µg/ml) solution, samples, and blank chromatograms of the solvent system were obtained using HPLC. This process aids in determining peak purity, distinguishing the analyte, and identifying any interference from other peaks.

Linearity and range: The linearity of the procedure was evaluated within the Linagliptin concentration range of 2-12 µg/ml. To assess linearity, different concentrations were prepared, injected into the HPLC system three times, and chromatograms were recorded to determine the PA for each injection. A standard calibration curve was then constructed by graphing the PA against Linagliptin concentration (µg/ml).

Precision: To assess the precision, three replicate injections of concentrations of 2, 6, and 10 µg/ml were made. Linagliptin injections in three replicates at varying concentrations were used for the intraday and interday precision study, and the percentage RSD for PA was calculated.

Ruggedness: The suggested method's robustness was evaluated by having another analyst examine the Linagliptin standard solution. For each peak in the chromatogram, the overall mean and percentage RSD were determined.

Robustness: By purposefully altering the chromatographic conditions while injecting the standard Linagliptin solution, the robustness of the procedure was assessed. We made changes to the mobile phase composition and FRs in our suggested technique.

Accuracy: The addition method was used to determine accuracy. Three distinct concentrations of standard Linagliptin (80%, 100%, and 120% of the sample solution) were added to it. To further verify the accuracy of the data, recovery and percentage RSD were computed.

ASSAY OF LINAGLIPTIN IN TABLET DOSAGE FORMS

An analysis was conducted on a commercial tablet containing 50mg of linagliptin to determine the applicability of the proposed RP-HPLC technology. Three copies of the analysis were done, and the assay value as a percentage was given.

Preparation of sample solution for assay

The average weight of each of the twenty Linagliptin tablets was determined. The tablets were then crushed and ground into a fine powder. Powder equivalent to 5 mg of lignin was precisely weighed using a calibrated balance and transferred to a dry, clean, 10 mL calibrated flask in order to create the sample solution for assay analysis. After adding roughly 5 mL of mobile phase and giving it a good shake to remove the Linagliptin from the tablet excipients, the mixture was sonicated for 20 minutes to ensure full drug extraction. Using mobile phase as a diluent, the volume was adjusted to the appropriate level after the VF contents were cooled to room temperature following sonication. Following a filtering process using Whatman filter paper, the solution containing 1000µg/ml of linagliptin was obtained. This solution was subsequently diluted to provide a sample solution for quantitative determination that with a concentration of 5µg/ml of Linagliptin.

RESULTS

Using a combination of trial and error and a literature search, preliminary experiments were conducted to determine the feasibility of developing a straightforward, accurate, and exact RP-HPLC method for the evaluation and QC of Linagliptin in both bulk and pharmaceutical tablet dosage forms. The first step in the development process involved choosing the mobile phase using several solvent combinations, including o-phosphoric acid, Methanol, and ACN. To improve Linagliptin separation, the buffer phase is experimented with at various concentrations. In the initial investigation, it was shown that the choice of 0.2% concentration of o-phosphoric acid resulted in a more rapid and efficient drug separation, with drug elution occurring at a reduced RT and sufficient low peak tailing and peak symmetry. In **Figure 40**, the optimized chromatogram was displayed. **Table 41** displays the parameters of HPLC.

Table 41: Developed HPLC method specifications for Linagliptin

Sr. No.	Parameters	Specifications
1	Technique	Chromatographic separation
2	Machine	Shimadzu Prominence – *i* series LC-2030c 3-D plus (Autosampler)
3	Model	Shimadzu
4	Brand	Prominence
5	Processing Software	Lab solutions Version 5.97 SP1
6	Stationary Phase	Shim-pack GIST C18 column
7	Solvent ratio	ACN and 0.2% OPA (31.7: 68.3 v/v)
8	FR	0.96 mL/min
9	λmax	226nm
10	Injection Volume	20 µl
11	Analyte	Linagliptin
12	RT	4.2 min

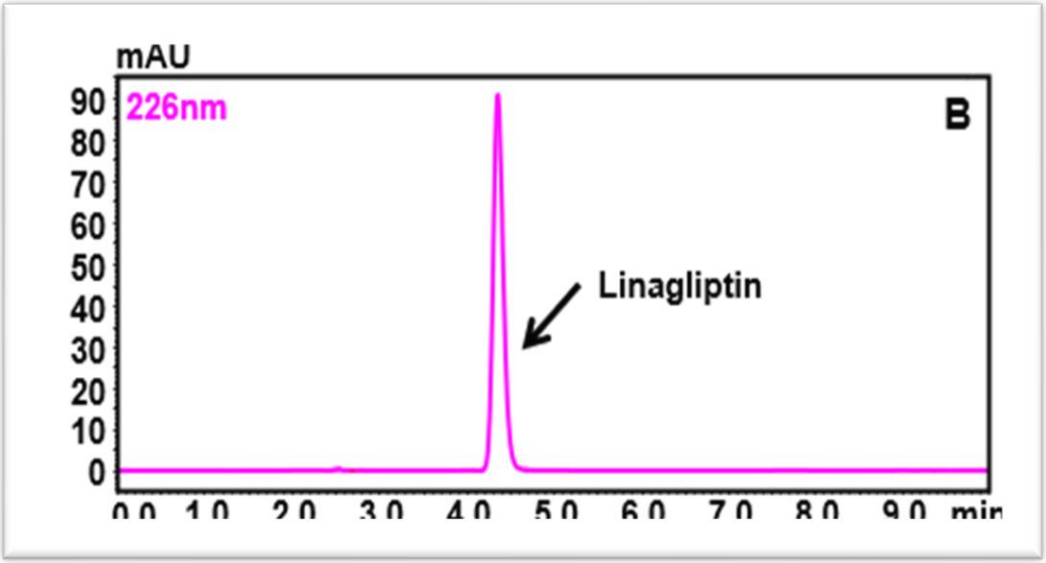


Figure 40: Optimized HPLC chromatogram of standard Linagliptin

OPTIMIZATION BY QBD APPROACH

Defining analytical profile

The requirements for the procedure that are anticipated to be measured are described in ATP. The analytical target profile is defined using the analytical process's scientific rationale and body of knowledge. The ATP for the suggested HPLC technique for linagliptin is shown in **Table 42.**

DoE optimization

The factorial design was employed in this investigation to optimize the chosen independent variables. The variables and their levels utilized in the 3^2 factorial design are compiled in **Table 43**. Three elements in the study the number of TP, the RT, and the TF are impacted by these independent variables. A total of 13 runs were obtained using the factorial design with this setup. **Table 44** displayed the 3^2 factorial design and the observed values. ANOVA results for the different responses are summarized in **Table 45.**

Regression analysis was examined for replies Y1, Y2, and Y3 in order to fit them to various polynomial models. Studies pertaining to SD, R2, adjusted and predicted R2, and %CV are included in the regression analysis. The R2 values of all three replies were high, ranging from 0.9831 to 1.000, indicating that the generated polynomials suited the response data the best. Less than 0.2 separated the modified R2 value from the projected R2 value, and these values' proximity to the real model R2 further supported the data's excellent fit. The quadratic model was suggested by the regression analysis." **Figure Table 46** provided the statistical analysis of the same. Throughout the experimental design process, response surface diagrams (3-D graphs) were generated to explore the interactions among the variables (Figures 41a-c). Optimal conditions were identified where maximum TP (TP), minimized RT (RT) of the analyte, and a TF (TF) closer to one were achieved at intermediate levels of the selected independent variables. Notably, the lowest RT was observed at the ideal concentration of 25% v/v, pH 4.92 of the buffer, and a FR of 1 mL/min. These findings unequivocally indicate the significant impact of the independent parameters chosen for optimization on the dependent variables. The application of the analytical Quality by Design (QbD) philosophy facilitated the optimization of method parameters, utilizing a factorial design method that incorporated variables and their respective levels.

Table 42: ATP for HPLC analysis of Linagliptin

Sl. No.	ATP Parameters	Target
1	Target Analyte	Linagliptin
2	Target Sample	Linagliptin Tablets
3	Method category	Chromatographic
4	Instrument	HPLC
5	Nature of analyte	Solid (Solution)
6	Standard stock solution	Dilution of main drug in linear manner
7	Application of Method	Estimation of Linagliptin
8	Validation parameters	Specificity, System suitability, Linearity, Range, Precision, Accuracy, Ruggedness, Robustness, Selectivity and Sensitivity

Table 43: Levels of 3^2 factorial design for HPLC analysis of Linagliptin

Factor	Levels used, actual (coded)		
	Low (-1)	Medium (0)	High (+1)
Independent variables			
X_1: Solvent concentration in mobile phase (% v/v)	25	30	35
X_2: Flow rate (ml/min)	0.80	1.00	1.20
Dependant variables			
Y_1: Number of theoretical plates	Maximize		
Y_2: Retention time	In the range		
Y_3: Tailing factor	Minimize		

Table 44: 3^2 factorial design and observed values for optimization for Linagliptin

Run No	Independent Variables				Dependent variables		
	X_1	X_2	X_1 (% v/v)	X_2 (ml/min)	Y_1	Y_2	Y_3
1	-1	+1	25.00	1.20	7445	7.223	1.065
2	-1	0	25.00	1.00	8522	8.625	1.077
3	+1	-1	35.00	0.80	5805	4.280	1.239
4	-1	-1	25.00	0.80	9962	10.721	1.090
5	+1	0	35.00	1.00	4801	3.440	1.233
6	0	0	30.00	1.00	6320	4.839	1.145
7	0	0	30.00	1.00	6306	4.839	1.141
8	+1	+1	35.00	1.20	4054	2.883	1.231
9	0	0	30.00	1.00	6310	4.834	1.147
10	0	-1	30.00	0.80	7543	6.017	1.154
11	0	0	30.00	1.00	6322	4.839	1.144
12	0	0	30.00	1.00	6301	4.838	1.145
13	0	+1	30.00	1.20	5412	4.037	1.142

X_1 Solvent concentration in mobile phase (% v/v), X_2 Flow rate (ml/min),

Y_1 Number of theoretical plates, Y_2 Retention time and Y3 Tailing factor

Table 45: ANOVA and adequate precision for various response for Linagliptin

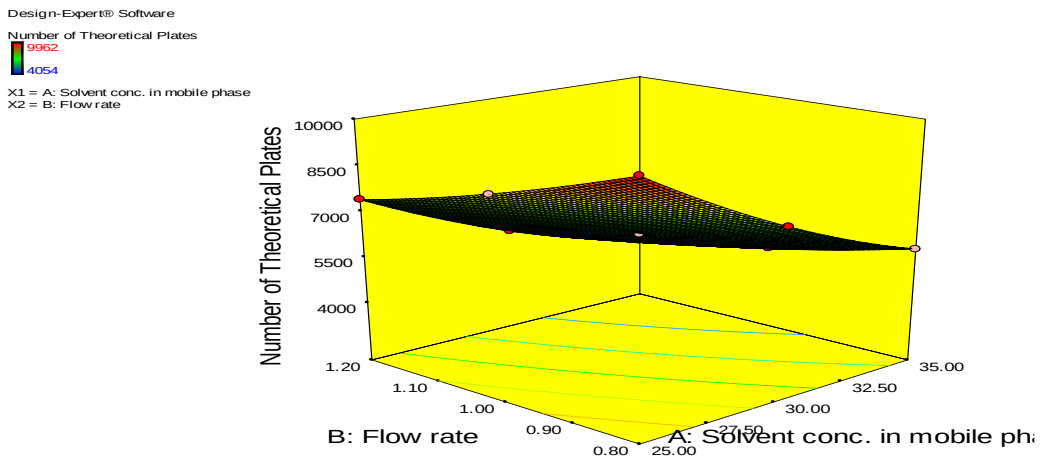
Source	Y_1 (Number of theoretical plates)		
	F-value	**p-value Prob > F**	**Adequacy precision**
Model	29956.90	< 0.0001	625.833
X_1	1.103E+005	< 0.0001	
X_2	35568.48	< 0.0001	
X_1X_2	764.52	< 0.0001	
X_1^2	1708.70	< 0.0001	
X_2^2	370.97	< 0.0001	
Source	**Y_2 (Retention time)**		
	F-value	**p-value Prob > F**	**Adequacy precision**
Model	897.58	< 0.0001	99.369
X_1	3340.15	< 0.0001	
X_2	619.32	< 0.0001	
X_1X_2	86.76	< 0.0001	
X_1^2	323.86	< 0.0001	
X_2^2	10.11	0.0155	
Source	**Y_3 (Tailing factor)**		
	F-value	**p-value Prob > F**	**Adequacy precision**
Model	1732.29	< 0.0001	121.272
X_1	8483.32	< 0.0001	
X_2	77.44	< 0.0001	
X_1X_2	16.58	0.0047	
X_1^2	56.78	0.0001	
X_2^2	3.85	0.0905	

X_1, *X_2* coded levels of independent variables,
X_1X_2 interaction term,
X_1^2, *X_2^2* quadratic terms

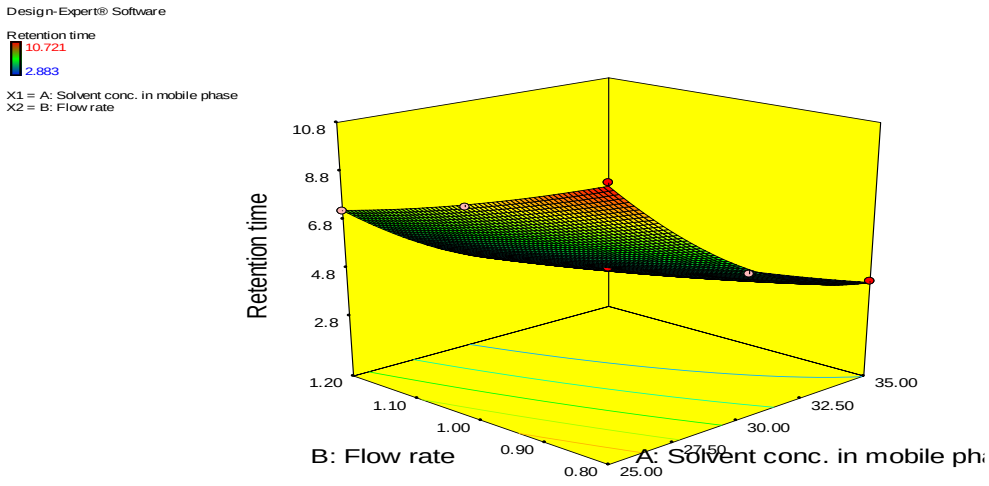
$Y_1 = + 6313.28 -1878.17\ X_1 - 1066.50X_2 + 191.50\ X_1X_2 + 344.53X_1^2 + 160.53\ X_2^2$
$Y_2 = + 4.83 - 2.66X_1 - 1.15X_2 + 0.53X_1X_2 + 1.22X_1^2 + 0.22X_2^2$
$Y_3 = +1.14 + 0.079\ X_1 - 7.500E\text{-}003X_2 + 4.250E\text{-}003\ X_1X_2 + 9.466E\text{-}003\ X_1^2 + 2.466E\text{-}003\ X_2^2$

Table 46: Data for regression analysis for HPLC analysis of Linagliptin

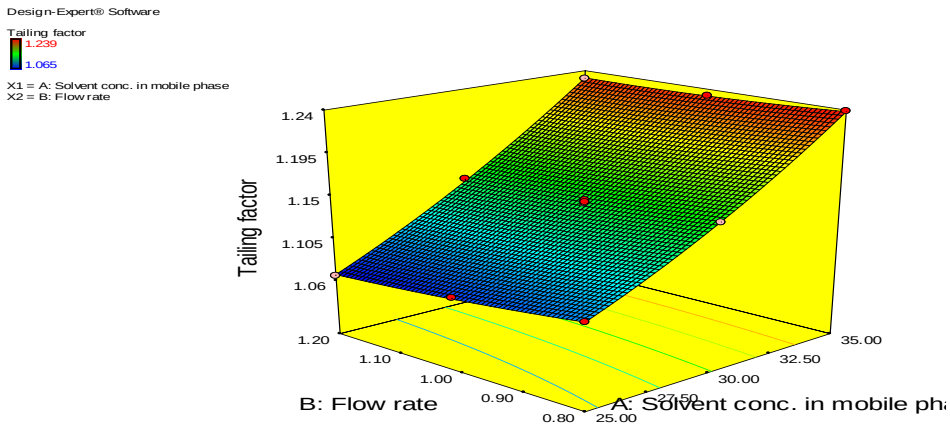
Models	SD	R^2	Adjusted R^2	Predicted R^2	PRESS	CV (%)	Remark
Response (Y_1)							
Linear	274.03	0.9739	0.9686	0.9426	1.649E+006	4.19	
2FI	259.11	0.9790	0.9720	0.8985	2.918E+006	3.96	
Quadratic	13.85	1.0000	0.9999	0.9996	10576.28	0.21	Suggested
Response (Y_2)							
Linear	0.83	0.8809	0.8571	0.7427	14.71	15.02	
2FI	0.80	0.9002	0.8669	0.5468	25.91	14.50	
Quadratic	0.11	0.9984	0.9973	0.9843	0.90	2.05	Suggested
Response (Y_3)							
Linear	6.851E-003	0.9876	0.9851	0.9746	9.593E-004	0.60	
2FI	6.642E-003	0.9895	0.9860	0.9575	1.605E-003	0.58	
Quadratic	2.088E-003	0.9992	0.9986	0.9965	1.304E-004	0.18	Suggested



41a: Effect of FR and solvent concentration in mobile phase on TP



41.b: Effect of FR and solvent concentration in mobile phase on RT



41c: Effect of FR and solvent concentration in mobile phase on TF

Figure 41: 3-D-response surface graphs for Linagliptin Showing effect of independent variable on dependant variables

Optimized solution

The resultant solution, which was eluted at a FR of 0.9 mL/min and contained ACN and 0.2% OPA (32: 69 v/v), produced acceptable values with all of the CAAs in the target range (**Table 47**). The design space for optimal parameters is shown in **Figure 42.**

Table 47: Optimized solution for Linagliptin HPLC analysis

Organic solvent	FR	Wavelength	RT	PA	TP	TF
31.70%	0.96 ml	226	4.2	82441	5896	1.12

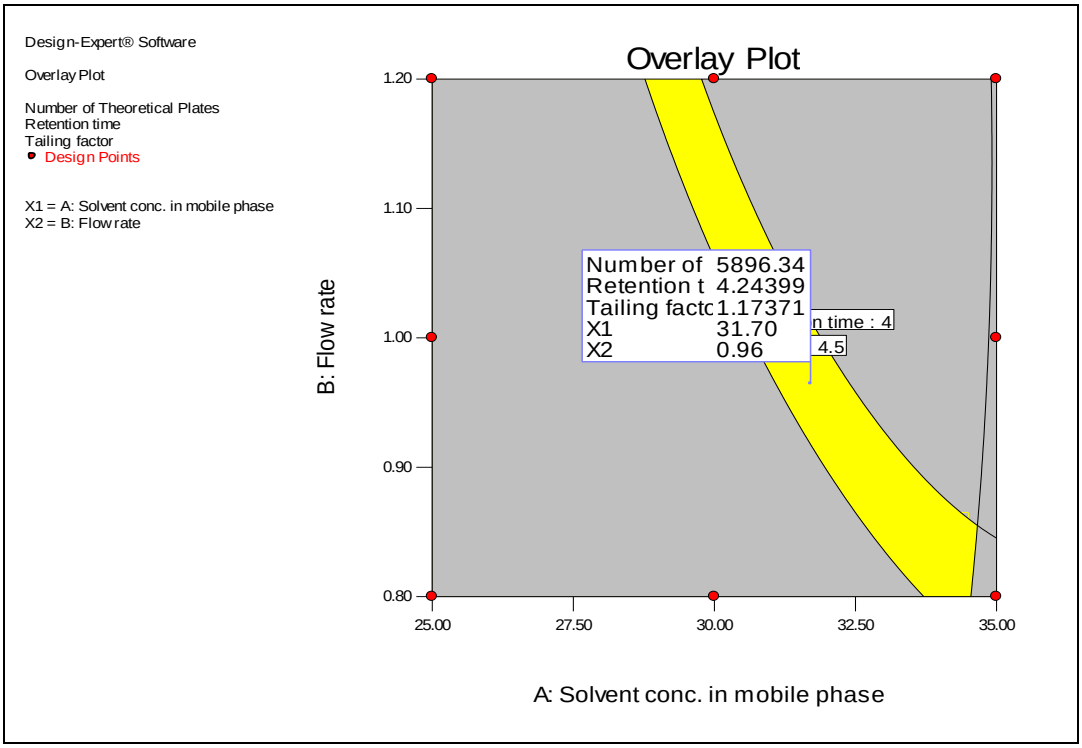


Figure 42: Design space of optimized parameters for Linagliptin by HPLC

METHOD VALIDATION

System suitability: Excellent %RSD for chromatographic parameters such as retention duration, PA, TF, and theoretical plate counts were achieved from the analytical investigation of the system suitability experiment. Each parameter's %RSD value was determined to be within 2%, indicating a greater level of accuracy and applicability for the HPLC method's analysis of Linagliptin (**Table 48**).

Table 48: System suitability data of Linagliptin by HPLC method

Run	RT	PA	TP	TF
1	4.363	460551	1.171	5897
2	4.362	460095	1.173	5912
3	4.362	459827	1.174	5931
4	4.361	459983	1.176	5912
5	4.358	460077	1.176	5900
6	4.359	459906	1.178	5909
Average	**4.360**	**460073**	**1.174**	**5910**
STDEV	**0.001**	**255.142**	**0.002**	**11.99**
%RSD	**0.044**	**0.05545**	**0.213**	**0.202**

Specificity and Selectivity: Figure 43 displays the standard chromatograms of Linagliptin and the sample solution derived from Linagliptin. It is evident that the RT of the standard Linagliptin and its sample preparation remained same, and no additional peaks were seen in the blank chromatogram. As a result, the finding highlights the analytical method's exceptional selectivity and specificity.

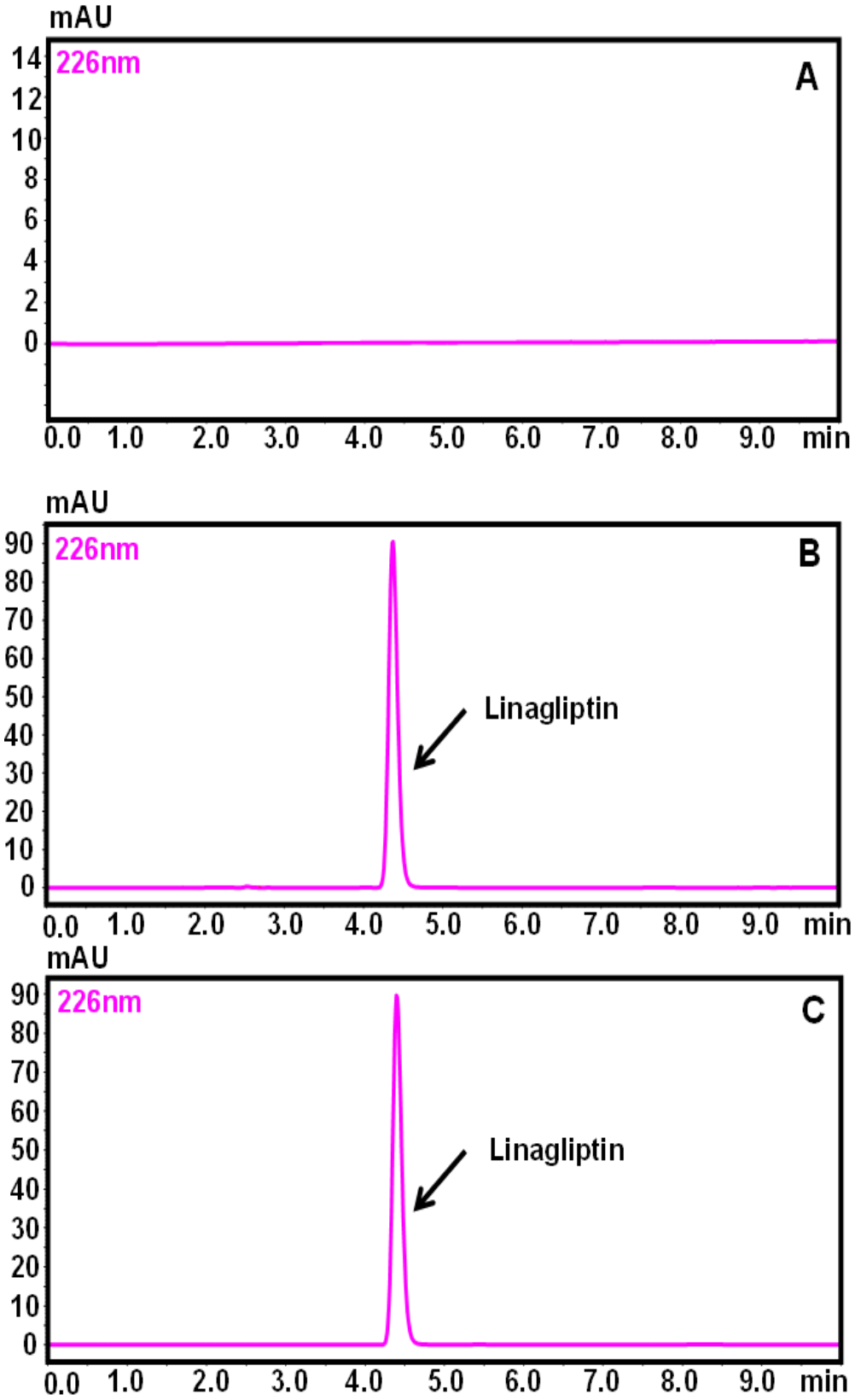


Figure 43a-c: Chromatogram of A) Blank, B) Standard C) Sample Linagliptin

Linearity and range: By creating a standard calibration curve that graphed the quantity of linagliptin (µg/ml) against the PA observed in chromatograms, the linearity of the suggested HPLC method was examined (**Figure 44**). With a r^2 of 0.9995, the technique appears to be linear (**Table 49**).

Table 49: Linearity and range data of Linagliptin by HPLC method

Concentration	I	II	III	Mean Area
2 µg/ml	136265	133852	139127	136414
4 µg/ml	278050	279864	267823	275245
6 µg/ml	459952	424023	434137	439370
8 µg/ml	601581	600454	549019	583684
10 µg/ml	740634	726211	735068	733971
12 µg/ml	901449	870147	843493	871696
	y = 74241x – 12959		**r^2 = 0.9995**	

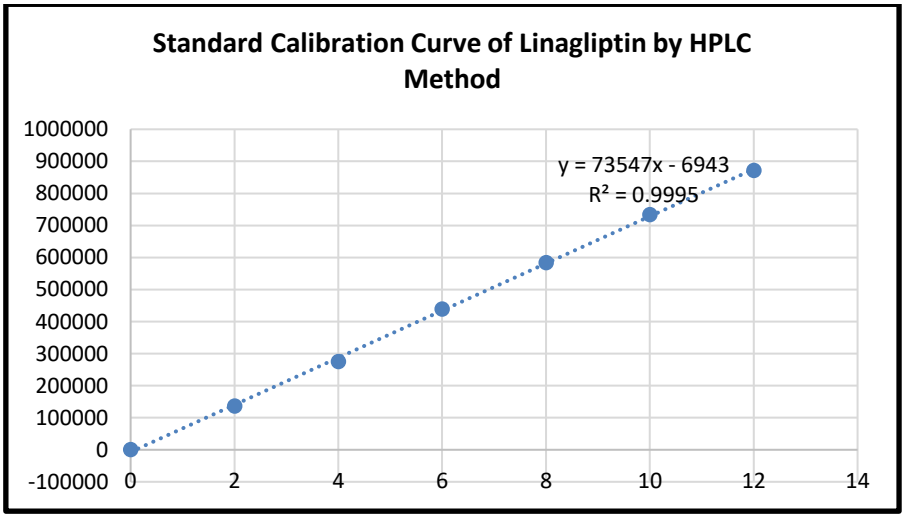


Figure 44: Calibration curve of Linagliptin by RP-HPLC method

Detecting limit and quantifying limit: The LOD and LOQ values of linagliptin were determined to be 0.307µg/ml and 0.932µg/ml, respectively. The findings show that the new RP-HPLC analytical method has a higher sensitivity for accurate detection and quantification.

Precision: The precision data is displayed in **Table 50**. The results indicate that the % RSD of the linagliptin peak regions was considerably under 1% for both the intraday and interday precision levels, indicating the significant precision of the RP-HPLC technology.

Ruggedness: Different analyst tested the method's robustness, and the results were determined to be satisfactory. **Table 51** shows the ruggedness of the suggested technique (%RSD of PA <2%).

Robustness: It was studied by varying the FR and mobile phase composition on a small scale and looking at the modest differences in TF, PA, and RT. The robustness of the approach is demonstrated by the data in **Table 52.**

Accuracy: For the proposed HPLC technique of Linagliptin, the reference analyte solution was spiked at 3 different levels: 80 %, 100 %, and 120 %, and the percentage recovery ranged from 99.68 to 100.44%, indicating an excellent high level of accuracy with a percent RSD of 0.03 to 0.05. **Table 53** shows the outcomes of accuracy data.

ASSAY OF LINAGLIPTIN IN TABLET DOSAGE FORMS

To assess the applicability of the proposed RP-HPLC technology, a commercial tablet containing 50mg of Linagliptin was tested. When marketed tablets were analysed, the results were 98.45±0.24% Linagliptin. The assay findings showed good compliance with the tablet's label claim.

Table 50: Precision data of Linagliptin by HPLC method

Precision	**Intraday Precision**	**Inter day Precision**		
Levels		**Day 1**	**Day 2**	**Day 3**
Content	% RSD	% RSD	% RSD	% RSD
2.00 µg/ml	0.07	0.02	0.05	0.05
6.00 µg/ml	0.11	0.05	0.02	0.06
10.00 µg/ml	0.05	0.03	0.02	0.02

Table 51: Ruggedness data of Linagliptin by HPLC method

Ruggedness Type	**Concentration**	**Mean PA**	**SD**	**%RSD**
Change in Analyst	2 µg/ml	132536.29	530.30	0.40
	6 µg/ml	441364.83	479.57	0.11
	10 µg/ml	706078.73	5633.01	0.80

Table 52: Robustness study data of Linagliptin by HPLC method

Parameters	**Modification**	**% RSD of PA**	**% RSD of RT**	**TF (T)**	**Percentage recovery**
FR	0.86 mL/min	0.03	0.12	1.17	111.63
	Optimized	0.09	0.15	1.17	100.00
	1.06 mL/min	0.06	0.11	1.16	91.32
Mobile phase composition	30.7 % ACN	0.08	0.06	1.15	100.33
	Optimized	0.09	0.15	1.17	100.00
	32.7 % ACN	0.04	0.10	1.18	100.45

Table 53: Accuracy data of Linagliptin by HPLC method

Levels (%)	**Sample µg mL^{-1}**	**Standard (µg)**	**Theoretical quantity (µg)**	**Recovered quantity (µg)**	**Recovery (%)**	**% RSD**
80	4	2	6	6.58	100.68	0.03
100	4	4	8	8.28	100.37	0.04
120	4	6	10	10.90	100.03	0.05

DISCUSSION

QbD analysis-based method for Linagliptin estimation in pharmaceutical preparations has been developed using HPLC. The RT, TP, and TF were incorporated in the ATPP for the Linagliptin HPLC analysis. Two important technique factors that were shown to have an impact on the ATPP are the composition of the mobile phase and its FR. The factorial design was utilized in order to maximize the designated independent variables. Two independent variables were coded at three distinct levels: low, medium, and high, indicated by the signs (-1), (0), and (+1), respectively, for the solvent concentration in the mobile phase and the mobile phase FR.[36] The number of TP, RT, and TF are the three study factors that are influenced by these independent variables. The QbD method was effectively applied in the creation of Linagliptin's HPLC procedure. A Shim-pack GIST C18 column was used to elute linagliptin at a FR of 0.96 mL/min using ACN and 0.2% OPA. The method demonstrated a linear relationship between 2 and 12 µg/ml, with a correlation coefficient of 0.999. The spiked samples' % recovery ranged from 100.03±0.05 to 100.68±0.03, satisfying ICH regulations. The ICH recommendations were followed in developing the strategy.

CONCLUSION

An experimental screening and optimization design was used along with a systematic QbD methodology to evaluate the quality of linagliptin using the HPLC method. Factorial design was used to optimize the composition of the mobile phase and FR; design expert software was used to generate the counter graphs and statistical computations. After a thorough analysis, the optimized procedure was confirmed. The ultimate technique was discovered to be a strong analytical approach that utilized very little organic solvent and required less time to produce. It also effectively separated materials. A comprehensive approach using the QbD methodology for Linagliptin is given, and the method is found to be robust for Linagliptin analysis. The fully validated method can be used effectively to check the quality of Linagliptin and its tablet formulation during pharmaceutical manufacturing.

METHOD 5: DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR ESTIMATION OF SITAGLIPTIN

MATERIALS AND METHODS

Chromatographic Conditions

An Agilent 1220 (Autosampler) HPLC system with Chemstation software was employed in the suggested study. Through chromatographic separation, sitagliptin was isolated on an Agilent C18 column. The solvent combination consisted of MW: Methanol in a 55:45 v/v ratio with 0.1% orthophosphoric acid. At 0.8 mL/min, sitagliptin was eluted and detected by UV at 267 nm. The injection volume was 10 µL, and the column temperature was maintained at room temperature. Before utilizing chromatographic grade water to formulate the solvent system composition, it underwent filtration using 0.45 µm nylon membrane filters.

METHOD DEVELOPMENT

To optimize a simple, cost-effective approach for assessing and quantifying Sitagliptin in its pharmaceutical tablet dose form, early tests were conducted using a literature search and trial and error. Initially, work began by selecting a mobile phase using various solvent combinations such as acetonitrile, Methanol, and MW with varying proportions of orthophosphoric acid.

Identification of the detecting wavelength

The Sitagliptin solution was scanned in the 200-400 nm range to find the λmax for analysis. The UV spectra of the standard medication exhibited a maximum absorption at 267nm. As a result, it served as the detecting wavelength.

Standard stock solution

To prepare the 1000 µg/ml standard stock solution, 10 milligrams of Sitagliptin were meticulously dissolved in 10 mL of the mobile phase diluent. This stock solution was then diluted to a sub-stock concentration of 100 µg/ml and 10 µg/ml solution.

METHOD OPTIMIZATION BY QUALITY BY DESIGN APPROACH

Following steps were applied for optimization of developed method based on Analytical QbD approach: [37]

Establishing analytical target profile

The development of ATP is critical for defining the factors required to ensure the quality and purpose of an analytical method. the nature of analyte, samples, and diverse analytical procedures are all part of the target profile for effective method development.

Determination of critical analytical attributes

To meet the requirements of the analytical procedure's target profile, distinct CAAs must be selected, which is a critical stage in the development of AQbD-based methods. It was established after a thorough examination of the literature.

Identification of critical method parameters and risk identification

The CMPs for developing an RP HPLC method include the percentage of organic modifier in the mobile phase, the pH of the mobile phase, the column temperature, the flow rate, and the injection volume. An Ishikawa fishbone diagram was created to identify potential threats to the performance of RP-HPLC **Figure 8**.

Application of Design of experiment approach

For DOE selection, the central composite experimental design was used to optimize and choose two critical components: the percentage organic phase modifier and the flow rate of HPLC technique. The central composite statistical screening strategy was used to investigate the various interaction and quadratic impacts of the percent organic phase modifier and flow rate on retention duration, peak area, theoretical plates, and tailing factor. A 2-factor design with % organic phase modifier and flow rate at three stages was used, with Design Expert® (Version 11.0, Stat-Ease Inc.) providing the optimum result for second-order polynomial exploration of quadratic response surfaces. $\mathbf{Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_{12} AB + \beta_{11} A_2 + \beta_{22} B_2 B + \beta_{22} B_2 A + \beta_{11} A_2}$ Were A and B are independent variables coded for levels, Y is the measured response associated with each combination of factor level, β0 is an intercept, and β1–β22 are regression coefficients calculated from the observed experimental values of Y. Interaction and quadratic terms denote the terms AB, A2, and B2. Because multivariable interactions of variables and process parameters have been investigated, the factors were chosen based on preliminary investigation. **Table 54** shows the independent variables, which are % organic phase modifier and flow rate. Retention time, peak area, theoretical plates, and tailing factor served as dependent factors for the suggested independent variables. [43]

Table 54: Coded values for independent variables as per CCD for Sitagliptin analysis by HPLC method

Independent Variable	Levels		
	+1	**0**	**-1**
Organic Modifier (%)	**50**	**55**	**60**
Flow rate (ml)	0.8	0.9	1.0

Evaluation of experimental results and selection of final method conditions

The CCD approach was used to examine these method conditions. The initial phase involved evaluating the circumstances for retention duration, peak area, theoretical plates, and tailing factor. This produced unique chromatographic conditions for sitagliptin. The proved acceptable ranges from resilient zones in which deliberate modifications in procedure parameters have no effect on quality. This ensures that the method does not fail during subsequent validation testing. If the modeling experiments do not produce the intended results, the variable must be tuned at various levels until the responses fall within acceptable limits. The Design Expert tools will be used to optimize the most appropriate chromatographic conditions.

ANALYTICAL METHOD VALIDATION [150]

System suitability: 6 replicate analyses of standard Sitagliptin (7 µg/ml) were conducted to determine system appropriateness. The standard deviation and % RSD of PA, TF, RT, and TP were calculated.

Specificity and selectivity: Test selectivity and specificity by injecting 10µl of working standard, sample, and blank solution separately and recording the chromatograms.

Linearity and range: The linearity was assessed by serially diluting of Sitagliptin stock solution from 7 to 35 µg/ml and graphing the calibration curve of PA versus Sitagliptin concentrations. The method's linearity was proved by running a regression statistical analysis.

Precision: The precision was tested with three distinct dosages of Sitagliptin (7 µg/ml, 21 µg/ml, and 35 µg/ml) at different time intervals on the same day and consecutive days. I calculated the percentage RSD for all data.

Ruggedness: The suggested method was tested for ruggedness by testing standard Sitagliptin solutions (7 µg/ml, 21 µg/ml, and 35 µg/ml) in triplicate using different analyst. The overall mean and percentage RSD for each PA were calculated using the chromatogram.

Recovery: The accuracy was calculated using the addition method. A sample of Sitagliptin (5 µg/ml) was spiked with 50% (7 µg/ml), 100% (14 µg/ml), and 150% (21 µg/ml) of the standard dose. Furthermore, recovery and % RSD were calculated to assure data accuracy.

ESTIMATING SITAGLIPTIN IN MARKETED TABLETS

The method described here was used to measure Sitagliptin in marketed tablets. The analysis was done in triplicate and the percentage assay value was provided.

Preparation of sample solution: Twenty tablets were weighed, and the average weight of the tablets was computed. The tablets were ground into a fine powder using a mortar and pestle. Tablet powder containing 10 mg of Sitagliptin was carefully weighed and placed to a 100 mL clean and dry volumetric flask. Then, 70 mL of diluent was added and sonicated for 15 minutes. To achieve a concentration of 100 µg/ml, add diluent to the desired volume. To acquire the final concentration of 10 µg/ml, 1 mL of the aforesaid sample stock solution was pipetted into a 10 mL volumetric flask and diluted with diluent. The solution was then filtered through a 0.45 µ Millipore nylon filter.

RESULTS

METHOD DEVELOPMENT

The preliminary tests revealed that Methanol and 0.1% OPA in MW would be ideal mixes for the mobile phase, as well as lower peak tailings. **Figure 45** shows the UV spectrum of standard Sitagliptin. **Figure 46** shows the optimized chromatogram. The optimized technique parameters are shown in **Table 55.**

Table 55: Developed HPLC method specifications for Sitagliptin

Sr. No.	Parameters	Specifications
1	Technique	Chromatographic separation
2	Machine	HPLC
3	Model	Agilent
4	Brand	Agilent 1220
5	Processing Software	Chemstation possessing
6	Stationary Phase	C_{18} column
7	Solvent ratio	Methanol: 0.1 % OPA in MW (55:45 v/v)
8	FR	0.9 ml
9	λmax	267 nm
10	Injection Volume	10 µL
11	Analyte	Sitagliptin
12	RT	4.8 min

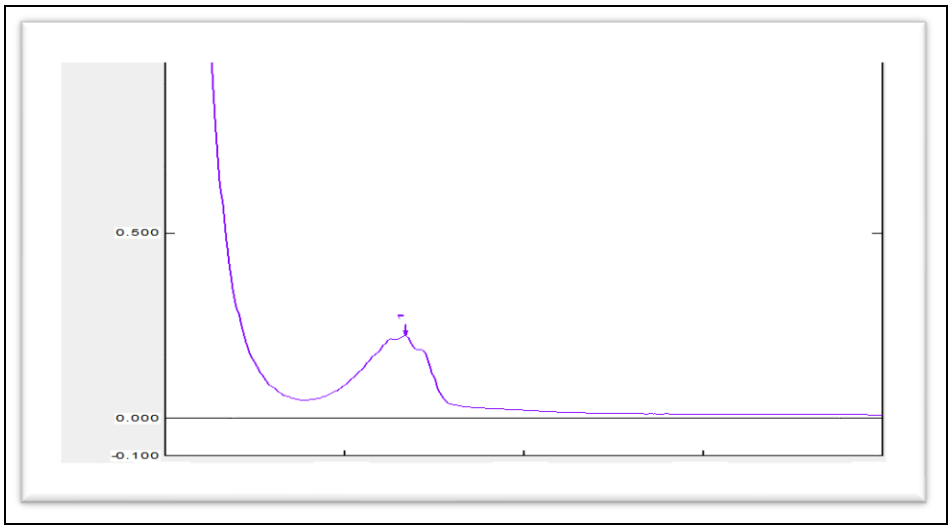


Figure 45: UV Spectrum of standard Sitagliptin

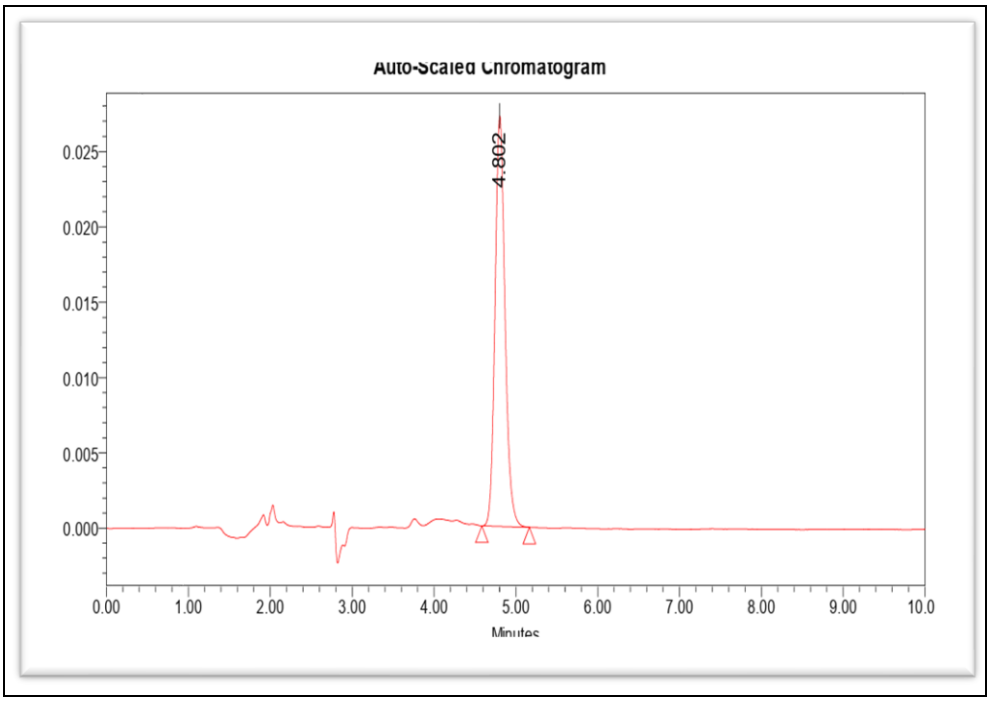


Figure 46: Optimized HPLC chromatogram of standard Sitagliptin

OPTIMIZING THE HPLC METHOD WITH QBD STRATEGY

Establishing target profile for analytical method

ATP defines the method requirements that are intended to be measured. The analytical target profile is defined using the information and scientific reasoning of the analytical process. **Table 56** depicts ATP for the suggested HPLC technique for Sitagliptin.

Determination of critical analytical attributes: In case of chromatographic development usually the critical quality attributes include the RT, TP, PA and TF peak spectral purity, and assay limit etc.

Identification of critical method parameters and risk identification: this investigation, an IFB diagram (**Figure 8**) was created to identify major risk variables that could affect the method's performance. The risk factors indicated may include instrumental parameters such as solvent system ratio, chromatographic mode, flow rate, and injection volume. The high-risk procedure variables were identified and subjected to further study using an appropriate experimental optimization design. The high-risk method variables used were flow rate of mobile phase and percentage composition of organic phase (methanol) in mobile phase.

Application of Design of experiment approach for method optimization: The central composite design was used to optimize various factors inside the design space. The CCD central composite design was chosen for the suggested HPLC technique development. **Table 57** shows the optimization of several parameters.

Design space: The response surface study type, central composite design, and quadric design model with 11 runs were utilized. The proposed CCD experimental design was used, and the percentage organic modifier and flow rate of the mobile phase were evaluated against the four responses, retention time, peak area, theoretical plates, and tailing factor, with the results presented. The quadratic model was used to analyze the data for both main and interaction effects. **Table 58** shows the optimized model parameters. The model generates a quadratic equation for each CAA parameter.

Figure 47a and equation retention time (for actual values) = 93.1047 - 1.90328A - 70.6476B + 0.6365 AB + 0.0110063 A2 + 17.9658 B2 suggest that as the amount of methanol in the mobile phase (A) decreases and the flow rate of the mobile phase (B) decreases, the value of retention time increases. Based on **Figure 47b** and the equation peak area (for actual values) = 9.07884 - 17032.6 A -771373 B + 6464.5 AB + 104.277 A2 + 196742 B2, it was concluded that as the amount of methanol in the mobile phase (A) decreases and the flow rate of the mobile phase (B) decreases, the value of peak area increases. Based on **Figure 47c** and the equation theoretical plates (for actual values) = -1339.76 + 70.6667 A + -96.6667 B, it was concluded that as the amount of methanol in the mobile phase (A) increases and the flow rate of the mobile phase (B) decreases, the value of theoretical plates increased. **Figure 47d** and equation tailing factor (for actual values) = 15.0577 + -0.38936 A + -4.24956 B + 0.045 AB + 0.00276842 A2 + 0.921053 B2, it was concluded that as the amount of methanol in the mobile phase (A) decreases and the flow rate of the mobile phase (B) decreases, the value of theoretical plates increased.

Table 56: ATP for HPLC analysis of Sitagliptin

Sl. No.	ATP Parameters	Target
1	Target Analyte	Sitagliptin
2	Target Sample	Sitagliptin Tablets
3	Method category	Chromatographic
4	Instrument	HPLC
5	Nature of analyte	Solid (Solution)
6	Standard stock solution	Dilution of main drug in linear manner
7	Application of Method	Estimation of Sitagliptin
8	Process Parameters	Specificity, System suitability, Linearity, Precision, Accuracy, Ruggedness, Selectivity and Sensitivity

Table 57: Optimization of parameters for analysis of Sitagliptin using CCD

		Factor 1	Factor 2
Std	Run	A: % Organic Modifier (%)	B: Flow Rate (ml)
5	5	50	0.9
3	7	50	1
1	11	50	0.8
7	1	55	0.8
11	3	55	0.9
9	6	55	0.9
8	8	55	1
10	10	55	0.9
6	2	60	0.9
2	4	60	0.8
4	9	60	1

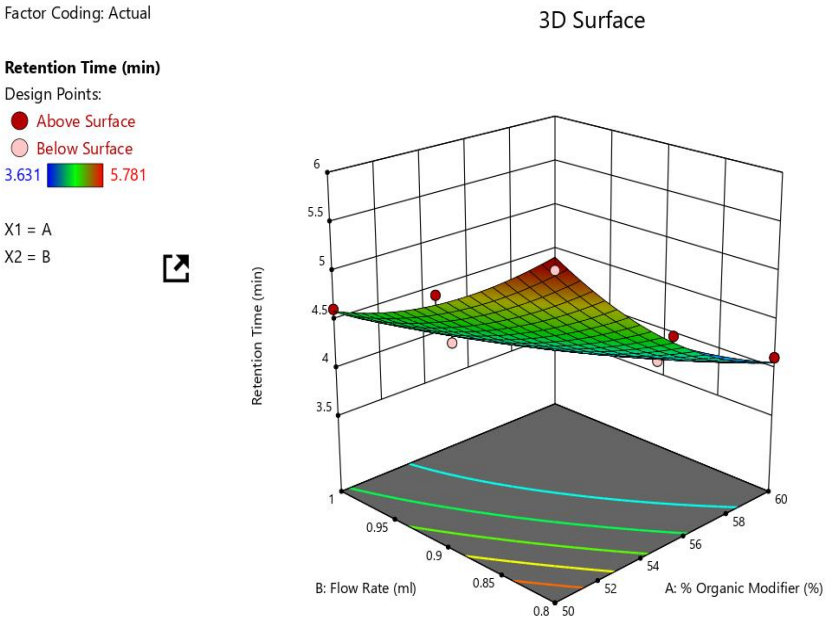


Figure 47a: 3D surface plot for effect of combination of factors on R1 retention time of Sitagliptin by using central composite design

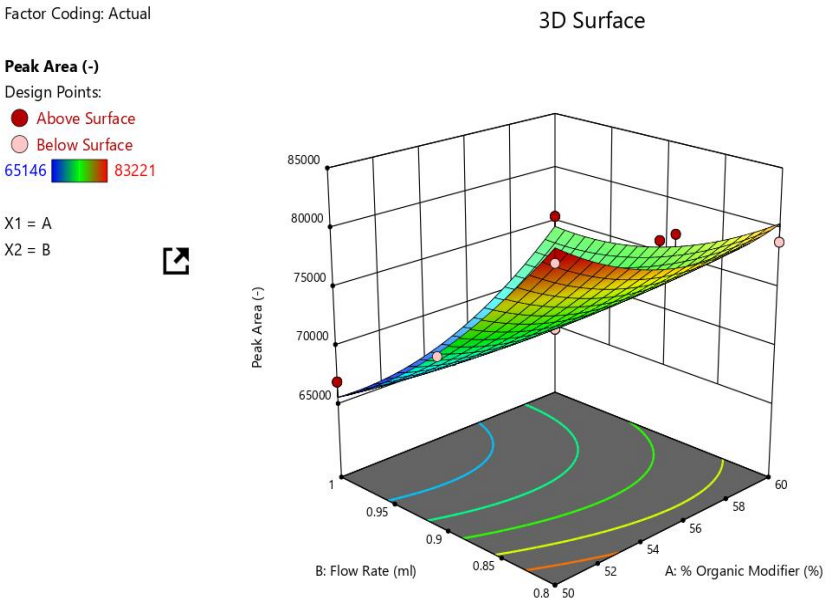


Figure 47b: 3D surface plot for effect of combination of factors on R2 peak area of Sitagliptin by using central composite design

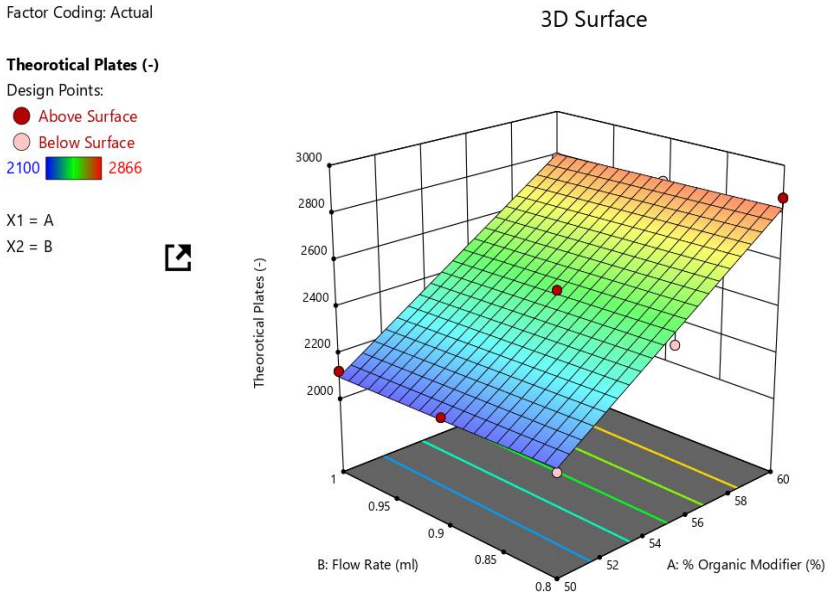


Figure 47c: 3D surface plot for effect of combination of factors on R3 theoretical plates of Sitagliptin by using central composite design

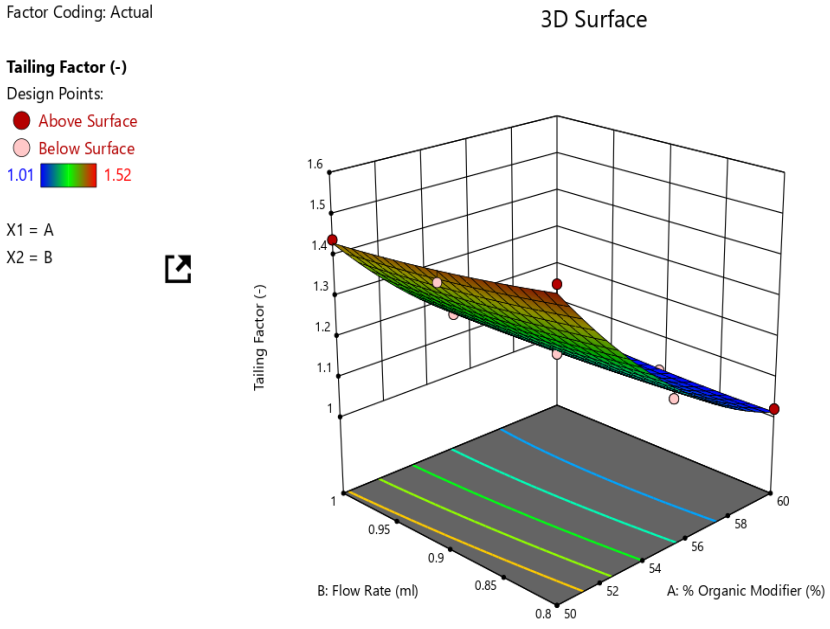


Figure 47d: 3D surface plot for effect of combination of factors on R4 tailing factor of Sitagliptin by using central composite design

Table 58: ANOVA responses of CCD for Sitagliptin HPLC method

Source	Sum of squares	df	Mean square	F-value	p-value	
For Retention Time						
Model	3.58	5	0.7152	20.66	0.0024	significant
A-Organic modifier	2.15	1	2.15	62.13	0.0005	
B-Flow rate	0.6541	1	0.6541	18.90	0.0074	
For Peak Area						
Model	3.165E+08	5	6.330E+07	16.53	0.0040	significant
A-Organic modifier	9.823E+06	1	9.823E+06	2.56	0.1702	
B-Flow rate	2.283E+08	1	2.283E+08	59.62	0.0006	
For Theoretical Plates						
Model	7.496E+05	2	3.748E+05	369.29	< 0.0001	significant
A-Organic modifier	7.491E+05	1	7.491E+05	738.04	< 0.0001	
B-Flow rate	560.67	1	560.67	0.5524	0.4786	
For Tailing Factor						
Model	0.3119	5	0.0624	110.99	< 0.0001	significant
A-Organic modifier	0.2948	1	0.2948	524.57	< 0.0001	
B-Flow rate	0.0008	1	0.0008	1.45	0.2820	

Optimized solution

The resulting solution revealed that a mobile phase composition of 55:45%v/v Methanol: 0.1% OPA in Millipore water and a flow rate of 0.8 ml/min produced desirability values with all CQAs in the desired range. **Table 59**, and **Figure 48** depicts the design space for optimal parameters.

Table 59: Optimized solution for Sitagliptin HPLC analysis

Organic modifier	FR	Wavelength	RT	PA	TP	TF
55%	0.8 ml	267	4.8	82441	2416	1.16

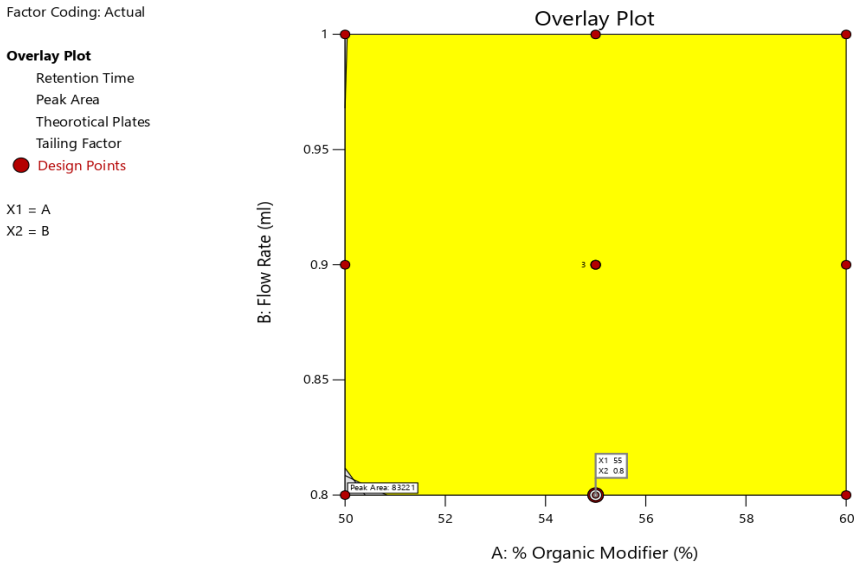


Figure 48: Design space of optimized parameters for Sitagliptin HPLC analysis

ANALYTICAL METHOD VALIDATION

System suitability: With six replicates, system suitability statistics demonstrate no significant variation in Sitagliptin peak area, retention time, theoretical plate, and tailing factor. Prior to doing the study, characteristics such as retention duration, peak area, theoretical plates, and tailing factor were assessed and %RSD was calculated. The RSD of all system suitability metrics was less than 2% (**Table 60, Figure 49**).

Specificity and Selectivity: The standard and sample HPLC chromatograms indicated a retention duration of 4.8 minutes (**Figures 50a-b**). The blank chromatogram exhibited no peak at Sitagliptin RT. There was no interference observed during the retention period of the Sitagliptin peak in the standard and sample chromatograms, which indicates methods specificity and selectivity.

Linearity and range: The linear calibration curve for Sitagliptin in concentrations ranging from 7 to 35 µg/ml demonstrated good linearity with a correlation coefficient of 0.998 (**Table 61, Figure 51**).

Sensitivity: The LOD and LOQ values for Sitagliptin were 0.023 µg/ml and 0.072 µg/ml, respectively. The acquired results demonstrate the excellent sensitivity of the RP-HPLC method.

Precision: The percentage RSD of intraday and interday precision for Sitagliptin was determined to be less than 2% after three consecutive measurements. These findings supported the high level of precision of the developed approach. **Table 62** shows the precision data for the procedure.

Ruggedness: Different analyst tested the procedure's reliability, and the results were judged to be acceptable. **Table 63** shows the robustness of the suggested technique (%RSD <2%).

Accuracy: **Table 64** shows that the developed approach is highly accurate, with percentage recovery ranging from 97.37±0.39 to 100.54±0.11% at all three levels and a % RSD value of less than 2%.

ESTIMATION OF SITAGLIPTIN IN MARKETED TABLETS

For the Sitagliptin label claim, the percentage test of drug content was found to be 99.20±0.38% (n=3). **Figure 52**, the assay result demonstrates the selectivity of the devised HPLC technique for Sitagliptin quantification in pharmaceutical dosage form.

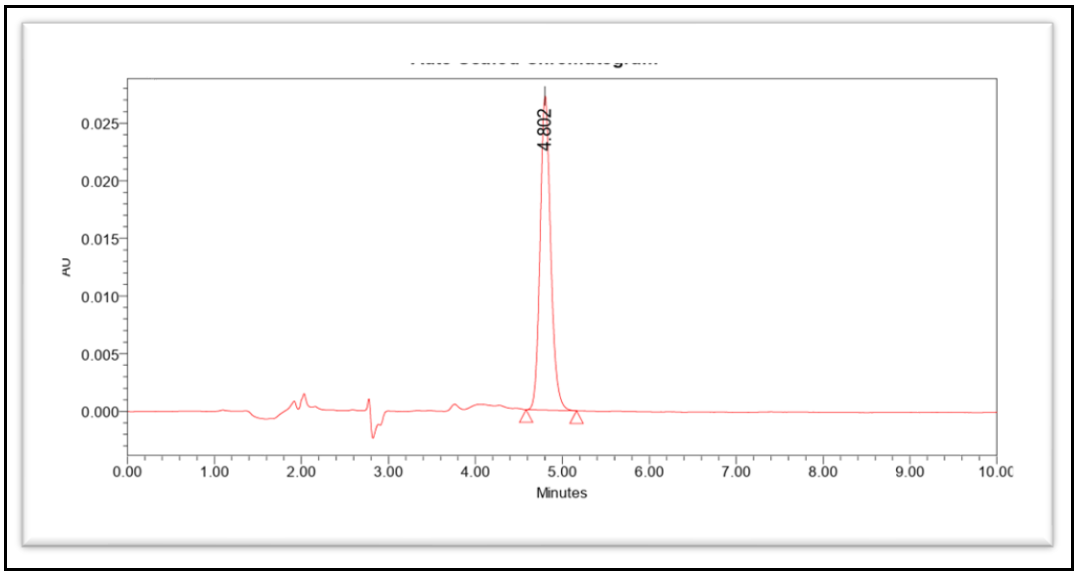


Figure 49: System suitability chromatogram of Sitagliptin

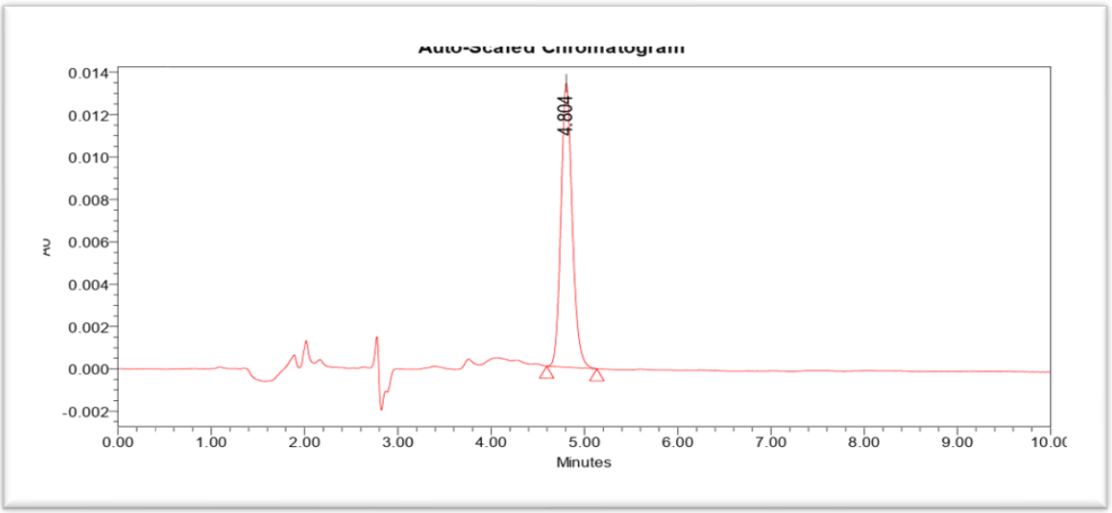


Figure 50a: Chromatogram of standard Sitagliptin

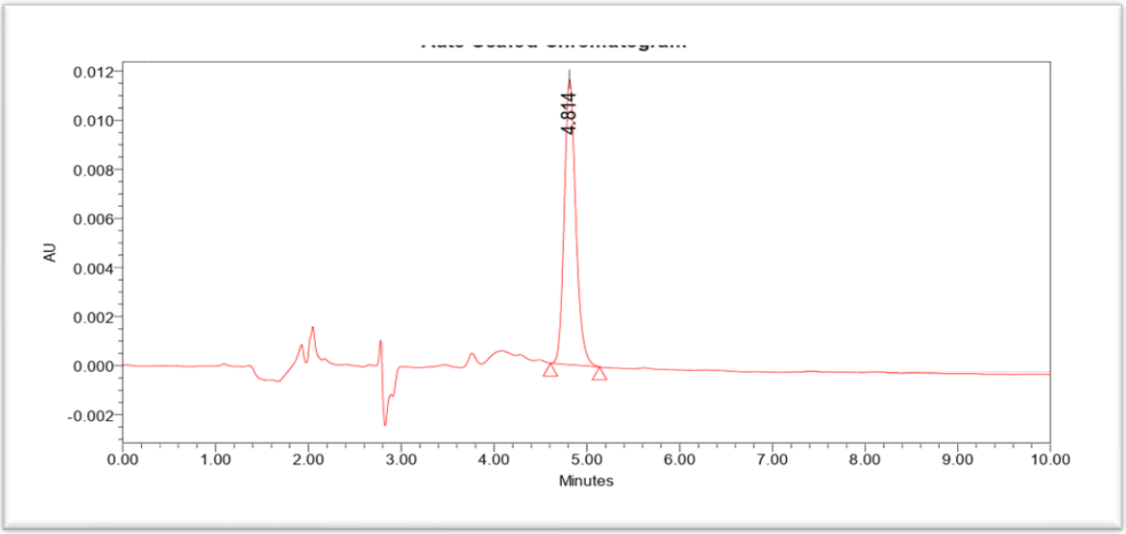


Figure 50b: Chromatogram of sample Sitagliptin

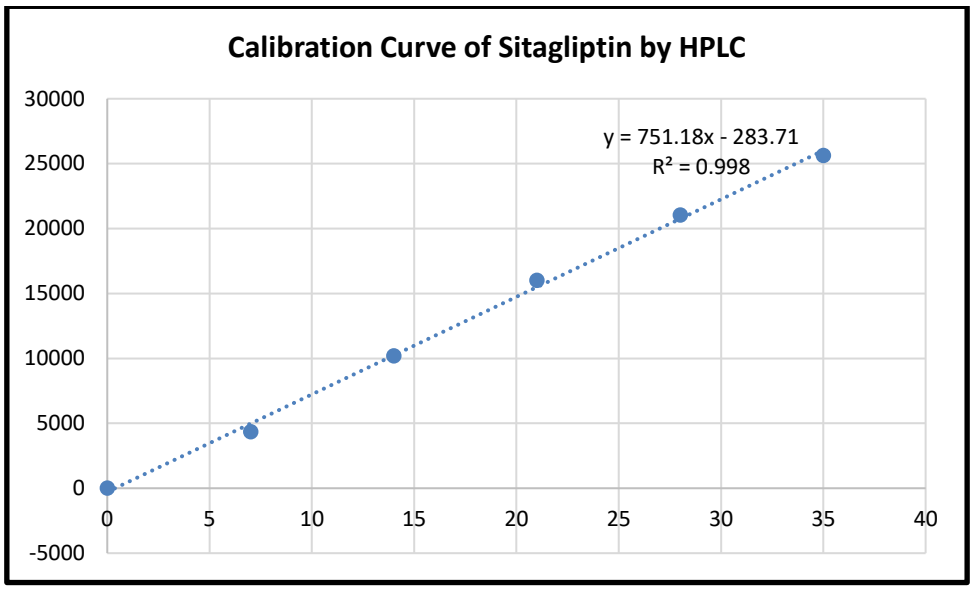


Figure 51: Calibration curve of Sitagliptin by HPLC method

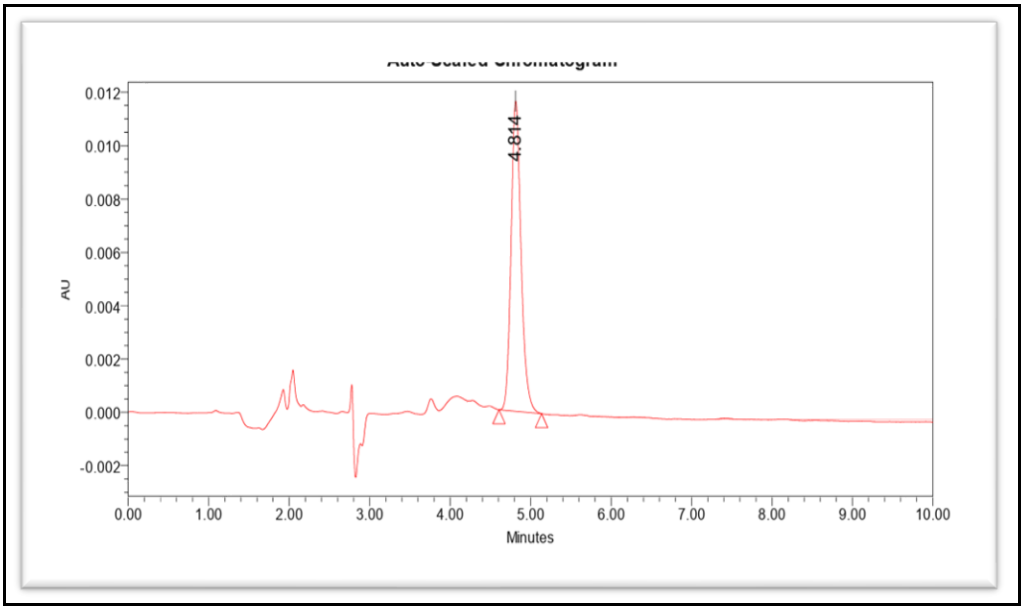


Figure 52: Assay chromatogram of Sitagliptin

Table 60: System suitability data of Sitagliptin by HPLC method

Parameter	Retention Time	Peak Area	Theoretical Plates	Tailing Factor
Mean	4.84	4368.87	3161.61	1.12
SD	0.04	36.51	20.37	0.01
%RSD	0.82	0.84	0.64	0.80

Table 61: Linearity and range data of Sitagliptin by HPLC method

Sl. No.	Parameters	Results
1	Linearity Range	7 to 35 µg/ml
2	Correlation coefficient (r^2)	0.998
3	Calibration Equation	y = 751.18x - 283.71
4	Slope	751.18
5	Intercept	283.71
6	Limit of Detection	2.27 µg/ml
7	Limit of Quantification	6.88 µg/ml

Table 62: Precision data of Sitagliptin by HPLC method

Type	Concentration	Mean PA	SD	%RSD
Intraday	7 µg/ml	4343.66	10.69	0.25
	21 µg/ml	16008.66	10.79	0.07
	35 µg/ml	25607.97	49.79	0.19
Interday	7 µg/ml	4519.60	30.60	0.68
	21 µg/ml	16141.56	70.44	0.44
	35 µg/ml	27845.67	164.68	0.59

Table 63: Ruggedness data of Sitagliptin by HPLC method

Ruggedness	Concentration	Mean PA	SD	%RSD
Change in Analyst	7 µg/ml	4545.14	51.32	1.13
	21 µg/ml	17692.97	191.33	1.08
	35 µg/ml	27417.39	493.29	1.80

Table 64: Recovery data of Sitagliptin by HPLC method

Levels %	Amount of test taken (µg/ml)	Amount of std. added (µg/ml)	Total amount (µg/ml)	Mean Amount Recovered (µg/ ml) ± SD	Mean % Recovery (µg/ ml) ± SD
50	5	7	12	12.01 ± 0.09	100.20 ± 0.67
100	5	14	19	19.10 ± 0.02	100.54 ± 0.11
150	5	21	26	25.32 ± 0.10	97.37 ± 0.39

DISCUSSION

For the purpose of estimating sitagliptin in pharmaceutical preparations, an analytical QbD HPLC approach has been devised. The two variables that were found to be critical method attributes that influence the CAAs are the mobile phase composition and the solvent phase FR. Using the Design Expert Software, the core composite design was applied for two factors at three separate levels. The key factors influencing the analytical target profile were determined by the risk assessment study. The HPLC technique for sitagliptin was successfully developed using the quality-by-design methodology. Agilent C18 column was utilized in the improved technique for Sitagliptin determination. The solvent system is made up of 55:45 v/v of Methanol, 0.1% orthophosphoric acid in MW. With UV identification at 267 nm, sitagliptin was eluted at a rate of 0.8 mL/min. It was discovered that sitagliptin had a RT of 4.8 minutes. With a correlation coefficient of 0.9980, the technique demonstrated linearity within the 7-35µg/ml range. The percentage recovery of the spiked samples was determined to be between 97.37±0.39 and 100.54±0.11%, meeting the ICH guidelines' acceptance criteria. The technique was successfully used for quantifying sitagliptin in marketed tablets after it was validated in accordance with ICH requirements.

CONCLUSION

The proposed research outlines a QbD approach to HPLC process development. The ATP plays a pivotal role in defining the method objectives. The experimental design delves into the exploration and characterization of the mobile phase and FR, both critical components of the HPLC technique. The Application of AQBD methodology is extended to optimize the Sitagliptin HPLC method.

METHOD 6: DEVELOPMENT AND VALIDATION OF UV-SPECTROPHOTOMETRIC METHOD FOR SITAGLIPTIN ESTIMATION

MATERIALS AND METHODS

Instruments and apparatus

The analyte was weighed using a weighing balance (SARTORIUS), absorbance was detected using a UV-Spectrophotometer (Shimadzu 1800), and sonication was accomplished using an Ultrasonic bath Sonicator.

METHOD DEVELOPMENT

The development of the UV-spectrophotometric method began with the selection of a solvent solution and the determination of the analytical wavelength. Dapagliflozin's solubility was investigated in a variety of solvents, and it was discovered to be highly soluble in Methanol. As a result, we used Methanol as the first stock in the dilution process. We used Methanol: Distilled Water (DW) (50:50% v/v) as a solvent system to create the secondary stock solution and working standards for Dapagliflozin. The solvent system was optimized based on DoE software and QbD principles.

Selection of solvent and wavelength of analysis

To determine the analysis wavelength, a solution with a concentration of 10 micrograms/ml underwent scanning in the ultraviolet (UV) region, ranging from 200 to 400 nms. Upon examination of the resulting spectrum, it was observed that the wavelength with the highest absorption occurred at 226 nm.

Preparation of standard solutions

A standard stock solution of Dapagliflozin was prepared at a concentration of 1000 micrograms/ml by dissolving 10 mg of the substance in a 10 ml volumetric flask. The solution was then diluted to volume with methanol. To attain a concentration of 100 micrograms/ml, 1 ml of this stock solution was transferred and added to a separate 10 ml volumetric flask. The dilution was carried out using a mixture of Methanol and DW as solvents. The stock solution was further diluted serially to achieve concentrations ranging from 4 micrograms/ml to 20 micrograms/ml. In the optimization experiment, secondary and working stock solutions with a Dapagliflozin concentration of 100 micrograms/ml were prepared using different ratios of Methanol and water: 30:70, 50:50, and 70:30 v/v.

METHOD OPTIMIZATION BY QBD APPROACH [148,149]

QbD is defined as a methodical development approach that commences with well-defined objectives, emphasizing a comprehensive understanding of both the product and the process through robust scientific principles and effective quality risk management. In this study, we investigated numerous features of AQbD and applied them to a practical technique. It was implemented using the following steps:

- Define the analytical Target Profile
- Identify critical quality/analytical attributes
- Identify crucial method parameters
- Utilize the fishbone approach for risk assessment
- Optimization of methods using DOE software.

Defining analytical target profile: The QbD technique starts with creating an analytical target profile that corresponds to the QTPP. The ATP describes the purpose of the analytical method development process, linking the technique's results to achieving QTPP. The analytical target profile is defined based on the analytical process's data and scientific rationale. An exhaustive review of existing literature, encompassing reports and drug profiles detailing the physical and chemical properties, was undertaken to formulate an ATP. This profile provides a dynamic and comprehensive summary of the quality attributes essential for an analytical method.

Identify essential quality analytical attributes: The CQAs or CAAs are measurable analyte parameters that must fall within an adequate and acceptable limit, range, or threshold in order for the analytical technique to perform as intended. The UV technique can be identified as Absorbance.

Identification of critical method parameters: CMPs are the sensitivity levels associated with a particular analytical procedure. Variations in solvent used, detection wavelength, scan speed, sampling interval, sample integrity, and slit width are all key technique characteristics to consider while developing UV methods.

Risk Assessment by employing fish-bone approach: Risk assessment identifies likely interactions with CMPs and assesses the likelihood of subsequent failure. In this regard, a fishbone diagram was created by highlighting various method factors that could potentially influence the method features of the UV spectrophotometric method of Dapagliflozin.

Method optimization by DoE software: Following the completion of a risk analysis to identify the Critical Method Variables, the attention shifted towards optimizing two pivotal method variables: scanning speed and solvent ratio. The remaining method variables were kept constant at either their lowest or optimum values. Employing the CCD within the framework of DoE with an alpha value of one, the objective was to determine the optimal levels for scanning speed (A) and solvent ratio (B) in order to enhance the method's robustness. The CCD approach entailed 9 runs for the two selected variables, including one centered point. The analysis of the selected independent variables, namely solvent ratio (A) and scanning speed (B), focused on one response variable: absorbance at 226nm for Dapagliflozin. To ensure reliability and accuracy, all experiments conducted during the optimization studies were performed in triplicate. The Design Expert software was then employed to input data and generate a coded equation (Equation 1), representing a suitable mathematical model.

$$\mathbf{Y = X_0 + X_1 A + X_2 B + X_3 AB + X_4 A^2 + X_5 B^2} \qquad \textbf{Equation 1}$$

In the coded equation, the symbol Y represents the response variable, X^0 denotes the intercept, and X_1 and X_2 are the coefficients associated with factors A (solvent ratio) and B (scanning speed), respectively. Furthermore, X_3 represents the coefficient of the correlation term between factors A and B, indicating their interaction. Additionally, X_4 and X_5 represent the coefficients of the quadratic terms for the selected factors, highlighting any non-linear relationships between the independent solvent ratio (A) and scanning speed (B).

Data analysis and model validation: The ANOVA was carried out using the Design Expert software to extract crucial parameters such as p-value, f-value, R^2 value, adequate precision, etc. Multiple polynomial equations were formulated, each demonstrating a significant p-value below 0.5. Various graphs, including normal graphs, predicted vs actual graphs, Residual vs Run Graphs, Residual vs Predicted graphs, and Cook's Distance graphs, were generated for both critical method variables. The purpose of these graphs was to assess the impact of scanning speed and solvent ratio, treated as independent variables, on the dependent variables or responses (Absorbance at 226nm). This comprehensive analysis provides valuable insights into the relationships and influences of the selected method variables on the analytical outcomes. To visually represent the relationship between the chosen independent method response variables, 2-D contour graphs and 3-D response surface graphs were generated. These graphs offer a graphical depiction of how changes in scanning speed and solvent ratio, as independent variables, influence the response variable (Absorbance at 226nm). Additionally, overlay graphs were created to illustrate the design space across all experimental areas. These visual representations serve as valuable tools for understanding the complex interactions and optimal conditions within the multidimensional design space, aiding in the interpretation and optimization of the analytical method.

Design space: In accordance with the guidelines set forth in ICH Q8, the term "Design space" or "working space" refers to a multifaceted amalgamation of input factors (method variables) and process parameters that have been demonstrated to ensure quality assurance. The creation of this space involves the utilization of optimization techniques, coupled with response surface methods. The design space

encompasses the operational range of the selected method variables, namely scanning speed and scanning interval. The implementation of the CCD in the experimental design led to 9 experimental runs for the two chosen method variables. This approach facilitates the exploration and identification of optimal conditions within the specified design space to ensure quality in the analytical method.

Model validation: To evaluate the efficacy of the developed model on a laboratory scale, the point prediction feature was utilized. This entailed obtaining response values at specific method variable values within the design space, facilitating further method optimization. The optimized response values were then compared to the response values obtained through actual laboratory-scale experiments conducted at the same method variable values. This comparative analysis served as a validation step, assessing the accuracy and reliability of the polynomial model-based predictions in real experimental conditions within the analytical laboratory.

ANALYTICAL METHOD VALIDATION [150]

Selectivity and specificity: Specificity testing was executed to eliminate the possibility of solvent interference, particularly in the region of Dapagliflozin's highest absorbance peak. The determination of Dapagliflozin's specificity and selectivity involved the comparison of spectra obtained from running both the solvent and the drug solution.

Linearity and range: The normal stock solution of Dapagliflozin was diluted to achieve concentrations ranging from 4µg/ml to 20µg/ml. The produced solutions were examined for UV analysis.

Precision: The precision was determined by measuring the absorbance of Dapagliflozin solutions at three distinct concentrations. The intraday and interday precision were assessed by doing analyses on the same day at two different intervals and three distinct days, respectively. After each analysis, the %RSD was determined for the absorbance values obtained.

Ruggedness: The ruggedness indicates variation within the laboratory conditions (different analyst/instrument). Three replicates of 4µg/ml, 12µg/ml and 20µg/ml of Dapagliflozin solutions were prepared by different analyst and they were subjected for UV analysis on same instrument. The % RSD values were calculated.

Accuracy: Recovery studies were conducted at three distinct levels to determine the accuracy of the established approach. Samples were spiked with 50%, 100%, and 150% standard solution, and the mixture was analyzed using a UV analyser to assess recoveries. The recovery analysis was performed in triplicate.

ESTIMATION OF DAPAGLIFLOZIN IN MARKETED TABLETS

Twenty commercially available Dapagliflozin tablets were weighed, and the mean weight was determined. Subsequently, a quantity equivalent to 10 mg of Dapagliflozin in powder form was weighed, transferred into a 10 mL volumetric flask, and subjected to extraction using a solvent solution comprising Methanol and water. This solution underwent sonication for 10 minutes and was then filtered. Serial dilutions were carried out to achieve a Dapagliflozin concentration of 10µg/ml, which was subsequently measured using absorbance. The entire process was replicated three times (n=3).

RESULTS AND DISCUSSION

The solvent development process uses a Methanol: DW (50:50% v/v) solvent solution, in which Dapagliflozin exhibited a spectrum with a maximal absorption at 226nm (**Figure 53**). The approach was optimized using QbD principles, as described in the methodology section. **Table 65** shows the optimized procedure parameters.

Table 65: Developed UV-Spectrophotometric method for Dapagliflozin

Sr. No.	Parameters	Specifications
1	Method	Spectrophotometric
2	Instrument	UV-Spectrophotometer
3	Model	Shimadzu
4	Make	UV-1800
5	Software	UV-Probe
6	Analyte	Dapagliflozin
7	Solvent	Methanol: Distilled water (50:50% v/v)
8	Lambda Max.	226nm

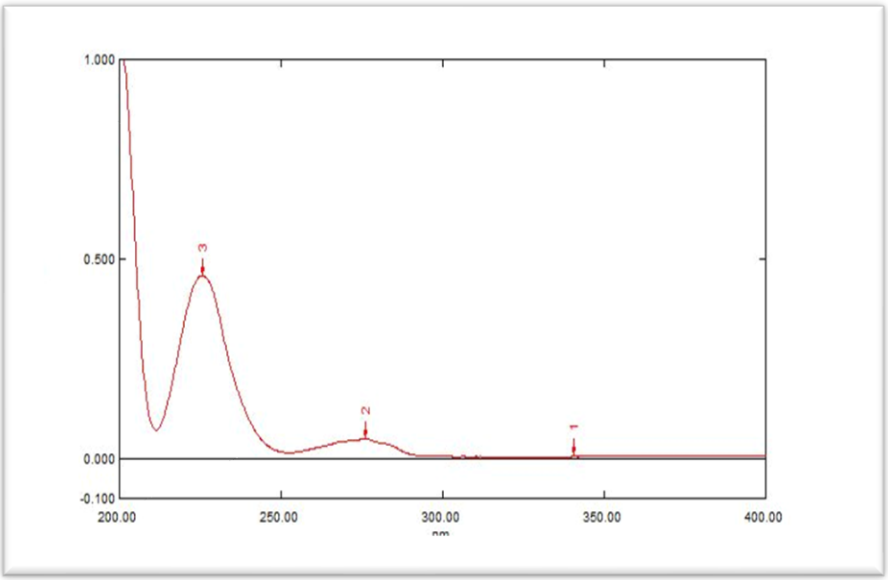


Figure 53: UV-Spectrum of Dapagliflozin (10µg/ml)

METHOD OPTIMIZATION BY QBD APPROACH

The various steps involved in QbD-based method optimization were carried out in a systematic manner. The results are presented as follows:

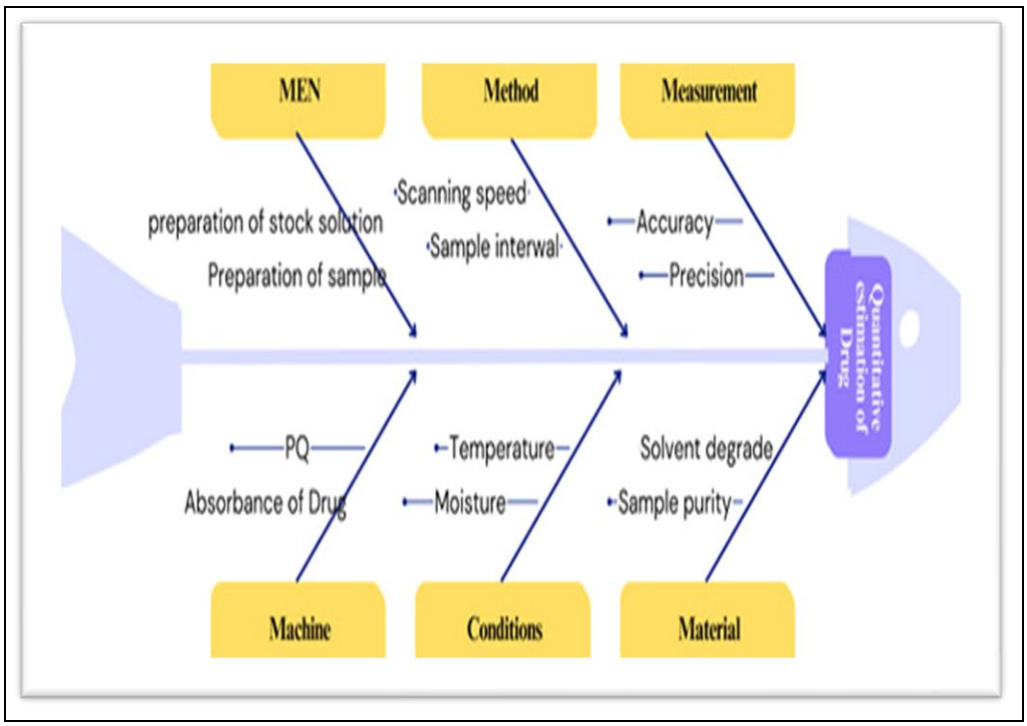
Defining the ATP: ATP outlines the expected method requirements for the measurement. The analytical target profile is defined using knowledge and scientific reasoning about the analysis process. As a result, depending on the primary goal of this investigation, a UV spectrophotometric approach was used for quick measurement of Dapagliflozin. The argument for choosing the UV spectrophotometric method was based on its simplicity and speed of drug analysis when compared to other complex analytical methods. **Table 66** represents ATP for proposed UV-spectrophotometric method for Dapagliflozin.

Identification of critical quality or analytical attributes: In proposed UV method, absorbance produced by drug was identified as critical quality attribute of analyte.

Risk Assessment by employing fish-bone approach: Risk assessment examines potential interactions with CMPs and determines the likelihood of subsequent failure. An Ishikawa fishbone diagram was built to identify potential dangers and underlying causes that affect the performance of the UV method by highlighting many method variables that may influence the method attributes of Dapagliflozin's UV spectrophotometer. **Figure 9** represents the Ishikawa fishbone diagram.

Table 66: ATP for UV-spectrophotometric analysis of Dapagliflozin

Sl. No.	ATP Parameters	Target
1	Target Analyte	Dapagliflozin
2	Target Sample	Dapagliflozin Tablets
3	Method category	UV-spectrophotometric method
4	Instrument requirement	UV-spectrophotometer
5	Nature of analyte	Solid (Solution)
6	Standard stock solution preparation	Dilution of main drug in linear manner
7	Application of Method	Estimation of Dapagliflozin
8	Validation parameters	Accuracy, Selectivity, ruggedness, precision, linearity, specificity, LOD and LOQ



Ishikawa fishbone diagram for UV-Spectrophotometric method

Method optimization by DoE Software: The approach was further developed using the CCD inside the framework of DoE with an alpha value of one. The goal was to establish the best settings for scanning speed (A) and solvent ratio (B) in order to improve the method's resilience. The selection of levels for CCD design is given in **Table 67.** The CCD method involved 9 runs for the two specified variables, including one centered point (**Table 68**). After doing the experiments indicated by CCD, the results were entered into the software and evaluated further.

Data analysis: The important analytical variables were optimized using a statistical design, specifically the CCD. Using the Design Expert program, a design plan was generated that included 9 experimental runs for the identified independent parameters, scanning speed and solvent ratio, using the CCD technique. The specifics of this experimental design are detailed in **Table 68**. The absorbance at 226nm was selected as the response or dependent variable for examination. The experimental data was examined using multiple linear regression analysis. Following the development of the mathematical model utilizing software, the data was examined using a second-order quadratic model established via a coded equation.

Table 67: Selection of levels for CCD of Dapagliflozin by UV method

Independent Variable	**Levels**		
	+1	**0**	**-1**
Scanning Speed (A)	**+1 (High)**	**0 (Medium)**	**-1 (Low)**
Solvent Ratio (B)	70 %	50%	30%

Table 68: Experimental design matrix by CCD for Dapagliflozin UV method

Run	Factor 1 (A: Solvent Ratio)	Factor 2 (B: Scanning Speed)	Response 1 (Absorbance)
1	30	0	0.137
2	70	0	0.201
3	70	1	0.204
4	50	1	0.158
5	70	-1	0.207
6	50	0	0.157
7	30	-1	0.132
8	50	-1	0.153
9	30	1	0.133

Responses equation in coded form: Coded factor equations were employed to predict the correlation between independent and dependent variables at different levels, categorized as low (-1) and high (+1).

Absorbance at 226 nm = 0.156 + 0.0350A+0.0005B-0.0010AB+0.0130 A^2-0.0005B^2

In the context of the equations, A represents solvent ratio, and B stands for scanning speed. Statistical analysis of the chosen factors involved the use of ANOVA. The lack of fit, R^2, and adjusted R^2 values were employed to evaluate the significance of the model, with a p-value below 0.05 indicating the model's adequacy. A significant "Lack of fit" suggests that the model does not sufficiently explain the differences between predicted and observed data points. ANOVA R^2 values close to 1 indicate how well the anticipated model aligns with the experimental model, with values

approaching 1 being desirable. **Table 69** presents various ANOVA analyses for the chosen response, Absorbance at 233nm, considering parameters such as p-value, Lack of fit, and R^2 value. The outcomes in Table 4 indicate that both the model and the factors have p-values below 0.05, signifying the significance of the developed model. The F-values for the response are 3646.09, underscoring the significance of the model. The chance of error based on the F-value is determined to be 0.01% in the developed model.

Table 69: ANOVA results for UV method of Dapagliflozin at 226 nm

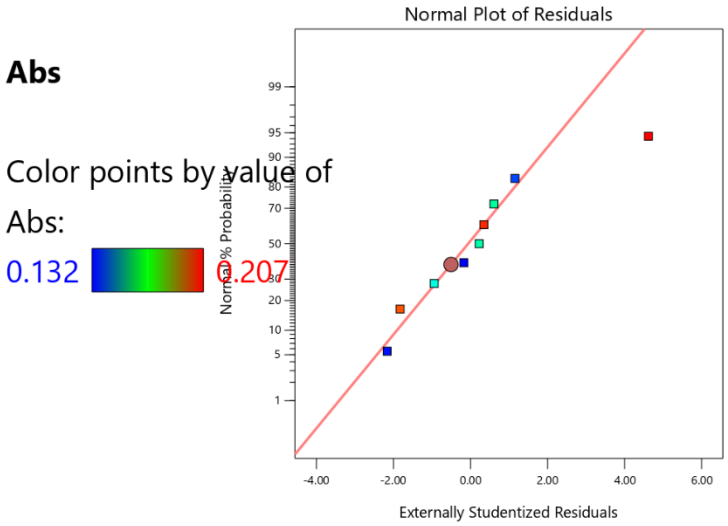
Source	**Sum of Squares**	**df**	**Mean Square**	**F-value**	**p-value**	
Model	0.0077	5	0.0015	115.41	0.0013	**Significant**
A-solvent ratio	0.0073	1	0.0073	551.25	0.0002	
B-scanning speed	1.500E-06	1	1.500E-06	1.1125	0.7594	
AB	4.000E-06	1	4.000E-06	0.3000	0.6220	
A^2	0.0003	1	0.0003	25.35	0.0151	
B^2	5.000E-07	1	5.000E-07	0.0375	0.8588	
Residual	0.0000	3	0.0000			
Cor Total	0.0077	8				

Various graphs, including the predicted vs. actual graph, box-cox graph, and normal graph generated in the design, are depicted in **Figure 54a-c**, all demonstrating satisfactory response criteria in comparison to variables. To illustrate the relationship between variables and response, 2-D contour graphs and 3-D response surface graphs were generated using Design Expert software and presented in **Figure 55a-b**. The analysis of these graphs led to the conclusion that absorbance decreases with intermediate and low values of both selected variables, i.e., scanning speed and solvent ratio.

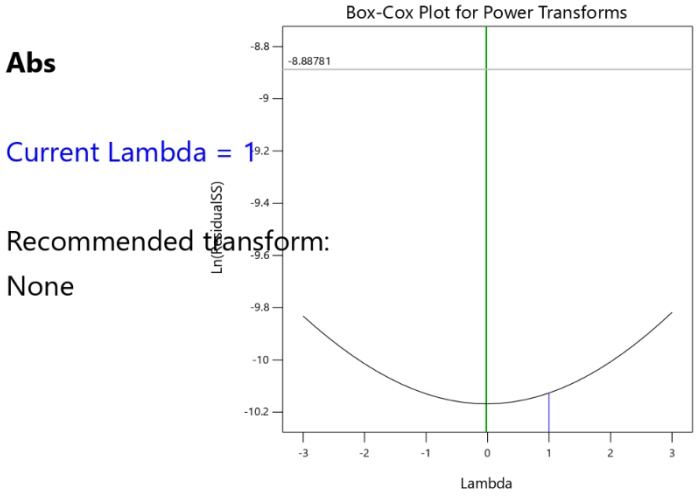
Design Space: The design space critical for optimizing the selected factors (solvent ratio and scanning speed) concerning the chosen response (absorbance at 226nm) is highlighted in **Figure 56.** The overlay graph delineates the design space in dark yellow within the experimental area in grey, while the region in light yellow signifies the area where various factors can be varied.

Validation of developed experimental model: Validation of the experimental design is critical for assessing the risk and practical value of any analytical technique development, particularly when QbD and Quality Risk Management (QRM) concepts are applied. When comparing the predicted values to the experimental values, it was discovered that the developed model had a high level of agreement with the experimental values. This validation confirms that the mathematical model is appropriate for determining the correlation of selected variables in order to get the required response within the given design space.

a. Normal Graph



b. Box-Cox



c. Predicted vs actual graph

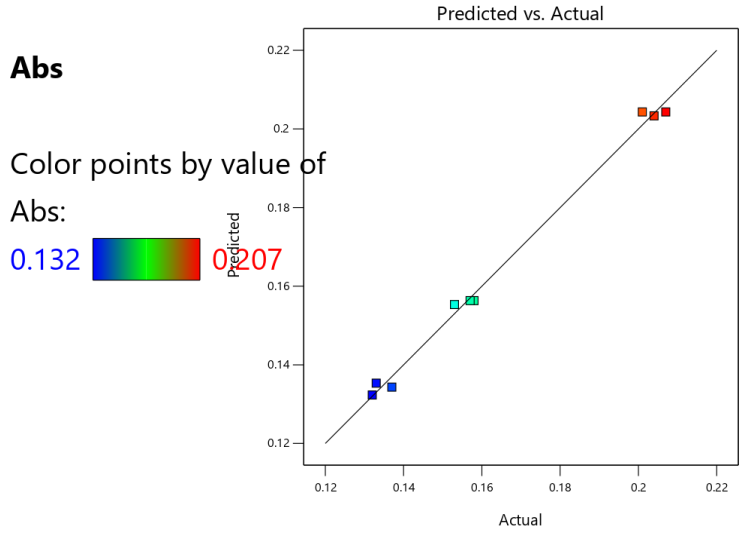
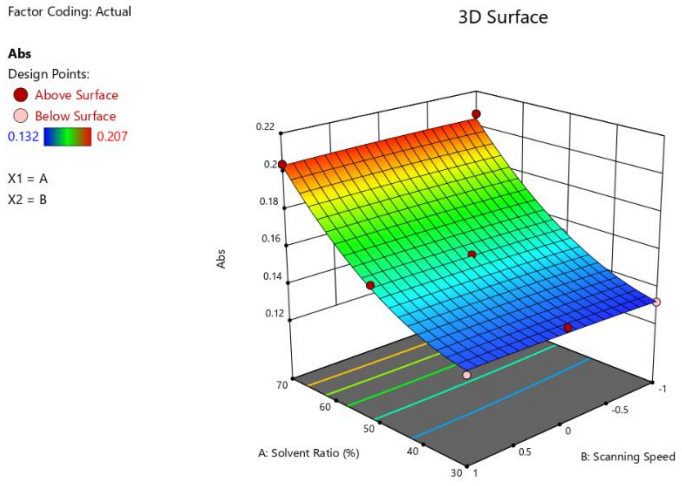


Figure 54: (a) Normal graph (b) Box-Cox graph (c) Predicted vs actual graph generated by Design Expert software for Dapagliflozin

(a) 3-D Response surface graph



(b) 2-D contour graph

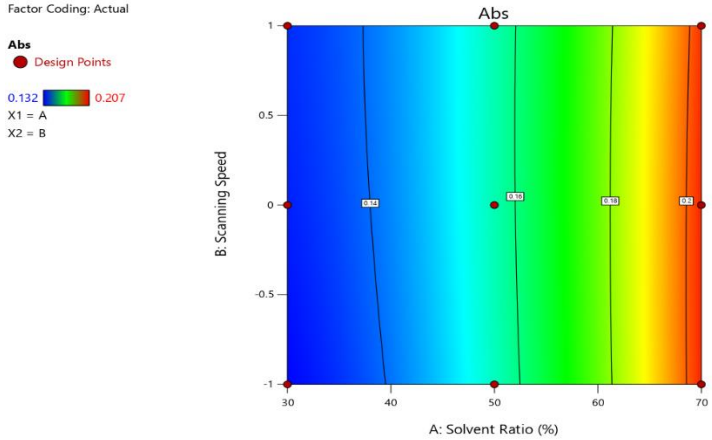


Figure 55: (a) 3-D Response surface graph (b) 2-D contour graph showing the corelation between the selected variables i.e., solvent ratio and scanning speed and response for Dapagliflozin by UV method

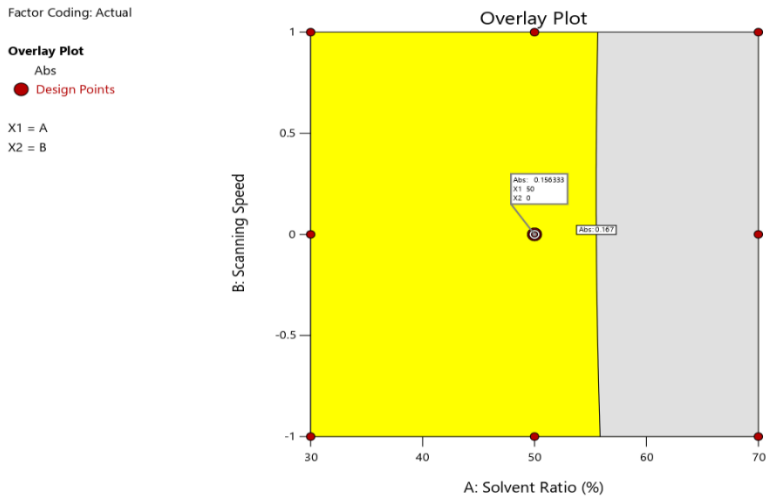


Figure 56: Overlay graph showing the design space from experimental area for Dapagliflozin UV method analysis

ANALYTICAL METHOD VALIDATION

Selectivity and specificity: The solvent spectrum displayed no interference of absorbance at 226nm, demonstrating the method's specificity and selectivity. **Figure 57a-b** shows the UV spectrum of the solvent and Dapagliflozin.

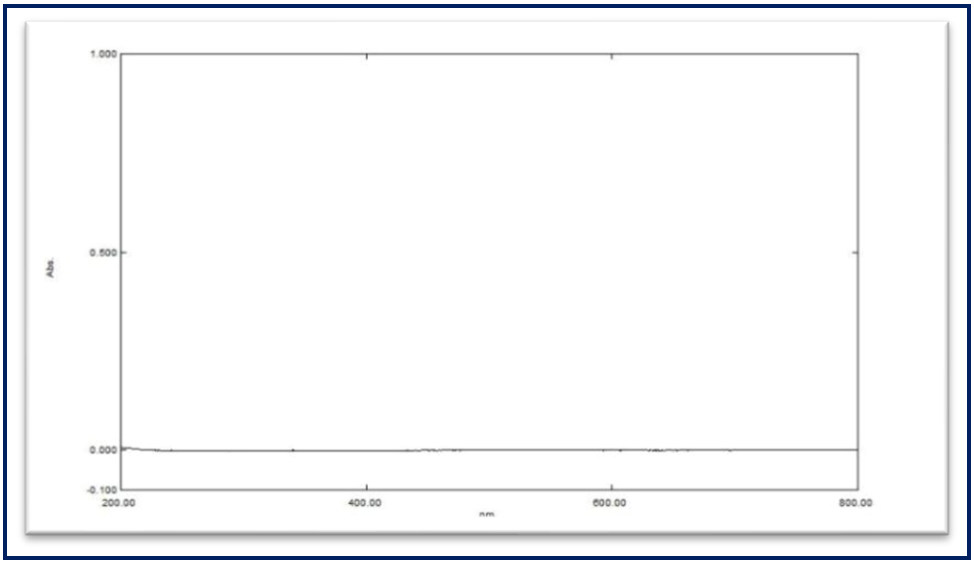


Figure 57a: UV Spectrum of blank solvent

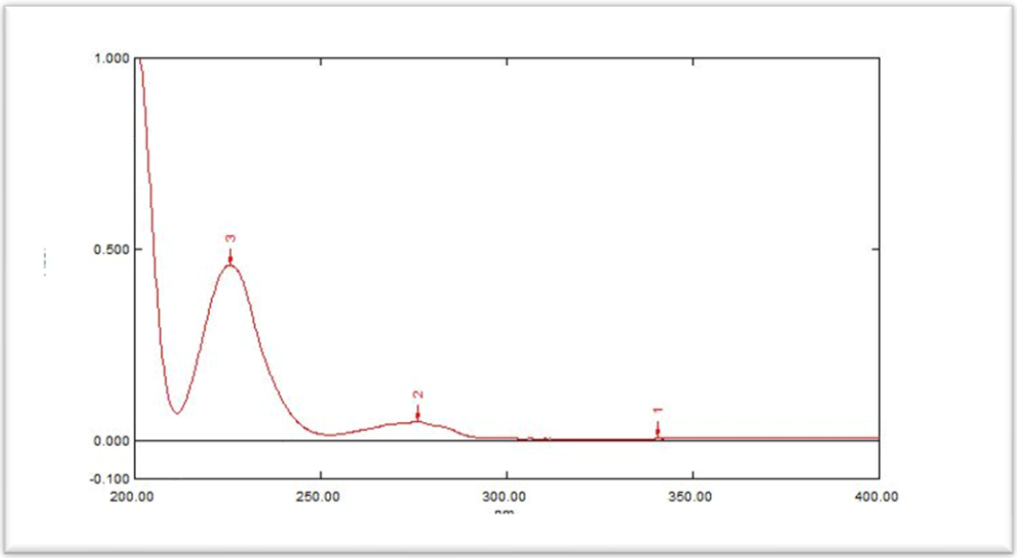


Figure 57b: UV-Spectrum of Dapagliflozin (10µg/ml)

Linearity and Range: A standard calibration curve was created by graphing concentration vs absorbance obtained from linear dilution of Dapagliflozin. The linear absorbance range for Dapagliflozin was 4, 8, 12, 16, and 20µg/ml, with a r^2 of 0.9998. **Table 70** presents linearity data. **Figure 58** shows a standard calibration curve. Figure 59 depicts the overlay spectrum of linearity for Dapagliflozin.

Sensitivity: The LOD and LOQ values were found to be 0.63µg/ml and 1.92µg/ml, respectively.

Precision: The precision of the method was demonstrated by a % RSD of less than 2% for three duplicate Dapagliflozin solutions at each precision level. **Table 71** shows the data for precision.

Ruggedness: The procedure was confirmed to be robust because the %RSD of each concentration conducted by various analysts and analyzed on separate instruments was within acceptable limits **Table 72.**

Accuracy: The approach was determined to be accurate, since all level recovery values are within acceptable limits. **Table 73** displays accuracy data.

ESTIMATION OF DAPAGLIFLOZIN IN MARKETED TABLETS

The suggested UV-Spectrophotometric approach yielded a drug content assay of 98.60±0.55% (n=3) for the Dapagliflozin label claim. The assay results demonstrate the selectivity of the established analytical approach for estimating Dapagliflozin in pharmaceutical dose form.

Table 70: Linearity data of Dapagliflozin by UV spectroscopic method

Sl. No.	Concentration (µg/ml)	Absorbance at 226nm
1	4	0.154
2	8	0.315
3	12	0.472
4	16	0.622
5	20	0.802
r^2		0.9998

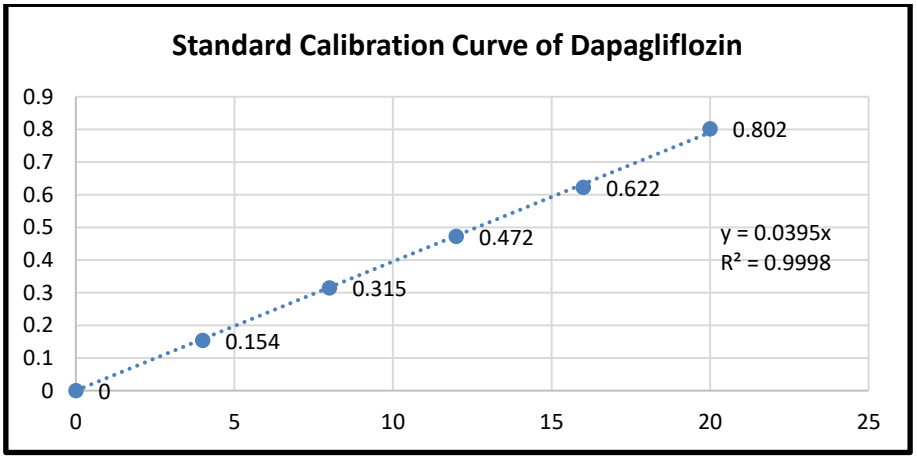


Figure 58: Calibration curve of Dapagliflozin by UV-Spectrophotometric method

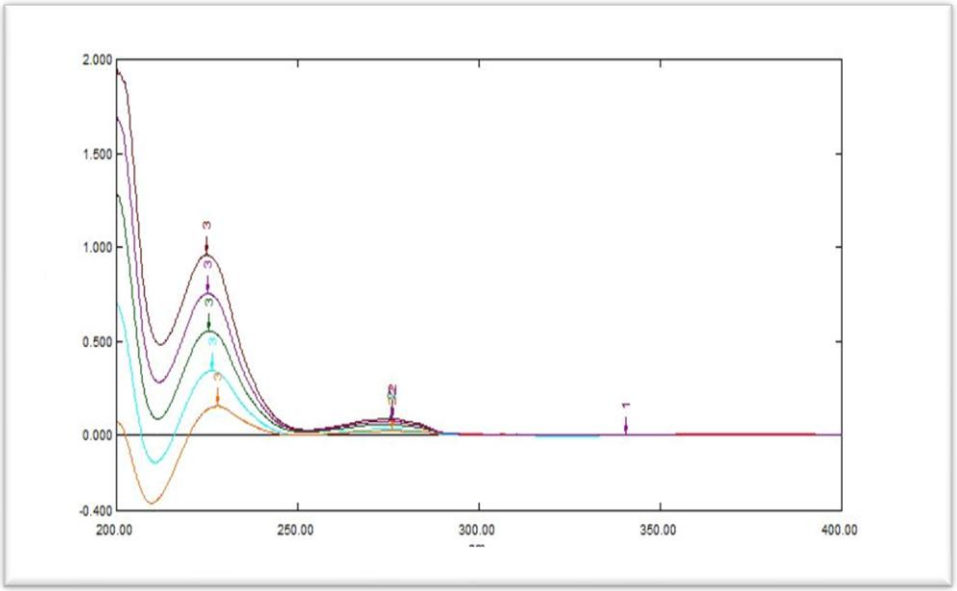


Figure 59: Linearity overlay spectrum of Dapagliflozin by UV method

Table 71: Precision data of Dapagliflozin by UV Spectroscopic Method

Precision	Concentration (µg/ml)	Mean Absorbance	Standard Deviation	%RSD
Intraday	4	0.153	0.001	0.75
	12	0.471	0.001	0.21
	20	0.802	0.003	0.37
Interday-1	4	0.157	0.001	0.73
	12	0.480	0.001	0.28
	20	0.805	0.006	0.75

Table 72: Ruggedness data of Dapagliflozin by UV Spectroscopic method

Precision Type	Concentration (µg/ml)	Mean Absorbance	SD	%RSD
Change in Analyst	4	0.162	0.001	0.62
	12	0.482	0.007	1.46
	20	0.823	0.002	0.24
Change in Instrument	4	0.167	0.002	1.04
	12	0.485	0.004	0.82
	20	0.883	0.002	0.24

Table 73: Recovery data of Dapagliflozin by UV Spectroscopic method

Levels %	Sample Added (µg/ml)	Standard Added (µg/ml)	Total quantity (µg/ml)	Mean Quantity Recovered (µg/ml) ± SD	Mean % Recovery (µg/ml) ± SD
50	2	6	8	7.79 ±0.02	97.43 ± 0.17
100	2	12	14	14.25 ±0.05	101.81 ± 0.35
150	2	18	20	19.89 ±0.04	99.43 ±0.18

CONCLUSION

This research underscores the utilization of cost-effective, reproducible UV-spectrophotometric techniques to estimation of Dapagliflozin. The study integrates QbD principles, identifying CMVs through risk assessment via the Ishikawa fishbone diagram. Employing risk surface methodology and CCD optimization aids in understanding the relationships and interactions among response factors. The developed analytical approach is subjected to validation following ICH guidelines. Results highlight the method's originality, linearity, accuracy, and precision, with LOD and LOQ values indicating high sensitivity. The incorporation of QbD and ICH guidelines supports the potential implementation of the optimized method in pharmaceutical QC labs for the estimation of Dapagliflozin. This method is considered suitable for the routine estimation of Dapagliflozin in marketed pharmaceutical preparations, displaying compatibility with common excipients in an economical and uncomplicated manner.

METHOD 7: DEVELOPMENT AND VALIDATION OF AREA UNDER CURVE UV-SPECTROPHOTOMETRIC METHOD FOR SITAGLIPTIN

METHOD DEVELOPMENT

The development of the UV-spectrophotometric method began with the selection of a solvent solution and the determination of the analytical wavelength. Dapagliflozin's solubility was investigated in a variety of solvents, and it was discovered to be highly soluble in Methanol. As a result, the initial stock dilution of the drug was made with Methanol as a solvent. Dapagliflozin secondary stock solution and working standards are made with a solvent mixture of Methanol and DW (50:50%v/v). [101,102]

Standard stock solution: A standard stock solution of Dapagliflozin at a concentration of 1000µg/ml was formulated by dissolving 10mg of the substance in a 10ml VF, and subsequently, the solution was diluted to volume using Methanol. To attain a concentration of 100µg/ml, 1ml was withdrawn from the stock solution and introduced into another 10ml VF, where the dilution was performed using a solvent mixture of Methanol and DW in a 50:50%v/v ratio. For the generation of various concentrations, the stock solution underwent serial dilution to achieve concentrations of 4 µg/ml -20 µg/ml. [112]

Selection of solvent and wavelength of analysis: Solubility assessments, coupled with a comprehensive literature review, revealed that Dapagliflozin exhibits solubility in Methanol. For the determination of the analysis wavelength, a solution with a concentration of 10µg/ml was subjected to UV scanning within the range of 200-400nm. The resulting spectrum was acquired, pinpointing the wavelength of maximum absorption at 226nmied as 226nm.

Area under curve measurement: To determine the spectrum, the absorbance method was utilized, and the area under the curve between two wavelengths of 220-234nm was analyzed.

Method specification: The solvent development process employs the use of DW, in which Dapagliflozin exhibited a spectrum with highest absorption at 226nm. Table 74 shows the developed procedure parameters.

Table 74: AUC UV-Spectrophotometric method for Dapagliflozin

Sr. No.	Parameters	Specifications
1	Technique	Spectrophotometric
2	Instrument	UV-Spectrophotometer
3	Model	Shimadzu
4	Make	UV-1800
5	Software	UV-Probe
6	Analyte	Dapagliflozin
7	Solvent	Methanol: Distilled water (50:50%v/v)
8	Lambda Max.	226nm
9	Area Under Curve	220-234nm

ANALYTICAL METHOD VALIDATION [150]

For validation experiments all the concentrations were prepared as per described in **Method 6**. The area under curve between two wavelengths 220-234nm was used for calculations.

Specificity and Selectivity: The solvent spectra obtained revealed no interference at Area Under Curve region produced by standard Dapagliflozin. This shown the specificity and selectivity of the proposed approach. **Figure 60** shows the UV spectra of the solvent and Dapagliflozin.

Linearity and range: The linear calibration curve for Dapagliflozin from 4 to 20 µg/ml showed good linearity, with a correlation coefficient of 0.999. (**Figure 61, Table 75**). **Figure 62** depicts the AUC graphs.

LOD and LOQ: The area under curve UV-Spectrophotometric Method had LOD and LOQ of 0.41µg/ml and 1.41µg/ml, respectively.

Precision: The %RSD for Dapagliflozin on three measurements was found to be less than 2% for each spectrum's area under curve. These findings provided evidence for the developed approach's high level of precision. **Table 76** displays the precision data for the process.

Ruggedness: The procedure was confirmed to be rugged because the %RSD of each concentration done by various analysts on different instruments for Area Under Curve of each spectrum was within acceptable limits. **Table 77** exhibits the method's precision statistics.

Accuracy: The developed technique demonstrated remarkable accuracy, with percentage recovery ranging from 98.04 to 101.90% at all three levels. The percentage RSD value was less than 2%. **Table 78** displays the results and statistical data for accuracy.

ESTIMATION OF DAPAGLIFLOZIN IN MARKETED TABLETS

To perform the experiment, Dapagliflozin was extracted in a manner similar to that described in Method 6. The Dapagliflozin label claim had a drug content percentage test of 98.20±0.26% (n=3). The assay findings show the selectivity of the developed area under curve UV spectrophotometric approach for calculating Dapagliflozin in pharmaceutical preparation.

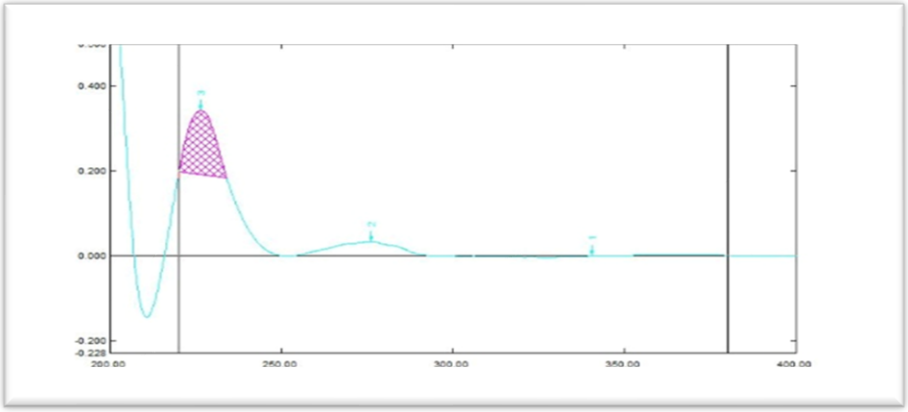


Figure 60: Selectivity UV-Spectrum of Dapagliflozin AUC method

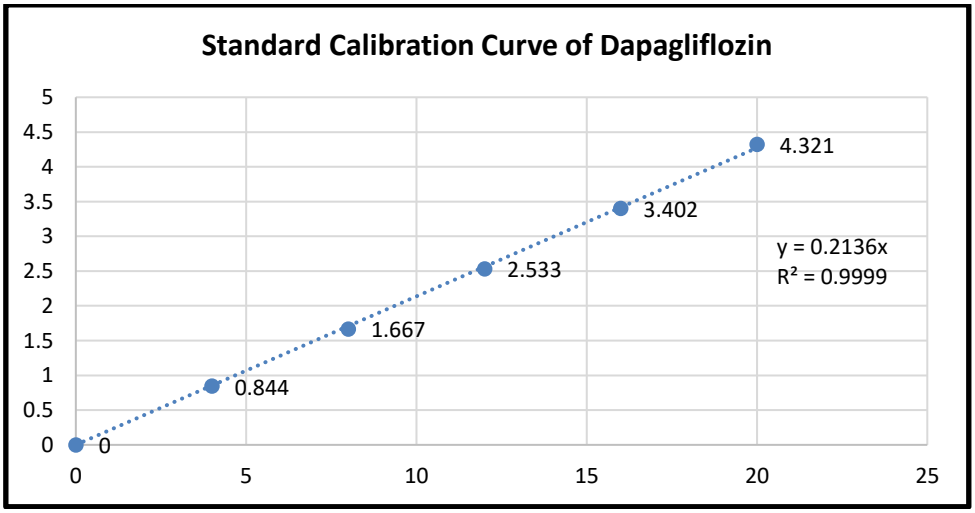
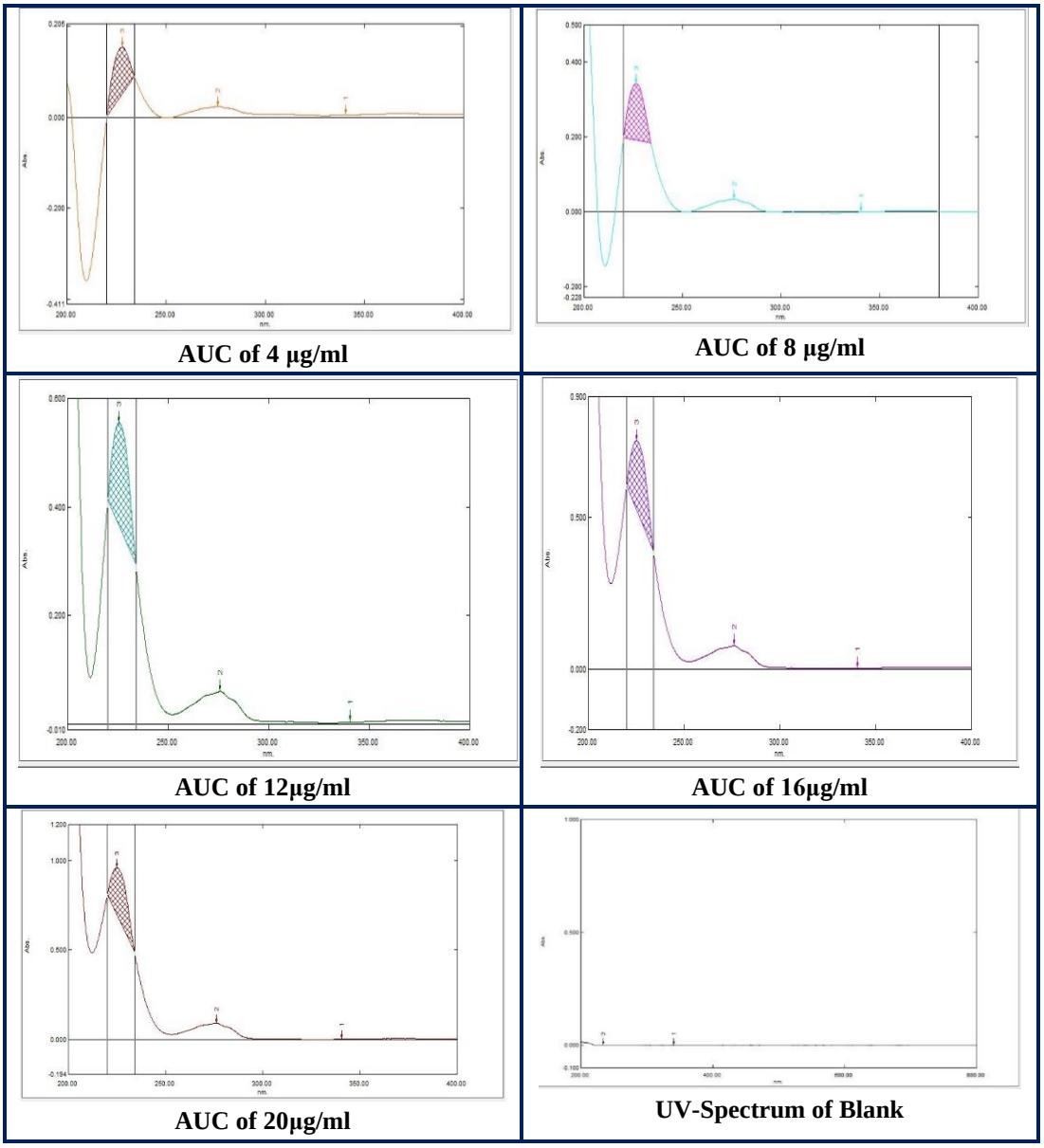


Figure 61: Calibration curve of Dapagliflozin by AUC UV method



AUC of 4 µg/ml

AUC of 8 µg/ml

AUC of 12µg/ml

AUC of 16µg/ml

AUC of 20µg/ml

UV-Spectrum of Blank

Figure 62: Linearity AUC graphs of Dapagliflozin by UV method

Table 75: Linearity data of Dapagliflozin by AUC UV-spectroscopic method

Sl. No.	Concentration	Area Under Curve (220nm– 234nm)
1	4µg/ml	0.844
2	8µg/ml	1.667
3	12µg/ml	2.533
4	16µg/ml	3.402
5	20µg/ml	4.321
r^2		**0.9999**

Table 76: Precision data of Dapagliflozin by AUC UV method

Precision	Concentration	Mean AUC	SD	%RSD
Intraday	4 µg/ml	0.850	0.005	0.60
	12 µg/ml	2.539	0.020	0.79
	20 µg/ml	4.325	0.004	0.08
Interday	4 µg/ml	0.850	0.007	0.84
	12 µg/ml	2.520	0.009	0.36
	20 µg/ml	4.326	0.004	0.10

Table 77: Ruggedness data of Dapagliflozin by AUC UV Method

Precision Parameter	Concentration µg/ml	Mean AUC	Standard Deviation	%RSD
Change in Analyst	4 µg/ml	0.853	0.010	1.15
	12 µg/ml	2.520	0.009	0.36
	20 µg/ml	4.363	0.067	1.54
Change in Instrument	4 µg/ml	0.861	0.009	1.05
	12 µg/ml	2.626	0.022	0.84
	20 µg/ml	4.366	0.072	1.65

Table 78: Recovery data of Dapagliflozin by AUC UV method

Levels %	Sample Added	Standard Added	Total quantity (µg/ml)	Mean Quantity Recovered (µg/ml) ± SD	Mean % Recovery (µg/ml) ± SD
50	2	6	8	7.87 ±0.05	98.04 ± 0.72
100	2	12	14	14.26 ±0.17	101.90 ± 1.20
150	2	18	20	19.68 ±0.20	98.38 ±0.99

CONCLUSION

The proposed area under curve UV-spectrophotometric method found to be precise, accurate, and unique for measuring Dapagliflozin in marketed dose forms. The validation reports support the preciseness, ruggedness and accuracy of method for quantification of Dapagliflozin in marketed samples. Hence the devised approach can be used for he routine QC of pharmaceuticals loaded with Dapagliflozin.

METHOD 8: DEVELOPMENT AND VALIDATION OF UV- METHOD FOR ESTIMATION OF EMPAGLIFLOZIN

METHOD DEVELOPMENT

The initiation of the UV-spectrophotometric method development involved the careful selection of a solvent solution and the determination of the analysis wavelength. Solubility assessments for Empagliflozin were conducted across various solvents, revealing its favorable solubility in Methanol. Consequently, Methanol was chosen as the primary solvent for the initial stock solution preparation. The subsequent generation of secondary stock solutions and working standards for Empagliflozin employed a solvent system comprising Methanol and MW in a 50:50% v/v ratio. The optimization of this solvent system was achieved through the utilization of DoE software and adhering to QbD principles. [102]

Selection of solvent and wavelength of analysis: To determine the analysis wavelength, a solution with a concentration of 10µg/ml was subjected to UV scanning within the range of 200-400nm. The resulting spectrum was captured, and the wavelength exhibiting the maximum absorption was identified as 233nm. [112]

Standard stock solution: To make an empagliflozin standard stock solution with a concentration of 1000µg/ml, 10mg was dissolved in 10ml of VF and diluted with Methanol. To achieve a concentration of 100µg/ml, 1ml of the stock was added to 10ml of VF and the volume was adjusted to the mark using a 50:50% v/v solvent system. The stock was serially diluted to prepare concentrations of 4 - 20 µg/ml. [151]

METHOD OPTIMIZATION BY QUALITY BY DESIGN APPROACH [148, 149]

In this study, we focused on several aspects of AQbD and applied them to a practical technique. It was implemented by performing the steps as mentioned in **Method 6.**

ANALYTICAL METHOD VALIDATION

The developed method was validated in terms of specificity, linearity, sensitivity, precision, ruggedness and accuracy as per ICH guidelines. It was performed in similar way as per the procedures discussed in Method 6.

ESTIMATION OF EMPAGLIFLOZIN IN MARKETED TABLETS

Twenty marketed formulations of Empagliflozin were weighed and average weight was calculated. Then powder equivalent to 10 mg of Empagliflozin was weighed and transferred to 10 mL volumetric flask and extraction was carried out using suitable solvents (Methanol and Millipore water) and sonication for 15 minutes. After extraction serial dilution was made to obtain concentration of 12µg/ml and absorbance was measured to calculate the % assay in marketed sample.

RESULTS AND DISCUSSION

The solvent development step involves the use of Methanol: Millipore water (50:50% v/v) as solvent system, in which Empagliflozin showed spectrum with maximum absorbance at 233nm (**Figure 63**). The method was optimized using quality by design principles as mentioned in the methodology part. The Optimized method parameters are presented in **Table 79**.

Table 79: Developed UV-Spectrophotometric method for Empagliflozin

Sr. No.	Parameters	Specifications
1	Method	Spectrophotometric
2	Instrument	UV-Spectrophotometer
3	Model	Shimadzu
4	Make	UV-1800
5	Software	UV-Probe
6	Analyte	Empagliflozin
7	Solvent	Methanol: Millipore water (50:50% v/v)
8	Lambda Max.	233nm

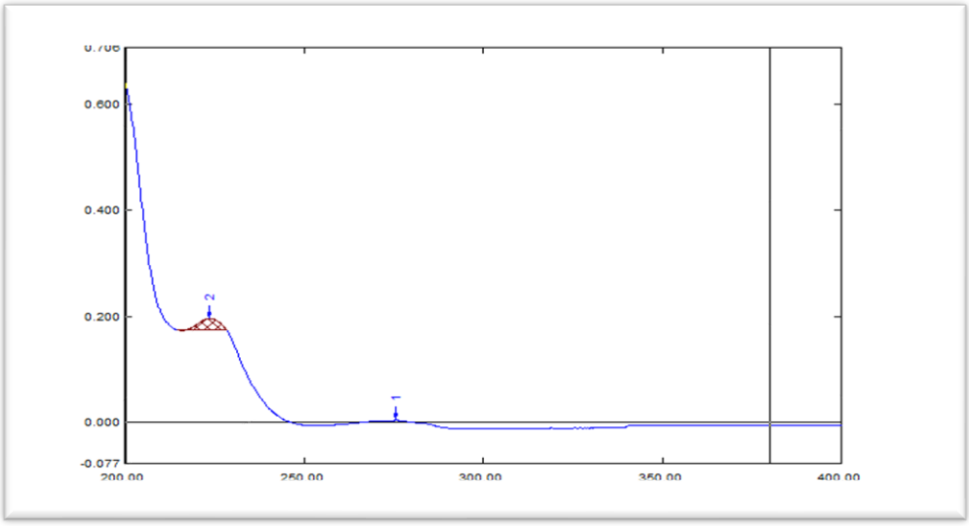


Figure 63: UV-Spectrum of Empagliflozin (10µg/mL)

METHOD OPTIMIZATION BY QUALITY BY DESIGN APPROACH

Defining the analytical target profile: Table 80 represents ATP for proposed UV-spectrophotometric method for Empagliflozin.

Table 80: ATP for UV-spectrophotometric analysis of Empagliflozin

Sl. No.	ATP Parameters	Target
1	Target Analyte	Empagliflozin
2	Target Sample	Empagliflozin Tablets
3	Method category	UV-spectrophotometric method
4	Instrument requirement	UV-spectrophotometer
5	Nature of analyte	Solid (Solution)
6	Standard stock solution preparation	Dilution of main drug in linear manner.
7	Application of Method	Estimation of Empagliflozin
8	Process parameters	Specificity, selectivity, linearity, range, precision, robustness, ruggedness, accuracy, LOD, LOQ.

Method optimization by DoE software: The approach was further refined using the Central Composite Design inside the framework of DoE with an alpha value of one. The goal was to establish the best settings for scanning speed (A) and solvent ratio (B) in order to improve the method's resilience. The selection of levels for CCD design is given in **Table 81.**

Table 81: Selection of levels for CCD for UV method of Empagliflozin

Independent Variable	Levels		
	+1	**0**	**-1**
Scanning Speed (A)	+1 (High)	0 (Medium)	-1 (Low)
Solvent Ratio (B)	70 %	50%	30%

Table 82 shows that the CCD technique required 9 passes for the two selected variables, one of which was centered. After doing the experiments indicated by CCD, the results were entered into the software and evaluated further.

Table 82: Experimental design matrix by CCD for Empagliflozin UV method

Run	Factor 1 (A: Solvent Ratio)	Factor 2 (B: Scanning Speed)	Response 1 (Absorbance)
1	30	-1	0.189
2	50	0	0.210
3	70	1	0.257
4	30	1	0.186
5	50	-1	0.207
6	70	0	0.260
7	30	0	0.185
8	70	-1	0.267
9	50	1	0.210

Data analysis: The optimization of key analytical variables was carried out using a statistical design, specifically the CCD. Using the Design Expert program, a design plan was generated that included 9 experimental runs for the identified independent parameters, scanning speed and solvent ratio, using the CCD technique. **Table 82** details this experimental design. The absorbance at 233nm was selected as the response for examination. The experimental data was examined using multiple linear regression analysis. Following the development of the mathematical model utilizing software, the data was examined using a second-order quadratic model established via a coded equation. This model predicts the link between the factors and the response, which makes it easier to comprehend the experimental results.

Responses equation in coded form: Coded factor equations were used to predict the correlation between independent and dependent variables at various levels, which were classified as low (-1) and high (+1). **Absorbance at 233 nm = 0.2083+0.0373A-0.0017B-0.0018AB+0.0150A^2+0.0010B^2.** In the formulae, A indicates the solvent ratio and B denotes scanning speed. The chosen factors were statistically analyzed using ANOVA. The lack of fit, R2, and adjusted R2 values were used to assess the model's significance, with a p-value of less than 0.05 confirming adequacy. ANOVA R2 values close to 1 reflect how closely the predicted model matches the experimental model, with values near 1 being preferable. **Table 83** shows a variety of ANOVA studies for the chosen response, absorbance at 233nm, taking into account characteristics such as p-value, lack of fit, and R2. The results in **Table 83** show that both the model and the factors have p-values less than 0.05, indicating the importance of the developed model. The F-values for the answer are 3646.09, demonstrating the model's relevance. In the created model, the F-value-based error rate is calculated to be 0.01%. The analysis also supports the second-order quadratic model.

Table 83: ANOVA results of CCD for Empagliflozin by UV method

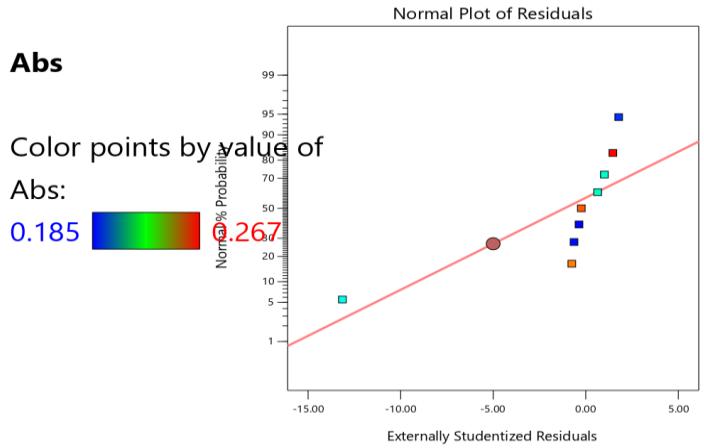
Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	0.0088	5	0.0018	145.71	0.0009	significant
A-solvent ratio	0.0084	1	0.0084	688.92	0.0001	
B-scanning speed	0.0000	1	0.0000	1.37	0.3259	
AB	0.0000	1	0.0000	1.01	0.3891	
A^2	0.0005	1	0.0005	37.07	0.0089	
B^2	2.000E-06	1	2.000E-06	0.1648	0.7120	
Residual	0.0000	3	0.0000			
Cor Total	0.0089	8				

The model F-value of 145.71 indicates that the model is significant. There is only a 0.09% probability that a significant F-value will occur owing to noise. P-values less than 0.05 suggest that the model terms are significant. In this example, A and A2 are significant model terms. Values above 0.1000 imply that the model terms are not significant. If there are a lot of significant model terms, model reduction may help. **Figure 64a-c** depicts a variety of plots, including the expected vs. actual plot, box-cox plot, and normal plot developed in the design, all of which show appropriate response requirements in comparison to variables. **Figure 65a-b** depicts 2D contour plots and 3D response surface plots created with Design Expert software to demonstrate the link between factors and response.

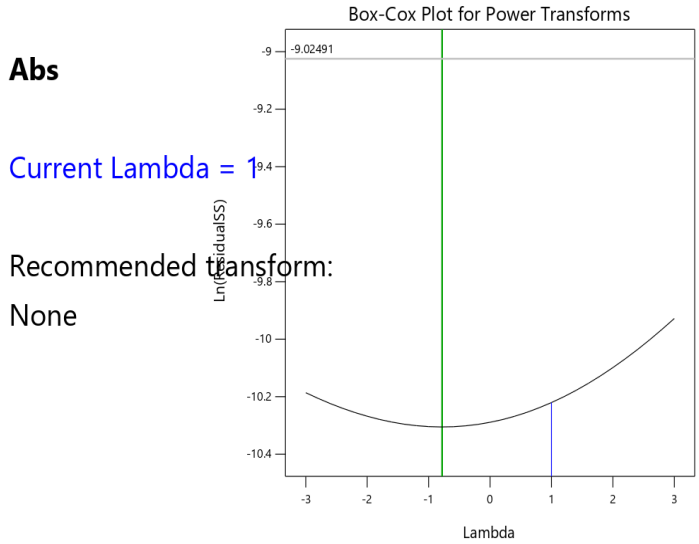
Design Space: Figure 66 depicts the essential design space for optimizing the specified parameters (solvent ratio and scanning speed) with respect to the chosen response (absorbance at 233nm). The overlay plot shows the design space in dark yellow within the experimental area in grey, while the light-yellow region represents the area where many elements can be changed.

Validation of developed experimental model: Validation of the experimental design is an important step in assessing the risk and practical utility of any analytical method development. When the predicted values were compared to the experimental values, it was discovered that the developed model demonstrated a high level of agreement with the experimental values. This validation confirms that the mathematical model is appropriate for determining the correlation of selected variables in order to get the required response within the given design space.

d. Normal Plot



e. Box-Cox



f. Predicted vs actual plot

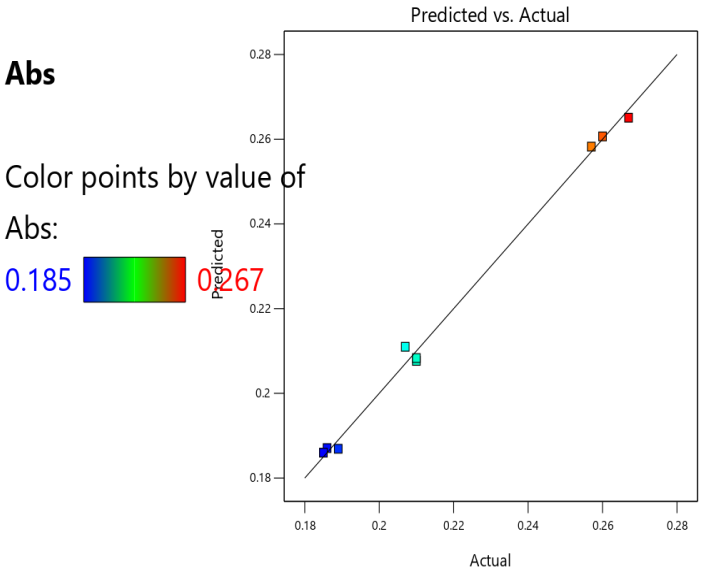
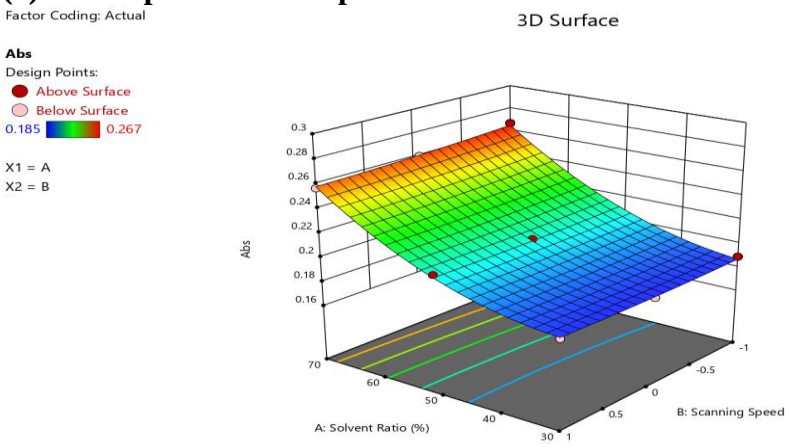


Figure 64: (a) Normal plot (b) Box-Cox plot (c) Predicted vs actual plot generated by Design Expert software for UV method of Empagliflozin

(a) 3D Response surface plot



(b) 2D contour plot

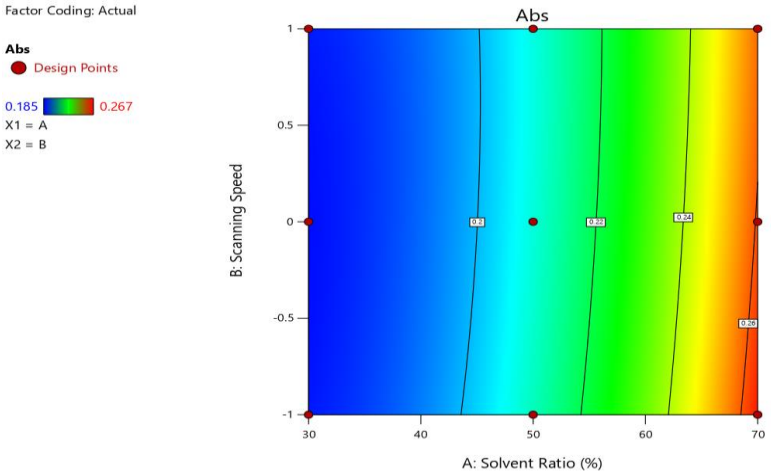


Figure 65: (a) 3D Response surface plot (b) 2D contour plot showing the corelation between the selected variables i.e., solvent ratio and scanning speed and response for UV method of Empagliflozin

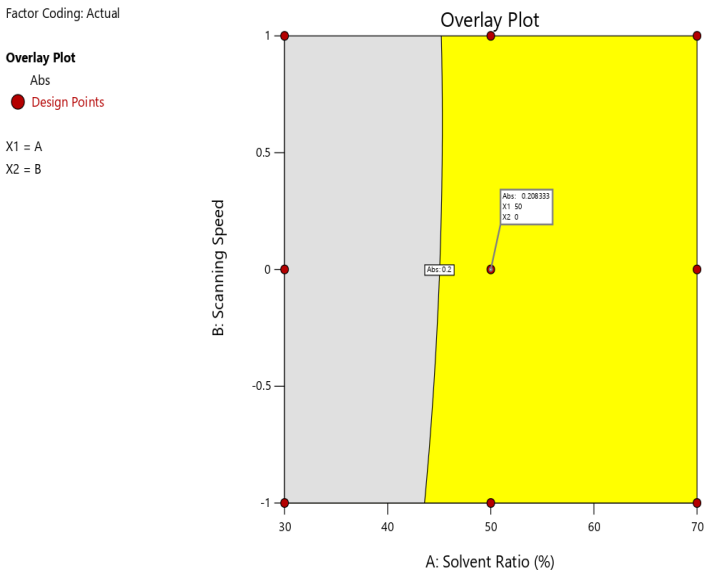


Figure 66: Design space from experimental area for UV method of Empagliflozin

ANALYTICAL METHOD VALIDATION

Specificity and Selectivity: The solvent spectrum obtained showed no interference of absorbance 233nm which show the specificity and selectivity of method. UV spectrum of solvent and Empagliflozin were showed in **Figure 67.**

Linearity and range: The standard calibration curve was plotted using concentration *vs* absorbance obtained by linear dilution of Empagliflozin. Each concentration showed linear absorbance range between the concentration range of 4, 8, 12, 16, 20µg/mL with regression equation of 0.9998 for Empagliflozin. Linearity data was presented in **Table 84**. Standard calibration curve was presented in **Figure 68** The overlay spectrum of linearity of Empagliflozin was showed in **Figure 69.**

LOD and LOQ: The Limit of Detection and Limit of Quantification was found to be 0.36µg/ml and 1.10µg/ml respectively using absorbance method.

Precision: Method was found to be precise as the % RSD calculated for three replicates solution of Empagliflozin at each precision level was found to be less than 2%. **Table 85** represents the data of interday and intraday precision.

Ruggedness: The method was found to be rugged as the %RSD of each concentration performed by different analyst and on different instrument was within the acceptance. **Table 86** represents the ruggedness data.

Accuracy: The method was found to be accurate as all the level recovery values well within the acceptance (97.35 to 101.79%). **Table 87** represents the data of accuracy.

Assay: The % assay of drug content was found to be 99.80±0.12% (n=3) for label claim of Empagliflozin. The assay result indicates selectivity of the developed method.

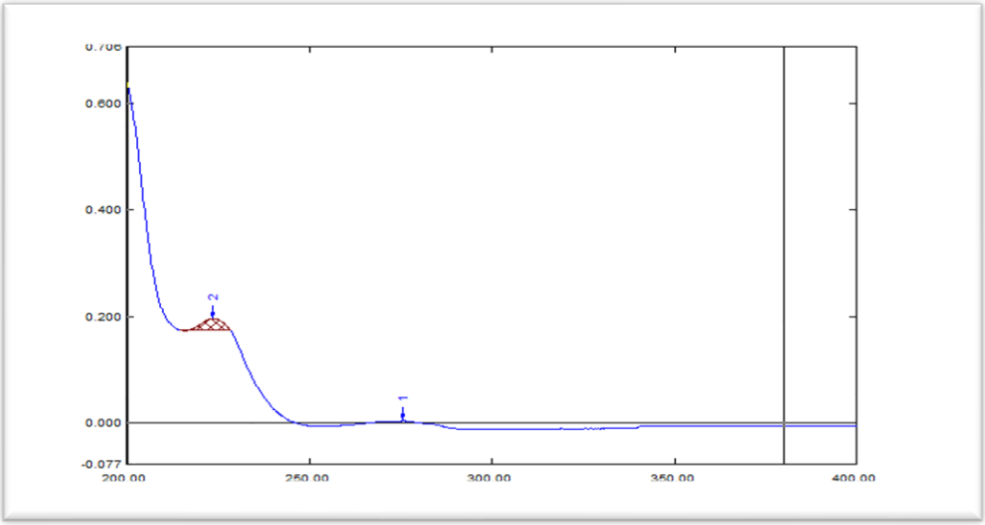


Figure 67: UV-Spectrum of Empagliflozin (10µg/mL)

Table 84: Linearity data of Empagliflozin by UV-spectroscopic method

Sl. No.	Concentration	Absorbance at 233nm
1	4µg/ml	0.208
2	8µg/ml	0.406
3	12µg/ml	0.618
4	16µg/ml	0.832
5	20µg/ml	1.025
	r^2	**0.9998**

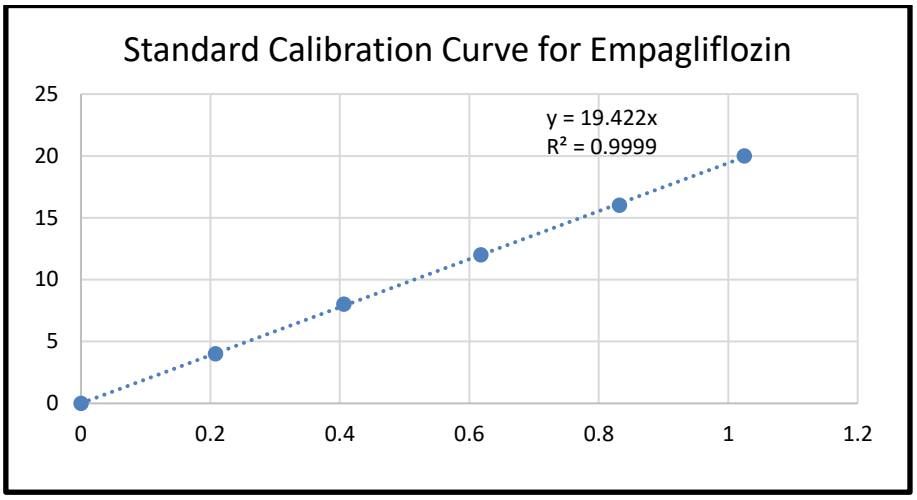


Figure 68: Calibration Curve of Empagliflozin by UV method

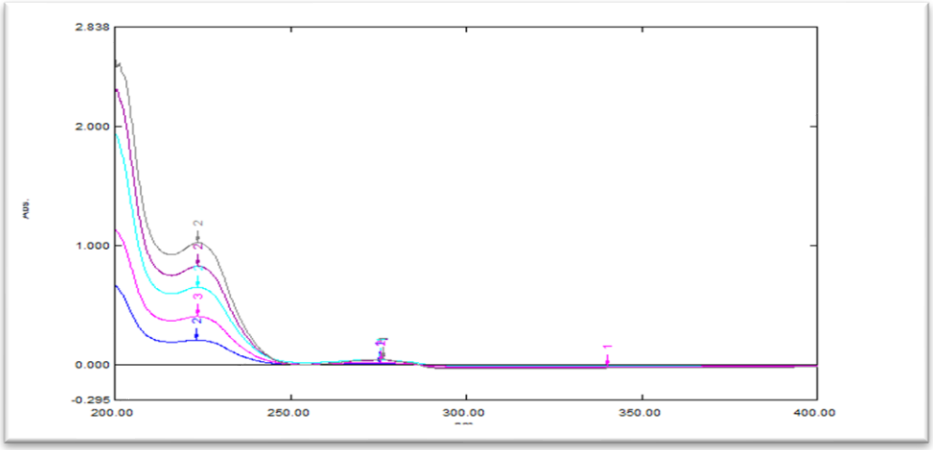


Figure 69: Linearity overlay spectrum of Empagliflozin

Table 85: Precision data of Empagliflozin by UV Spectroscopic method

Type	Intraday Precision			Interday Precision		
Conc	**4 µg/ml**	**12 µg/ml**	**20 µg/ml**	**4 µg/ml**	**12 µg/ml**	**20 µg/ml**
Mean Abs.	0.211	0.617	1.030	0.203	0.613	1.018
SD	0.003	0.001	0.003	0.001	0.002	0.001
%RSD	**0.19**	**0.18**	**0.31**	**0.49**	**0.24**	**0.11**

Table 86: Ruggedness data of Empagliflozin by UV spectroscopic method

Concentration	Absorbance (Change in Analyst)	Absorbance (Change in Instrument)
4µg/ml (n=6)	0.204	0.214
SD	0.004	0.003
%RSD	1.767	1.496

Table 87: Accuracy data of Empagliflozin by UV spectroscopic method

Levels %	Amount of sample (µg/ml)	Amount of standard (µg/ml)	Total amount (µg/ml)	Mean Amount Recovered (µg/ml) ± SD	Mean % Recovery (µg/ml) ± SD
50	2	6	8	8.17 ±0.05	101.79 ± 0.72
100	2	12	14	14.04 ±0.07	100.26 ± 0.48
150	2	18	20	19.47 ±0.17	97.35 ± 0.85

CONCLUSION

The current study highlights the creation of a simple, reproducible, sensitive, and cost-effective analytical UV-spectrophotometric approach for determining Empagliflozin using systematic QbD principles. Following adoption of the ATP and CAAs, CMVs were chosen using risk assessment studies based on the Ishikawa fishbone diagram. The experimental design methodology, which utilized a CCD for optimization, offered a thorough grasp of the link between distinct response components and their interactions. The new analytical method was validated using the various parameters provided in the ICH standards. The findings indicated that the suggested method has good linearity, accuracy, and precision characteristics. Furthermore, the calculated LOD and LOQ values indicated that the new approach is highly sensitive. By adopting QbD principles and following to numerous factors mentioned in ICH Q2 recommendations, the optimized analytical approach shows promising applicability in QC laboratories for assessing Empagliflozin in the industry.

METHOD 9: DEVELOPMENT AND VALIDATION OF UV- METHOD FOR ESTIMATION OF LINAGLIPTIN

METHOD DEVELOPMENT

Preliminary solubility study and determination of λmax of Linagliptin: The solubility of Linagliptin was performed in various solvent and reported as Linagliptin is soluble in Methanol, ethanol, very slightly soluble in water, isopropyl alcohol and acetone. The λmax of Linagliptin in Methanol is recorded as mentioned: 1µg/ml of solution containing standard Linagliptin was scanned between 400-200nm in UV-Spectrophotometer. A thorough literature survey revealed that Linagliptin is soluble in Methanol. In order to select the solvent, few trials were carried out in different solvents. Many literatures reported UV estimation using Methanol as solvent. In our research work we have planned to use DW for estimation. The solvent composition was optimized by using QbD approach. [101, 148, 149]

Preparations of Standard Stocks of Linagliptin: Linagliptin standard stock solution (1000µg/ml) was made in a 10ml VF by dissolving 10mg and diluting to volume with Methanol. To achieve a concentration of 100µg/ml, 1ml was taken from the stock and added to a 10ml VF with Methanol and DW as solvents. The stock was serially diluted to prepare concentrations of 1 µg/ml, 2 µg/ml, 3 µg/ml, 4 µg/ml, and 5 µg/ml. In the optimization investigation, secondary and working stock solutions containing 100µg/ml of Linagliptin were produced using Methanol and water in the following ratios: 30:70, 50:50, and 70:30 v/v.

METHOD OPTIMIZATION BY QBD [36, 146,147]

In this study, we investigated numerous features of AQbD and applied them to a practical technique.

Method optimization by DoE software: After completing a risk analysis to identify the Critical Method Variables, the focus switched to optimizing two critical method variables: solvent composition and wavelength of detection. The remaining method variables were held constant at either their minimum or maximum values. Using the CCD within the framework of DoE (DoE) with an alpha value of one, the goal was to establish the best levels for solvent ratio (A) and wavelength (B) in order to improve the method's reliability. The CCD method required nine runs for the two specified variables, including one centered point. The study of the selected independent variables, namely solvent ratio (A) and wavelength (B), centered on a single response variable: Linagliptin absorbance. Design Expert Software Version 11 by Stat-Ease Inc. was used to determine the link between the independent and dependent variables. To ensure dependability and precision, all experiments in the optimization studies were carried out in triplicate. The Design Expert software was then used to enter data and construct a coded equation (Equation 1) that represented the appropriate mathematical model. This equation accurately depicts the link between the independent method variables and the dependent variables or answers, resulting in a simple representation of the investigated data.

$Y= X_0 + X_1 A + X_2 B + X_3 AB + X_4 A^2 + X_5 B^2$ Equation 1

In the coding equation, Y is the response variable, X0 is the intercept, and X1 and X2 are the coefficients for variables A (solvent ratio) and B (wavelength), respectively. Furthermore, X3 shows the coefficient of the correlation term between variables A

and B, demonstrating how they interact. Furthermore, X4 and X5 are the coefficients of the quadratic terms for the chosen components, emphasizing any non-linear interactions between the independent solvent ratio (A) and wavelength (B). This coded equation is a simple representation of the mathematical model that describes the interaction between the independent and dependent variables during the optimization process.

Data analysis and model validation: ANOVA was performed using the Design Expert software to extract important metrics such as p-value, f-value, R2 value, acceptable precision, and so on. Multiple polynomial equations were developed, each with a significant p-value less than 0.5. For both crucial method variables, plots. The main aim of these graphs was to determine the effect of scanning speed and solvent ratio, which were considered as independent factors, on the dependent variables/Absorbance. This detailed study sheds light on the links and implications of the specified procedure variables on the analytical results. To visually show the link between the selected independent method variables and response variables, 2-D contour graphs and 3-D response surface graphs were created. These charts show how changes in scanning speed and solvent ratio, as independent factors, affect the absorbance. In addition, overlay graphs were generated to show the design space over all experimental areas. These visual representations are useful tools for comprehending the intricate relationships and ideal circumstances inside the multidimensional design space, as well as for interpreting and optimizing the analytical approach itself.

ANALYTICAL METHOD VALIDATION

Selectivity and specificity: To ensure the absence of solvent interference at the peak absorbance region of Linagliptin, specificity tests were conducted. This involved analysing spectra of both the solvent system used for dilution and the Linagliptin solution. By comparing these spectra, any irregularities in the Linagliptin signal at its maximum wavelength (lambda max) due to the solvent system could be identified, thus affirming the specificity and selectivity of the analysis.

Linearity and Range: To assess the linearity of the developed method, a serial dilution of the Linagliptin stock solution ranging from 1 to 5 µg/ml was analyzed in triplicate. The calibration curve, depicting absorbance against Linagliptin concentration, was plotted based on these measurements. Verification of the method's linearity involved conducting regression statistical analysis on the collected data using an MS Excel spreadsheet.

Ruggedness: It was carried out by having different analysts execute the same analysis on the same instrument as well as on another. Six replicate solutions containing 1 µg/ml and 3 µg/ml of Linagliptin were tested. Calculated the percentage RSD for the absorbance values obtained.

Accuracy: The assessment of accuracy employed the addition method, where a sample solution of Linagliptin (2 µg/ml) was spiked with 80% (6 µg/ml), 100% (12 µg/ml), and 120% (18 µg/ml) of standard Dapagliflozin. To validate the accuracy of the results, recovery and percent Relative Standard Deviation (RSD) were calculated, ensuring the correctness of the obtained data.

RESULTS AND DISCUSSION

In the solvent development stage, the chosen solvent system comprised Methanol: DW (50:50% v/v). Linagliptin exhibited a spectrum with the highest absorbance at 227nm, as illustrated in **Figure 70**. The optimization of the method was conducted following QbD principles, as detailed in the methodology section. The optimized parameters of the method are outlined in **Table 88**.

Table 88: Developed UV-Spectrophotometric method parameters for Linagliptin

Sr. No.	Parameters	Specifications
1	Method	Spectrophotometric
2	Instrument	UV-Spectrophotometer
3	Model	Shimadzu
4	Make	UV-1800
5	Software	UV-Probe
6	Analyte	Linagliptin
7	Solvent	Methanol: Millipore water (50:50% v/v)
8	Lambda Max.	227nm

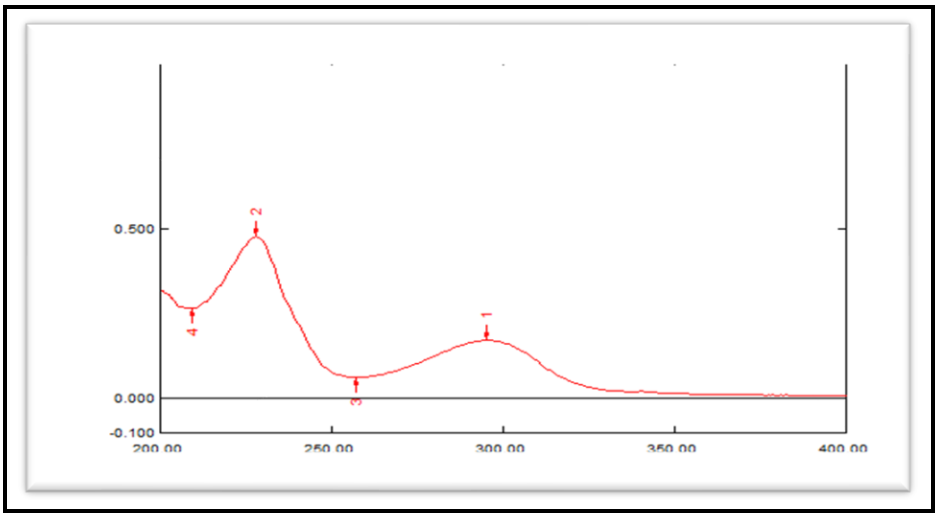


Figure 70: UV-Spectrum of Linagliptin showing λmax at 227nm

METHOD OPTIMIZATION BY QBD APPROACH

Analytical target profile: ATP outlines the expected method requirements for the measurement. The analytical target profile is defined using knowledge and scientific reasoning about the analysis process. **Table 89** represents ATP for proposed UV-spectrophotometric method for Linagliptin.

Table 89: ATP for UV-spectrophotometric analysis of Linagliptin

Sl. No.	ATP Parameters	Target
1	Target Analyte	Linagliptin
2	Target Sample	Linagliptin Tablets
3	Method category	UV-spectrophotometric method
4	Instrument requirement	UV-spectrophotometer
5	Nature of analyte	Solid (Solution)
6	Standard stock solution preparation	Dilution of main drug in linear manner.
7	Application of Method	Estimation of Linagliptin
8	Validation parameters	Accuracy, ruggedness, precision, linearity, specificity, LOD and LOQ

Method optimization by DOE Software: The approach was further refined using the CCD inside the framework of DoE with an alpha value of one. The goal was to establish the best levels for solvent ratio (A) and wavelength (B) in order to improve the method's resilience. **Table 90** shows the selection of levels for CCD design. **Table 91** shows that the CCD technique required 9 passes for the two selected variables, one of which was centered. After doing the experiments indicated by CCD, the results were entered into the software and evaluated further.

Table 90: Selection of levels for CCD for Linagliptin UV spectroscopic method

Independent Variable	Levels		
	+1	**0**	**-1**
Solvent Ratio (A)	70 %	50%	30%
Wavelength (B)	228	227	226

Table 91: Experimental design matrix by CCD for Linagliptin UV method

Run	A: Solvent Ratio	B: Scanning Speed	Absorbance
1	30	226	0.197
2	**50**	**227**	**0.268**
3	30	228	0.198
4	30	227	0.195
5	70	227	0.306
6	50	226	0.27
7	70	228	0.305
8	50	228	0.272
9	70	226	0.301

Data analysis: The essential analytical variables were optimized using a statistical design. Using the Design Expert program, a design plan was constructed that included 9 experimental runs for the identified independent parameters, solvent ratio and wavelength, using the CCD technique. **Table 91** details this experimental design. The chosen responses or dependant variables for evaluation at absorbance value. The experimental data was examined using multiple linear regression analysis. Following the development of the mathematical model utilizing software, the data was examined using a second-order quadratic model established via a coded equation.

Responses Equation in Coded form: Factor equations were employed in coding to anticipate the relationship between independent and dependent variables across different levels, categorized as either low (-1) or high (+1).

Abs= +0.2694+0.0537*A+0.0012*B+0.0007*AB-0.0197*A^2+0.0008*B^2

In the formulae, A denotes the solvent ratio and B stands for wavelength. The chosen factors were statistically analyzed using ANOVA. The lack of fit, R2, and adjusted R2 values were used to assess the model's significance, with a p-value of less than 0.05 confirming adequacy. A strong "Lack of fit" indicates that the model fails to adequately explain the differences between predicted and observed data points. ANOVA R2 values close to 1 reflect how closely the predicted model matches the experimental model, with values near 1 being preferable. **Table 92** shows multiple ANOVA analyses for the chosen response, taking into account criteria such as the p-value, lack of fit, and R2 values. The results in Table 92 show that both the model and the factors have p-values less than 0.05, indicating the importance of the developed model. The F-values for the answer are 729.39, demonstrating the model's relevance. In the created model, the F-value-based error rate is calculated to be 0.01%. The analysis also supports the second-order quadratic model, with an adjusted R2 of 0.999, which is close to one. The substantial model F-value of 729.39 signifies the significance of the model, with only a 0.09% probability of the F-value occurring by chance. Model terms are considered significant if their p-values are less than 0.05, and in this case, both A and A2 are found to be significant. Conversely, values exceeding 0.1000 indicate insignificance of the model terms. If there are numerous significant model terms, model reduction could be considered as a potential strategy.

Figure 71 shows various graphs generated in the design, including the expected vs. actual graph, that demonstrate satisfactory response criteria in comparison to variables. **Figures 72 (a) and (b)** show 2-D and 3-D graphs created with Design Expert software to demonstrate the link between factors and response. The study of these graphs revealed that absorbance declines with intermediate and low values of both selected variables, namely solvent ratio and wavelength.

Design space: Figure 73 depicts the essential design space for optimizing the selected factors (solvent ratio and wavelength) in relation to the desired response (absorbance). The overlay graph shows the design space in dark yellow within the experimental area in grey, while the light-yellow region represents the area where many elements can be changed

Table 92: ANOVA results for CCD of Linagliptin UV spectroscopic method

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	0.0181	5	0.0036	729.39	< 0.0001	significant
A-Solvent Ratio	0.0173	1	0.0173	3488.43	< 0.0001	
B-Wavelength	8.167E-06	1	8.167E-06	1.65	0.2893	
AB	2.250E-06	1	2.250E-06	0.4542	0.5486	
A^2	0.0008	1	0.0008	156.16	0.0011	
B^2	1.389E-06	1	1.389E-06	0.2804	0.6331	
Residual	0.0000	3	4.954E-06			
Cor Total	0.0181	8				

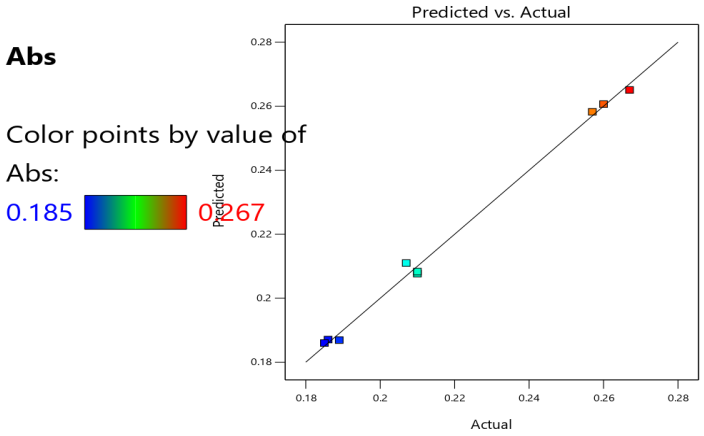
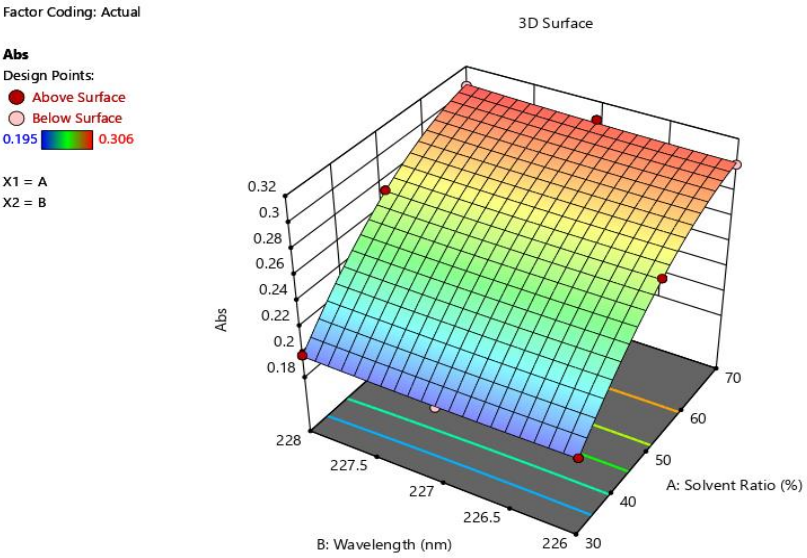


Figure 71: Predicted vs actual plot generated by Design Expert software for Linagliptin UV analysis

(a) 3D Response surface plot



(b) 2D contour plot

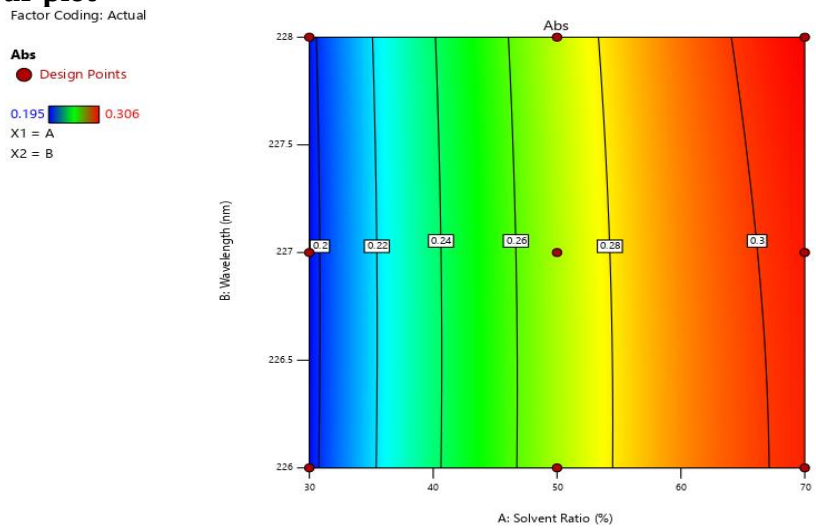


Figure 72: (a) 3D Response surface plot (b) 2D contour plot showing the corelation between the selected variables i.e., solvent ratio and wavelength and response for Linagliptin UV analysis

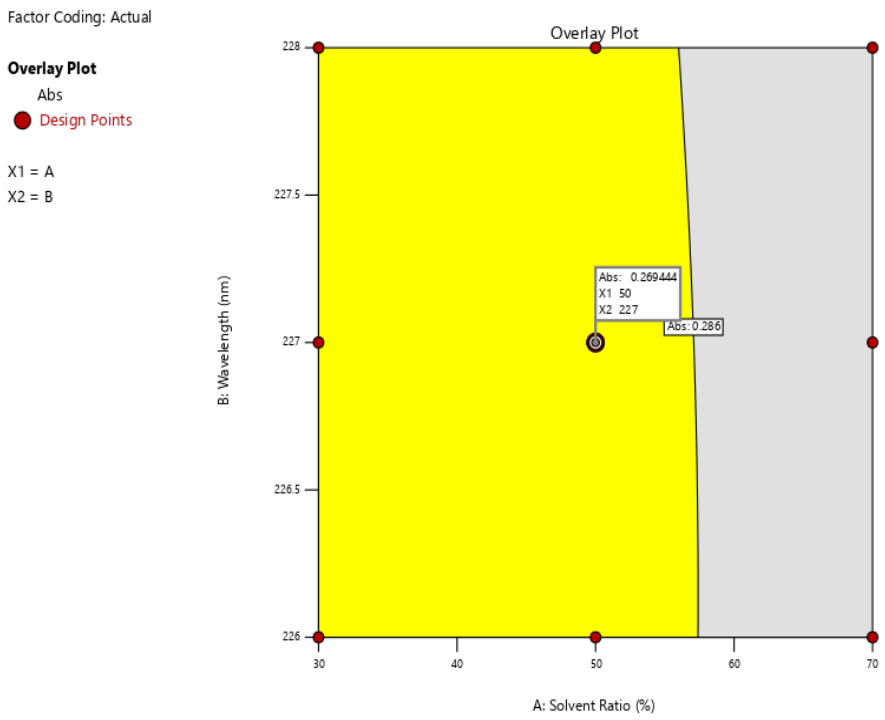


Figure 73: Overlay plot showing the design space from experimental area for Linagliptin UV analysis

ANALYTICAL METHOD VALIDATION

Specificity and selectivity: The solvent spectrum generated displayed no interference of absorbance at 227nm, confirming the specificity and selectivity of the method. **Figure 74b** presents the UV spectrum of linagliptin.

Linearity and range: The calibration curve was created by graphing the concentration vs. absorbance values obtained from each linear dilution of Linagliptin. The linear absorbance range for each concentration was 1, 2, 3, 4, and 5µg/ml, with regression equations of 0.9998 and 0.9998. **Table 93** contains linearity range data, Figure 75 depicts the overlay spectrum of Linagliptin's linearity, and **Figure 76** depicts a standard calibration curve.

LOD and LOQ: LOD value of Linagliptin was found to be 0.23 µg/mL. LOQ of value of Linagliptin was found to be 0.71 µg/mL.

Precision: The precision of the proposed method was established as high, as evidenced by the percentage Relative Standard Deviation (RSD) being less than 2% for six replicates of the Linagliptin solution at each precision level (refer to Table 94). The UV spectrum of precision studies is depicted in Figure 77.

Ruggedness: The percentage Relative Standard Deviation (% RSD) values for Linagliptin were consistently below 2%, indicating the robustness and reproducibility of the procedure. Table 95 demonstrates that the % RSD values for the absorbance of each replicate solution were within acceptable limits for both the analyst and the equipment used.

Accuracy: The accuracy of the method was confirmed, as all level recovery values fell comfortably within the acceptable range. Refer to Table 96 for detailed data on accuracy.

ESTIMATION OF LINAGLIPTIN IN MARKETED TABLETS

Sample extraction: Twenty commercially available Linagliptin tablets were weighed to determine their average weight. Subsequently, a quantity equivalent to 10 mg of Linagliptin in powder form was weighed and transferred to 10 mL of VF. Extraction was carried out using suitable solvents (Methanol and MW) along with sonication for 15 minutes. Following extraction, a serial dilution was performed to attain a concentration of 12 µg/ml, and the absorbance was measured. This information was then utilized to calculate the percentage assay in the marketed sample.

Estimation: The drug content's percentage assay for Linagliptin label claim was determined to be 98.24±0.58% (n=3). These assay results serve as evidence of the selectivity of the UV-Spectrophotometric technique developed through Quality by Design (QbD) for accurately estimating Linagliptin in pharmaceutical dose forms.

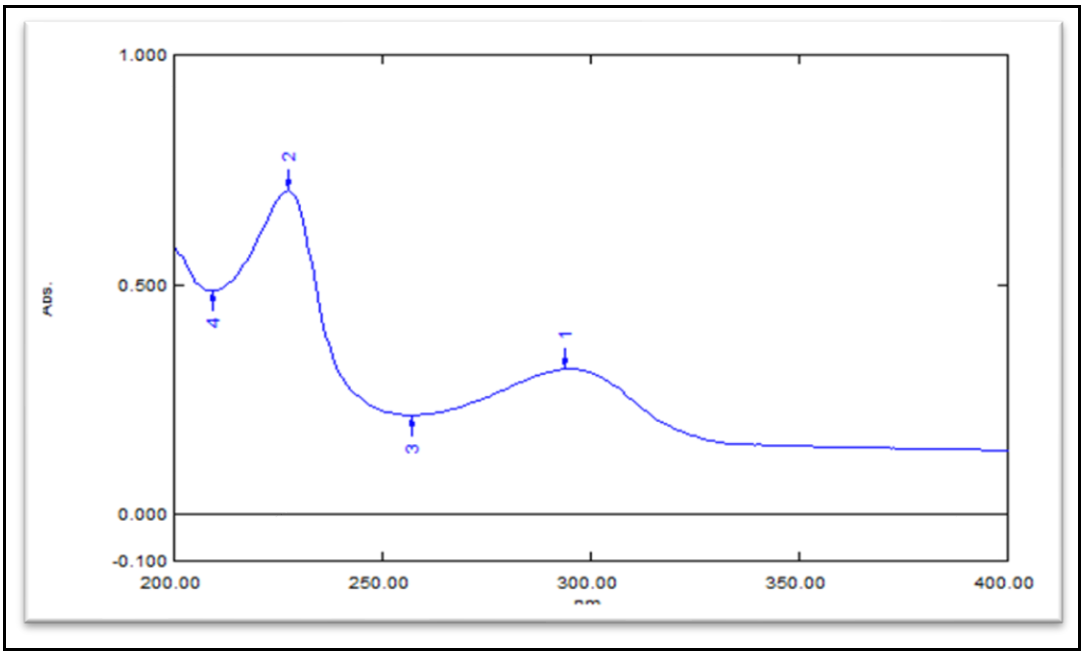


Figure 74: Spectrum of Linagliptin

Table 93: Linearity and range data of Linagliptin by UV method

Sr. No.	Concentration	Absorbance at 227 (n=3)
1	1µg/mL	0.138
2	2µg/mL	0.272
3	3µg/mL	0.433
4	4µg/mL	0.579
5	5µg/mL	0.743
r^2		**0.9998**

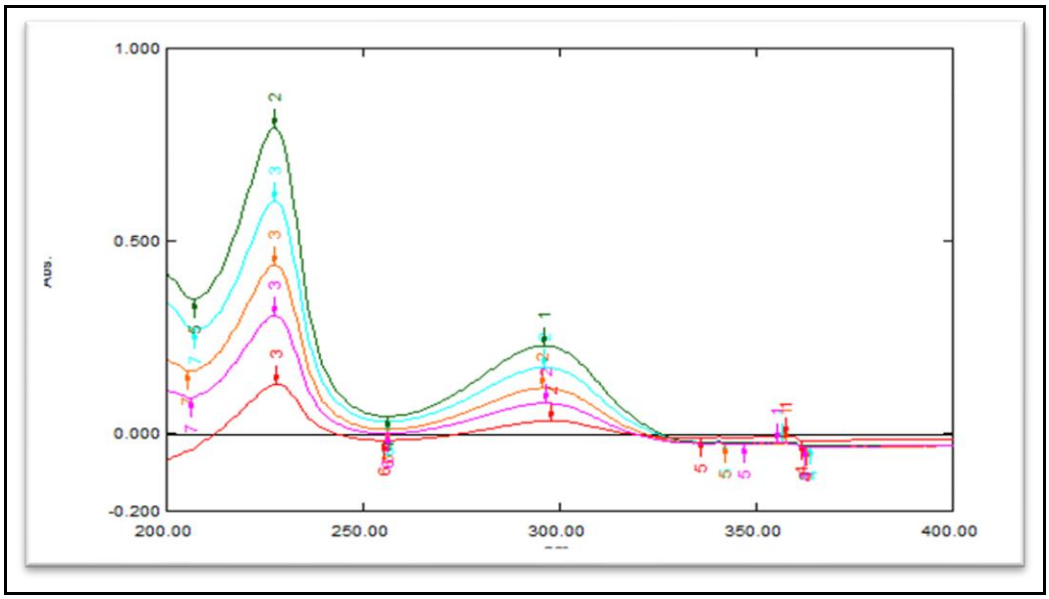


Figure 75: Overlay UV-Spectrum of Linagliptin

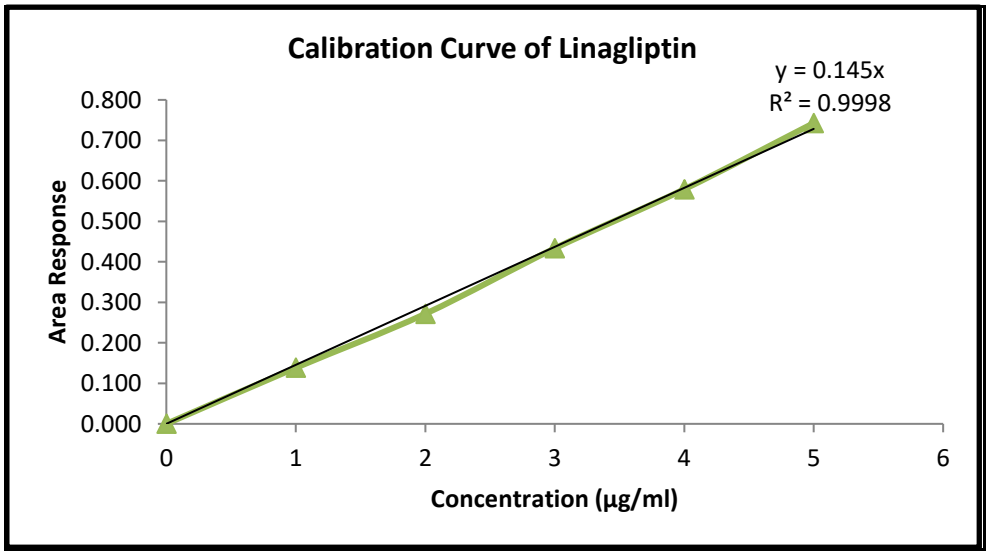


Figure 76: Standard Calibration Curve of Linagliptin

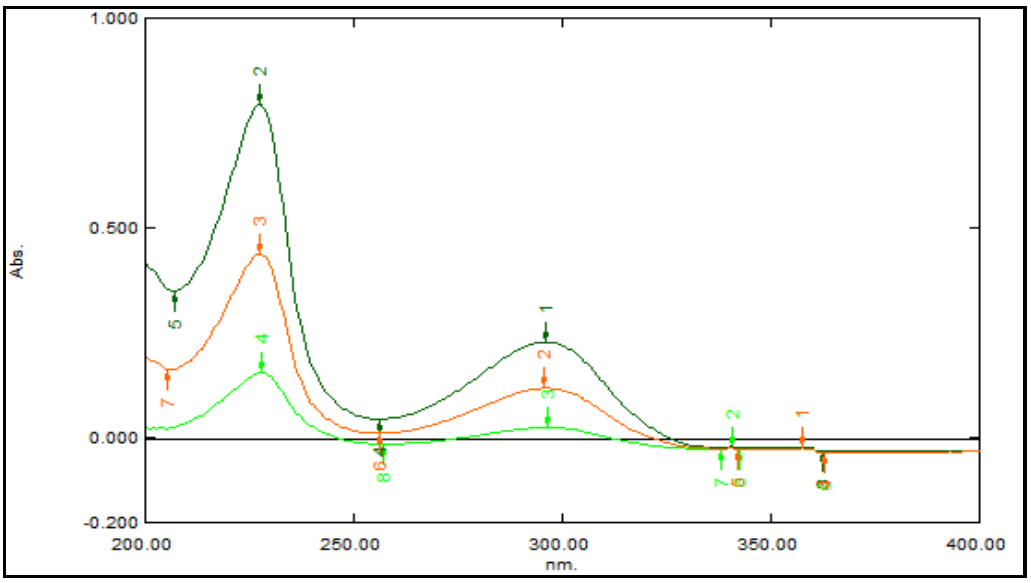


Figure 77: UV-Spectrum of Linagliptin for Precision Study

Table 94: Precision data of Linagliptin by UV spectroscopic method

Precision	Interday-1	Interday-2	Interday-3
Concentration	1 µg/mL	3 µg/mL	5 µg/mL
Mean	0.169	0.560	0.755
SD	0.001	0.001	0.008
% RSD	0.69	0.23	0.46
Precision	**Intraday 1 hr**	**Intraday 2 hr**	**Intraday 3 hr**
Concentration	1 µg/mL	3 µg/mL	5 µg/mL
Mean	0.188	0.472	0.773
SD	0.001	0.004	0.002
% RSD	0.29	0.78	0.26

Table 95: Ruggedness data of Linagliptin by UV spectroscopic method

Analysis by	Change in Analyst	Change in Instrument
Replicates	**Absorbance at 227 nm (1 µg/mL)**	**Absorbance at 227 nm (3 µg/mL)**
Mean	0.152	0.435
SD	0.002	0.001
% RSD	**1.29**	**0.23**

Table 96: Accuracy data of Linagliptin by UV spectroscopic method

Levels %	Amount of test taken (µg/ml)	Amount of std. added (µg/ml)	Total amount (µg/ml)	Mean Amount Recovered (µg/ ml) ± SD	Mean % Recovery (µg/ ml) ± SD
50	0.5	1.5	2	1.96 ±0.03	96.99 ± 1.73
100	0.5	3	3.5	3.51 ±0.03	100.19 ± 0.72
150	0.5	4.5	5	5.14 ±0.04	102.00 ± 0.70

CONCLUSION

The proposed UV-Spectrophotometric approach was found to be simple, precise, and accurate for measuring Linagliptin in marketed products. The study applies QbD concepts to identify CMVs through risk assessment using the Ishikawa fishbone diagram. Using risk surface technique with CCD optimization helps to explore the linkages and interactions between response components. The developed analytical approach is validated according to ICH requirements. The results demonstrate the method's uniqueness, linearity, accuracy, and precision, with LOD and LOQ values showing good sensitivity. The inclusion of QbD and ICH guidelines supports the prospective use of the optimized approach in pharmaceutical QC labs for Linagliptin estimate. This method is deemed appropriate for routine Linagliptin estimation in marketed pharmaceutical preparations.

METHOD 10: DEVELOPMENT AND VALIDATION OF CONDUCTOMETRIC METHOD FOR METFORMIN HCL

MATERIALS AND METHODS

METHOD DEVELOPMENT

The conductometric approach was developed with an electrical conductivity meter and MW. Different concentrations of Metformin HCl in water were produced and electrical conductivity was tested. The analyte concentrations resulted in different conductance values; as the concentration increased, the electrical conductance produced by the solution also found to be increased. To determine the suitability of the developed approach, the commercial tablet formulation of Metformin HCl was examined. The electrical conductivity meter (ELICO CM 183EC-TDS analyser version 2.3) was utilized for the analysis. [122]

Standard stock solution of Metformin HCl: To get 1000 µg/mL of Metformin HCl, 50 mg was carefully weighed and transferred to a 50 mL calibrated VF. MW was then added to make up the volume up to the mark.[122]

Working standard solutions of Metformin HCl: To produce a series of concentrations ranging from 50-250 µg/mL of Metformin HCl, different volumes were pipetted from the original stock and transferred to 50 mL VFs and volume was made up to the mark using Milli-Q Water. [122]

QUALITY BY DESIGN APPROACH [36, 38, 151]

In this study, we focused on several aspects of AQbD and applied them to a practical technique. It was implemented in the following steps: defining the analytical target profile, identifying critical analytical features, determining critical technique parameters, and conducting risk assessments.

Defining analytical target profile: A thorough assessment of available literature reports and drug profiles was conducted in order to develop an analytical target profile that included a dynamic overview of the quality attributes of an analytical method.

Identification of CAAs:" CAAs for analytical methods include method parameters and method features. In the suggested conductometric study, a significant analytical attribute was identified as electrical conductance.

Identification of critical method parameters : The CMPs are the sensitivity levels connected with the analytical procedure. In the instance of the suggested conductometric approach, the solvent utilized for analysis and the temperature were recognized as crucial parameter.

Risk Assessment: For the suggested conductometric measurement of Metformin HCl, it was revealed that the important technique variables, such as the temperature and the solvent utilized in the study, were highly risky.

METHOD VALIDATION [122, 150]

Selectivity and specificity: By measuring the electrical conductance of a blank and standard solution of metformin HCl (100 µg/mL), the specificity and selectivity of the proposed conductometric approach were examined.

Linearity and range: The electrical conductivity of various concentrations of metformin HCl was measured in order to determine the linearity and range of the suggested analytical approach. Electrical conductance of each dilution, comprising 50–250 µg/mL of metformin HCl, was measured in triplicate. Graphing the concentration against electrical conductivity (µS) yielded the calibration curve, from which the correlation coefficient (r2) value was calculated.

Precision: The precision was tested both within and between days. Three replicate solutions of metformin HCl at three distinct concentrations were tested for electrical conductivity, and each solution's relative standard deviation (RSD) was calculated.

Ruggedness: Ruggedness was tested by changing out the analyst in order to verify that standard results could be repeated. Six identical solutions with 50 µg/mL of metformin HCl were made by several analysts and put through conductometric testing.

Accuracy: Recovery studies were used to determine the accuracy of the sample recovery percentage at three different levels: 50%, 100%, and 150%. A percentage mean recovery was obtained after three determinations were made at each level.

ESTIMATION OF METFORMIN HCL IN MARKETED TABLETS

In order to see the applicability of proposed method, a marketed tablets containing Metformin HCl were estimated. Twenty Metformin HCl 500mg tablets were weighed and powdered. A powder equivalent to 500 mg of Metformin HCl was weighed and put to a 100 mL VF. MW was used to form the mark of 100 mL, and the solution was sonicated for 20 minutes before being filtered through Whatman filter paper. The filtrate was diluted to a concentration of 100µg/mL. The produced solutions were used to measure the conductance and calculate the assay.

RESULTS AND DISCUSSION

METHOD DEVELOPMENT

We have selected Metformin HCl, an oral anti-diabetic medication that is mainly sold as tablets, for the suggested study. In this study, we generated concentrations of metformin HCl ranging from 50 to 250 µg/mL and evaluated the conductance for each concentration using a unique approach. It was observed that the conductance value increased linearly with the analyte concentration. **Table 97** displays the parameters of the optimized approach.

Table 97: Developed Conductometric method for analysis of Metformin HCl

Sr. No.	Parameters	Description
1	Technique	Conductometric
2	Machine	Electrical Conductometer
3	Model	Elico
4	Drug	Metformin HCl
5	Diluent	MW

QUALITY BY DESIGN APPROACH

Defining analytical target profile: ATP outlines the necessary steps for the approach that will be measured. With the aid of scientific reasoning and analytical process knowledge, the analytical goal profile is specified. Thus, in order to quickly analyse metformin HCl, a conductometric method was used in accordance with the main goal of this investigation. The selection of **Table 98** as the representative ATP for the suggested Conductometric technique for Metformin HCL measurement was justified rationally.

Table 98: ATP for Conductometric analysis of Metformin HCl

Sl. No.	ATP Parameters	Target
1	Target Analyte	Metformin HCl
2	Target Sample	Metformin HCl Tablets
3	Method category	Electrochemical Method
4	Instrument requirement	Conductometer
5	Nature of analyte	Solid (Solution)
6	Standard stock solution preparation	Dilution of main drug in linear manner.
7	Application of Method	Estimation of Metformin HCl
8	Validation parameters	Accuracy, ruggedness, precision, linearity, specificity, detecting limit, and quantifying limit

METHOD VALIDATION

Specificity and selectivity: The results of the investigation showed that the suggested approach was both selective and specific, with the conductance of the 100µg/ml solution of metformin HCL measuring 60.66 µS, and the conductance of water, used as a blank, measuring 1.18µS.

Linearity and range: Metformin HCL showed a linear relationship between electrical conductivity (µS) and drug concentration at 50 to 250 µg/ml. It was found that the equation of the straight line, with a regression coefficient of r2, is 0.9999. The results are shown in **Table 99,** and the standard calibration is displayed in **Figure 78.**

Detecting limit and quantifying limit: The detecting limit and quantifying limit values of metformin were found to be as 3.12 µg/ml and 9.47 µg/ml, respectively.

Precision: The percent RSD values calculated for three replicates of the Metformin HCL solution at each accuracy level were found to be less than 2%, indicating the precision of the proposed conductometric approach. **Tables 100** and **Table 101** provide the interday and intraday precision data.

Ruggedness: Upon testing the process's ruggedness by another expert, it was discovered that the process's percentage RSD values for the specified conductance were less than 2%. **Table 102** presented the toughness experiment statistics.

Accuracy: The three recovery levels 100%, 150%, and 50% were used in the studies, and the individual percentage mean recovery was found to range from 99 to 100%, suggesting that the suggested method for estimating metformin HCL is accurate. The accuracy experiment's data and percentage mean recovery values are shown in **Table 103**.

CONDUCTOMETRIC ASSAY METFORMIN HCL

500 mg of Metformin HCL tablets were used to test the suitability of a recently developed and approved conductometric technique. The test result for commercially available tablets of metformin HCL was discovered to be 99.78±0.20%.

Table 99: Linearity analysis of Metformin HCL by Conductometric method

Sl. No.	Concentration (µg/mL)	Mean Electrical conductivity (µS) (n=3)
1	50	30.54
2	100	61.35
3	150	90.87
4	200	120.45
5	250	150.07
	r^2	**0.9999**

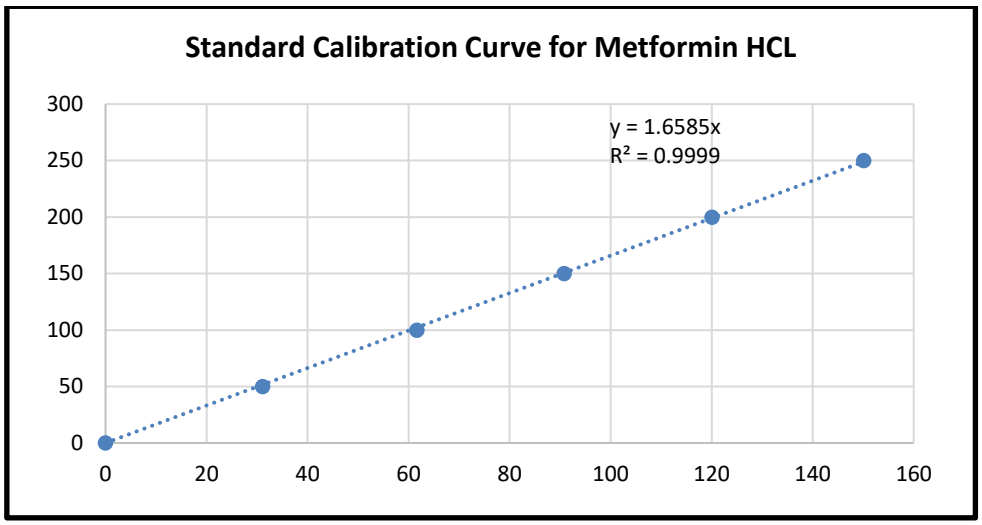


Figure 78. Calibration curve of Metformin HCl by conductometric method

Table 100: Intraday precision of Metformin HCl by Conductometric approach

Concentration	Time	Conductance (µS)($\bar{x}$)	SD	%RSD
50 µg/mL	1st hr	30.62	0.51	1.65
	2nd hr	30.74	0.36	1.18
	3rd hr	30.86	0.23	0.75
150 µg/mL	1st hr	91.03	0.07	0.08
	2nd hr	90.83	0.06	0.07
	3rd hr	91.02	0.18	0.20
250 µg/mL	1st hr	150.78	0.59	0.39
	2nd hr	150.56	0.47	0.31
	3rd hr	150.77	0.40	0.27

Table 101: Interday precision of Metformin HCl by Conductometric approach

Concentration	Day	Conductance (µS)($\bar{x}$)	SD	%RSD
50 µg/mL	1	31.18	1.12	0.58
	2	30.94	0.16	0.51
	3	30.82	0.17	0.55
150 µg/mL	1	90.76	0.05	0.06
	2	91.02	0.19	0.21
	3	90.97	0.08	0.08
250 µg/mL	1	150.480	0.33	0.22
	2	151.040	0.12	0.08
	3	150.790	0.60	0.40

Table 102: Ruggedness data of Metformin HCl by Conductometric method

Replicates	Concentration	Analyst-1	Concentration	Analyst-2
Mean (n=6) 50 µg/mL MET HCl		**31.063**	**Mean**	**91.863**
SD		**0.401**	**SD**	**1.173**
%RSD		**1.292**	**%RSD**	**1.277**

Table 103: Accuracy data of Metformin HCl by Conductometric method

Sl. No.	Level	Replicate	% Recovery	Mean % Recovery	%RSD
1	50%	1	99.90	99.90	0.05
		2	99.95		
		3	99.85		
2	100%	1	100.10	100.04	0.06
		2	100.05		
		3	99.98		
3	150%	1	99.86	99.98	0.15
		2	100.15		
		3	99.94		

GREENNESS ASSESSMENT [152,153]

We have converted each of the twelve Green Analytical Chemistry (GAC) codes into a score in order to methodically evaluate the greenness of analytical procedure. The following principles were followed:

1. Analyte treatment must be avoided by using direct analytical procedures
2. Utilizing a small number of samples and a small sample size
3. It is necessary to complete in situ analysis
4. Using cutting-edge strategies and tactics that minimize the usage of chemicals and conserve energy
5. It is necessary to design programmed and minimized techniques.
6. Avoid derivatization.
7. Avoid producing a lot of analytical waste and take the necessary precautions when it does
8. The multi-sample analysis approach is recommended.
9. Use the least quantity of energy possible
10. Reagents and chemicals made from renewable sources should be used whenever possible.
11. It is preferable to use harmless reagents
12. The safety of analysts needs to be considered

The method's greenness was assessed using the Greenness Calculator and the GAC principles as a reference. The GAC fundamentals and the suggested strategy's score are shown in **Table 104.**

Table 104: Score for the conductometric method's greenness assessment

Sl. No.	Green Analytical Chemistry Principles	Computed Rating
1	Analyte treatment must be avoided by using direct analytical procedures	0.60
2	Utilizing a small number of samples and a small sample size	1.0
3	It is necessary to complete in situ analysis	1.0
4	Using cutting-edge strategies and tactics that minimize the usage of chemicals and conserve energy	1.0
5	It is necessary to design programmed and minimized techniques.	0.5
6	Avoid derivatization.	1
7	Avoid producing a lot of analytical waste and take the necessary precautions when it does	1.0
8	The multi-sample analysis approach is recommended.	0.0
9	Use the least quantity of energy possible	1
10	Reagents and chemicals made from renewable sources should be used whenever possible.	1
11	It is preferable to use harmless reagents	0.059
12	The safety of analysts needs to be considered	0.8
	Final score	**0.75**

DISCUSSION

There are no additional solvents or reagents recommended for standard and sample processing in the suggested technique. The method suggests the use of MW as the solvent for analysis. The method involves determining the analyte's electrical conductivity. This makes it possible to do simple examinations quickly and affordably, which is essential in the pharmaceutical sector. In order to assess the marketed drugs containing Metformin HCl and demonstrate their applicability and repeatability during real-time analysis, the suggested approach was applied for quantification in marketed formulation. The efficacy of the proposed approach was evaluated by comparing it with published literature methodologies. [152,153]

CONCLUSION

The proposed research concludes that the successful development of a cost-effective, simple, and accurate conductometric method for analyzing Metformin HCL. The use of water as the analysis solvent, avoidance of extra solvents or costly chemicals, and adherence to green chemistry principles contribute to the method's eco-friendliness. The method is suitable for pharmaceutical samples and can be applied for routine QC makes it valuable for practical applications. The simplicity and cost-effectiveness of the method can potentially lead to more efficient and sustainable processes in the pharmaceutical industry.

Chapter 5

Summary

SUMMARY

TABLE 105: SUMMARY OF RP-HPLC METHOD OF METFORMIN HCL

Sl. No.	Parameters	Reports
1	System Suitability	The developed HPLC method was found to be suitable for the analysis of Metformin HCl as the % RSD values obtained for Retention time, Peak area, Theoretical plates and Tailing factor was found to be less than 2%.
2	Specificity & Selectivity	The proposed method was found to be specific and selective as no interference was observed at the retention time of Metformin HCl peak in standard and sample chromatogram.
3	Linearity and Range	5 to 25 µg/ml with r^2 =0.999
4	Limit of Detection	0.30 µg/ml
5	Limit of Quantification	0.93 µg/ml
6	Precision	The % RSD of intraday and interday precision for Metformin HCl on three times measurements was found to be less than 2% (0.11 -1.41).
7	Ruggedness	The method was found to be rugged by change in the analyst as the % RSD values obtained for peak area was found to be less than 2%.
8	Robustness	The method was found to be robust as the %RSD values obtained for peak areas after making small deliberate variations in developed method parameters was found to be less than 2%.
9	Accuracy	The proposed method shows the high degree of accuracy as the recovery values obtained was found to be 98.98 to 99.79%.

TABLE 106: SUMMARY OF RP-HPLC METHOD OF DAPAGLIFLOZIN

Sl. No.	Parameters	Reports
1	System Suitability	The developed HPLC method was found to be suitable for the analysis of Dapagliflozin as the % RSD values obtained for Retention time, Peak area, Theoretical plates and Tailing factor was found to be less than 2%.
2	Specificity & Selectivity	The proposed method was found to be specific and selective as no interference was observed at the retention time of Dapagliflozin peak in standard and sample chromatogram.
3	Linearity and Range	5 to 25 µg/ml with r^2 =0.9994
4	Limit of Detection	1.26 µg/ml
5	Limit of Quantification	3.83 µg/ml
6	Precision	The % RSD of intraday and interday precision for Dapagliflozin on three times measurements was found to be less than 2%. (0.13 – 1.06%)
7	Ruggedness	The method was found to be rugged by change in the analyst as the % RSD values obtained for peak area was found to be less than 2%.
8	Robustness	The method was found to be robust as the %RSD values obtained for peak areas after making small deliberate variations in developed method parameters was found to be less than 2%. Change in mobile phase conc. = 0.12 – 0.73% Change in flow rate = 0.10 to 0.14 Change in wavelength = 0.11 to 0. 13
9	Accuracy	The proposed method shows the high degree of accuracy as the recovery values obtained was found to be 98.67 to 102.33%.

TABLE 107: SUMMARY OF RP-HPLC METHOD OF EMPAGLIFLOZIN

Sl. No.	Parameters	Reports
1	System Suitability	The developed HPLC technique proved to be suitable for the analysis of Empagliflozin, with % RSD values for Retention time, Peak area, Theoretical plates, and Tailing factor less than 2%.
2	Specificity & Selectivity	The suggested approach was found to be specific and selective, as no interference was detected at the retention period of the Empagliflozin peak in the standard and sample chromatograms.
3	Linearity and Range	5 to 25 µg/ml with r^2 =0.9991
4	Limit of Detection	1.18 µg/ml
5	Limit of Quantification	3.57 µg/ml
6	Precision	The percentage RSD of intraday and interday precision for Empagliflozin was found to be less than 2% after three measurements. (0.13-1.28%)
7	Ruggedness	The approach was found to be rugged by changing the analyst, with % RSD values for peak area was 1.46%.
8	Robustness	The approach was confirmed to be adaptable because the percentage RSD values obtained for peak regions after making slight deliberate modifications in the defined method parameters were less than 2%. Change in mobile phase. Conc.=0.50-0.98% Change in wavelength: 0.19 to 1.46% Change in flow rate = 1.01 to 1.46 percent.
9	Accuracy	The suggested approach demonstrates a high level of accuracy, with recovery values ranging from 98.80 to 101.05%.

TABLE 108: SUMMARY OF RP-HPLC METHOD OF LINAGLIPTIN

Sl. No.	Parameters	Reports
1	System Suitability	The developed HPLC technique proved to be suitable for the analysis of Linagliptin, with % RSD values for Retention time, Peak area, Theoretical plates, and Tailing factor less than 2%.
2	Specificity & Selectivity	The suggested approach was found to be specific and selective, as no interference was detected at the retention period of the Linagliptin peak in the standard and sample chromatograms.
3	Linearity and Range	2 to 12 µg/ml with r^2 =0.9995
4	Limit of Detection	0.307 µg/ml
5	Limit of Quantification	0.932 µg/ml
6	Precision	The percentage RSD of intraday and interday precision for Empagliflozin was found to be less than 2% after three measurements. (0.02 to 0.06%)
7	Ruggedness	The approach was found to be rugged by changing the analyst, with % RSD values for peak area was 0.11 to 0.80%.
8	Robustness	The approach was confirmed to be adaptable because the percentage RSD values obtained for peak regions after making slight deliberate modifications in the defined method parameters were less than 2%. Change in mobile phase. Concentration (0.04-0.09%) and change in flow rate (0.04 – 0.09%).
9	Accuracy	The suggested approach demonstrates a high level of accuracy, with recovery values ranging from 99.68 to 100.44%

TABLE 109: SUMMARY OF RP-HPLC METHOD OF SITAGLIPTIN

Sl. No.	Parameters	Reports
1	System Suitability	The developed RP-HPLC technique proved to be suitable for the analysis of Sitagliptin, with % RSD values for Retention time, Peak area, Theoretical plates, and Tailing factor less than 2%.
2	Specificity & Selectivity	The suggested approach was found to be specific and selective, as no interference was detected at the retention period of the Sitagliptin peak in the standard and sample chromatograms.
3	Linearity and Range	7 to 35 µg/ml with r^2 =0.998
4	Limit of Detection	0.023 µg/ml
5	Limit of Quantification	0.072 µg/ml
6	Precision	The percentage RSD of intraday and interday precision for Empagliflozin was found to be less than 2% after three measurements. (0.07 to 0.68%)
7	Ruggedness	The approach was found to be rugged by changing the analyst, with % RSD values for peak area was 1.08 to 1.80%.
8	Accuracy	The suggested approach demonstrates a high level of accuracy, with recovery values ranging from 97.37 to 100.54%.

TABLE 110: SUMMARY OF DAPAGLIFLOZIN UV SPECTROSCOPIC METHOD

Sl. No.	Parameters	Reports
1	Specificity & Selectivity	Solvent spectrum obtained showed no interference of absorbance 226nm which show the specificity and selectivity of method.
2	Linearity and Range	4 to 20 µg/ml with r^2 =0.9998
3	Limit of Detection	0.63 µg/ml
4	Limit of Quantification	1.92 µg/ml
5	Precision	The percentage RSD of intraday and interday precision for absorbance values of Dapagliflozin was found to be less than 2% after three measurements. (0.21 – 0.75%)
6	Ruggedness	The approach was found to be rugged by changing the analyst, with % RSD values for absorbance was found to be 0.24 – 1.46%.
7	Accuracy	The suggested approach demonstrates a high level of accuracy, with recovery values ranging from 97.43 – 101.81%.

TABLE 111: SUMMARY OF AUC UV METHOD FOR DAPAGLIFLOZIN

Sl. No.	Parameters	Reports
1	Specificity & Selectivity	The solvent spectra obtained revealed no interference at Area Under Curve region produced by standard Dapagliflozin. This shown the specificity and selectivity of the proposed approach.
2	Linearity and Range	4 to 20 µg/ml with r^2 =0.9999
3	Limit of Detection	0.41µg µg/ml
4	Limit of Quantification	1.41 µg/ml
5	Precision	The percentage RSD of intraday and interday precision for absorbance values of Dapagliflozin was found to be less than 2% after three measurements. (0.08-0.84%)
6	Ruggedness	The approach was found to be rugged by changing the analyst, with % RSD values for absorbance was found to be 0.36 – 1.65%.
7	Accuracy	The suggested approach demonstrates a high level of accuracy, with recovery values ranging from 98.04 – 101.90%.

TABLE 112: SUMMARY OF EMPAGLIFLOZIN UV-SPECTROSCOPIC METHOD

Sl. No.	Parameters	Reports
1	Specificity & Selectivity	Solvent spectrum obtained showed no interference of absorbance 233nm which show the specificity and selectivity of method.
2	Linearity and Range	4 to 20 µg/ml with r^2 =0.9998
3	Limit of Detection	0.36 µg/ml
4	Limit of Quantification	1.10 µg/ml
5	Precision	The percentage RSD of intraday and interday precision for absorbance values of Dapagliflozin was found to be less than 2% after three measurements. (0.11-0.49%)
6	Ruggedness	The approach was found to be rugged by changing the analyst, with % RSD values for absorbance was found to be 1.49 - 1.76%.
7	Accuracy	The suggested approach demonstrates a high level of accuracy, with recovery values ranging from 97.35 - 101.79%.

TABLE 113: SUMMARY OF UV-SPECTROSCOPIC METHOD FOR LINAGLIPTIN

Sl. No.	Parameters	Reports
1	Specificity & Selectivity	Solvent spectrum obtained showed no interference of absorbance 227nm which show the specificity and selectivity of method.
2	Linearity and Range	1 to 5 µg/ml with r^2 =0.9998
3	Limit of Detection	0.23 µg/ml
4	Limit of Quantification	0.71 µg/ml
5	Precision	The percentage RSD of intraday and interday precision for absorbance values of Dapagliflozin was found to be less than 2% after three measurements. (0.23-0.69%)
6	Ruggedness	The approach was found to be rugged by changing the analyst, with % RSD values for absorbance was found to be 0.23 – 1.29%.
7	Accuracy	The suggested approach demonstrates a high level of accuracy, with recovery values ranging from 96.99 – 102.00 %.

TABLE 114: SUMMARY OF CONDUCTOMETRIC METHOD OF METFORMIN

Sl. No.	Parameters	Reports
1	Specificity & Selectivity	The suggested approach was found to be specific and selective, as no interference was detected by blank solvent at the conductance of standard and sample Metformin HCl.
2	Linearity and Range	50 to 250 µg/ml with r^2 =0.9992
3	Limit of Detection	3.12 µg/ml
4	Limit of Quantification	9.47 µg/ml
5	Precision	The percentage RSD of intraday and interday precision for Metformin HCl was found to be less than 2% after three measurements. (0.07 – 1.65%)
6	Ruggedness	The approach was found to be rugged by changing the analyst, with % RSD values for peak area was 1.27 – 1.29%.
7	Accuracy	The suggested approach demonstrates a high level of accuracy, with recovery values ranging from 99.85 to 100.15%.

Chapter 6

Conclusion

CONCLUSION

The proposed study concludes that the newly designed, optimized, and validated QbD assisted HPLC, UV-Spectrophotometric, and conductometric methods were found to be simple, specific, sensitive, precise, robust, rugged, and accurate for estimating Metformin HCL, Dapagliflozin, Empagliflozin, Linagliptin, and Sitagliptin in bulk and pharmaceutical preparations. Hence, the newly developed and techniques can be employed in routine QC analysis of selected anti-diabetic drugs in their preparations in the pharmaceutical industries. The proposed research also concludes that QbD is a systematic approach to development that begins with defined goals and focuses on product and process understanding via strong science and quality risk management. Thus, the principle of QbD can be successfully used to develop and optimize analytical methodologies. The various included in the QbD concept, such as establishing an analytical target profile, crucial quality attributes, significant method variables, and assessment of risk, can assist in understanding the method goals and the risk factors associated with the study. The BBD, CCD, and 3^2 factorial designs are important in method optimization as they assist to understand the impact of important method parameters and how they interact with analytical quality standards.

Chapter 7

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Chapter 8

Annextures

LIST OF RESEARCH PUBLICATIONS

1. Suryawanshi SS, Palled MS. Box-Behnken Design Assisted Optimization and Standardization of Chromatographic Methodology for Quality Assessment of Metformin: Analytical Quality by Design Avenue. Indian J of Pharmaceutical Education and Research. 2022;56(2s):s152-s162.

2. Shailendra S. Suryawanshi, Mahesh S. Palled, Shailendra S. Suryawanshi. A green chemistry approach to establish a conductometric technique for quantifying Metformin HCl in pharmaceutical samples and its greenness assessment using an analytical greenness metric calculator. Journal of Applied Pharmaceutical Sciences Vol. 13(11), pp 016-023, November, 2023.

LIST OF BOOK AND BOOK CHAPTERS

1. A Text Book of Quality Control and Standardization of Phytomedicines. BY Dr. Sunil S. Jalalpure, Dr. Bhaskar Kurangi, Mr. Shailendra S. Suryawanshi, Nirali Prakashan.

2. A text Book of Computer Aided Drug Design of Phytochemicals. BY Dr. Sunil S. Jalalpure, Mr. Shailendra S. Suryawanshi, Nirali Prakashan.

3. Fundamental Research Trends of Green Nanoscience and Nanotechnology. Archana S. Patil, Shailendra S. Suryawanshi. Book Green Nano architectonics. Edition1st Edition. First Published2022. Imprint Jenny Stanford Publishing. Pages26. eBook ISBN9781003318606. (Book Chapter)

LIST OF POSTER AND ORAL PRESENTATIONS

Sl. No.	Title of the Poster/Oral Presentation	Name of the Conference/Event and Venue	Organized By
1	Development & validation of advanced analytical techniques for quantification of Metformin HCl in pharmaceutical products using innovative QbD technology	Advances in analytical techniques for material & biomedical application	Rani Chennemma University, Belagavi. Karnataka
2	Innovative QbD technology in development of advanced chromatographic technique for quantification of Dapagliflozin	Advances in analytical techniques for material & biomedical application	Rani Chennemma University, Belagavi. Karnataka
3	QbD approach in development & standardization of chromatographic method for quality assessment of Linagliptin	Emerging trends and artificial intelligence in pharmaceutical, biomedical sciences & technology	Career Point School of Pharmacy, Kota, Rajashtan.
4	Establishing green analytical technology for development of conductometric method for quantification of Salbutamol sulphate in pharmaceutical samples	Worldwide trends & current challenges in herbal and pharmaceutical technology	Mallige College of Pharmacy, Bengaluru, Karnataka.
	Establishing green analytical technology for sustainable development of conductometric method for quantifying Metformin HCl in pharmaceutical samples: analyst and environment friendly practical approach	Prerana Utsav	Dr. Shivajirao Kadam College of Pharmacy, Sangali, Maharashtra
5	Development of plant derived anti-bacterial agents from medicinal plants	Microbiology: Recent research, technology development & future aspects.	Microbiological Association for Science and Technology Development, Lucknow,
6	Applications of spectrophotometric method for simultaneous Estimation of Ciprofloxacin & Curcumin	Novel trends in drug design & natural product chemistry	Yashwantrao Bhonsale College of Pharmacy, Sawantwadi, Maharashtra
7	Development & validation of RP-HPLC method for simultaneous estimation of Aceclofenac & Pantoprazole in bulk and tablet dosage forms	Prosecution to litigation: part III asserting exclusive rights to IP	Goa College of Conference, Goa

LIST OF PATENTS

Sl. No.	Title of Invention	Application no.	Date Month Year	Grant Patent No
1	IOT Enabled Ultrasonicator with Digital Control	392022-001	12 Oct 2023	392022-001
2	RP-HPLC Method for Simultaneous Estimation of Embelin and Piperine for Routine Analysis of Marketed Polyherbal Capsules and Tablets	202341064747 A	12 Jan 2024	-

LIST OF CONFERENCES ATTENDED

1. National Conference on Advances in Analytical Techniques for Material and Biomedical Application. Organized by Rani Chennemma University, Belagavi. (December 2022)

2. Prerana Utsava. Organized by Dr. Shivajirao Kadam College of Pharmacy, Kasbe Digraj. (March 2023)

3. International Conference on Emerging Trends and Artificial Intelligence In Pharmaceutical, Biomedical Sciences and Technology. Organized by Career Point School of Pharmacy, Kota, Rajashtan. (March 2022)

4. National conference on Emerging Approaches in Drug Discovery: Artificial Intelligence & Drug Repurposing Perspective. Organized by KLE College of Pharmacy, Vidyanagar, Hubballi. (March 2022)

5. International conference on Latest Trends and Innovation in Pharmaceutical and Bioscienecs. Organized by Career Point School of Pharmacy, Kota, Rajashtan. (March 2023)

6. International conference on Worldwide Trends and Current Challenges in Herbal and Pharmaceutical technology. Organized by Mallige College of Pharmacy, Bengaluru, Karnataka, India. (April 2023)

7. Microbiology: Recent Research, Technology Development and Future Aspects. Organized by Microbiological Association for Science and Technology Development (MASTD), Lucknow, UP, India. (April 2023)

LIST OF AWARDS AND RECOGNITIONS

1. Young Researcher Award by Career Point School of Pharmacy, Kota, Rajashtan at conference on Latest Trends and Innovation in Pharmaceutical and Bioscienecs. March 2023.

2. Best Article Award for title Now is the Time for Youth to Switch in to Intellectual Property Rights in Pharmaceutical Sciences (received Positioning among the Top Three in the Intellectual Property Review Article Competition 2023. Awalnut Consultants, UAE. June 2023.

3. Received Best Oral Presentation for Presentation Titled "Development & Validation of Advanced Analytical Techniques for Quantification of Metformin HCl in Pharmaceutical Products using Innovative Quality by Design Technology" at National Conference on Advances in Analytical Techniques for Material and Biomedical Application at Rani Chennemma University, Belagavi.

4. Third Best Poster Presentation for poster title Establishing Green Analytical Technology for Sustainable Development of Conductometric Method for Quantifying Metformin HCl in Pharmaceutical Samples at Prerana Utsava in Dr. Shivajirao Kadam College of Pharmacy, Kasbe Digraj, Sangali. March 2023.

5. Received Best Poster Presentation Award on topic Role of Pharmacy Professional in COVID-19 Crisis organized by National Level e-Poster Competition organized by Konkan Gnyanpith Rahul Dharekar Institute of Pharmacy and research Centre, Karjat on 30 May 2020.

6. Second Best Poster Presentation at Mallige College of Pharmacy, Bengaluru, Karnataka, India at conference on Worldwide Trends and Current Challenges in Herbal and Pharmaceutical technology. April 2023.

7. Received Best Post Presentation Award on topic Drug Targets for COVID-19, organized by National level e-poster competition on COVID-19 Pandemic, organized by Lore Academy, Maharashtra on 29 June 2020.

8. Received Second Best Poster Presentation Award Prize for Poster Entitled " Drug Repurposing of Selected Bioflavonoids Against Main Protease of COVID-19. Conference Emerging Approaches in Drug Discovery: Artificial Intelligence and Drug Repurposing Perspectives. KLE College of Pharmacy, Hubli.

9. Received First Prize for Poster Presentation titled "Role of Pharmacist in Health Care". Poster Presentation Competition Organized by Sarada Vilas College of Pharmacy, Mysuru and Association of Pharmaceutical Scientist and Educators Karnataka.

10. Received First Prize for Poster Presentation titled “Computer Aided Identification of Selected Phytochemicals from Muntingia Calabura against targets of colorectal cancer". International Conference on Start Herb: Journey from Innovation to Entrepreneurship in Herbals. Dr. DY Patil University School of Pharmacy, Mumbai.

11. Received Second Prize for Title Custom Software in Health Care in Innovation in Healthcare or Pharmacy Competition Organized on Occasion of Azadi Ka Amrit Mahotsava. Konkan Gyanpeeth Rahul Dharkar College of Pharmacy & Research Institute Karjat- Raigad.

12. Received Third Prize for title Development of Berberine Nano-micelle for its Improved Anti-inflammatory Activity in Innovation in Healthcare or Pharmacy Compition Organized on Occasion of Azadi Ka Amrit Mahotsava. Konkan Gyanpeeth Rahul Dharkar College of Pharmacy & Research Institute Karjat-Raigad.

13. Second Best Poster Presentation Award for Title Pharmacovigilance of Herbal Formulations at Drug Monitoring Centre, IIMT College of Pharmacy, Greater Noida, UP. Jan 2023.

14. Best Poster Presentation Award at Microbiological Association for Science and Technology Development, Lucknow, UP, India at conference on Microbiology: Recent Research, Technology Development and Future Aspects. April 2023.

15. Appointed as Assistant Editor for Journal in Editorial Board of International Journal of Pharmaceutical Science and Drug Analysis (2021).

Original Article

Box-Behnken Design Assisted Optimization and Standardization of Chromatographic Methodology for Quality Assessment of Metformin: Analytical Quality by Design Avenue

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ABSTRACT

Background: Metformin (MET) is an oral antidiabetic agent falls chemically under the category of biguanides and it is effectively utilized in the management of type 2 diabetes mellitus. It is marketed in the tablet dosage form and hence quality control and assessment of MET is very much essential and important. **Objectives:** The objectives of present research investigation are to implement the Box-Behnken Design (BBD) in the optimization and validation of Reverse Phase-High Performance Liquid Chromatographic (RP-HPLC) method for the quality assessment of MET and also to systematically apply the Analytical Quality by Design (AQbD) Approach. **Materials and Methods:** In methodology BBD was employed to identify and optimize the critical method aspects for augmenting the performance of proposed methodology. The optimum chromatographic elution was performed on Agilent C_{18} (5μm, 250 × 4.6 mm i.d.) column employing methanol and 0.1% ortho phosphoric acid in water with pH 2.8 in the proportions of 4:96 v/v as solvent system. The elution was carried out at 0.7 mL/min flow of mobile phase and identification at 231 nm using UV detector. The BBD driven optimized method was standardized as per guidelines of International Council for Harmonisation Q2 (R_1) in terms of validating method parameters such as specificity, linearity, detection and quantitation limit, precision, robustness, ruggedness and accuracy. **Results:** The method was linear in the concentration of 5-25μg/ml with R^2 = 0.999. The detection and quantitation limit were obtained at concentration of 0.30 and 0.93 μg /ml. The values of precision, robustness, and ruggedness parameters were found to be well within the required limit of acceptance with deviation of relative standard < 2%. The accuracy of MET was observed 99.22% to 100.25% at three different recovery experiments. At the end the proposed design of experiment (DoE) oriented methodology successfully applied for quantification of MET. **Conclusion:** The BBD and AQbD approaches are highly useful for the quality assessment of MET in bulk and its marketed tablet dosage forms.

Key words: Analytical Quality by Design, Box–Behnken Design, Chromatography, Metformin, Standardization.

INTRODUCTION

The quality assessment of drugs and its pharmaceutical formulations is very important and essential during product development stage in pharmaceutical industry. Analytical assessment is the critical approach in quality control of pharmaceutical products. The poor analytical assessment may lead to generation of inaccurate values, wrong data that may be risky to the drug and product formulation stage. In order to address such crucial issues and concerns, various pharmaceutical drug regulatory bodies, such as "US Food and Drug Administration" and "International Council for Harmonisation" (ICH) have been stated the quality by design (QbD) approaches and guideliens to overcome these issues. In the recent years, ICH has declared set of rules

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and guidance in ICH Q14 on development of analytical procedures and revision of Q2 (*R*1) analytical validation Q2 (*R*2)/Q14.[1]

The traditional chromatographic approach for assessment of drug molecules in bulk and dosage forms was done by a trial approach and error method. Example, by changing single parameter at single time and identify the change of the result until we get the suitable procedure was obtained. Traditional approach is very long approach or method and needed large quantity of manual result analysis. It required basically few practical runs, and in few cases, the reported procedures requires again change in method when scaled up, and in actual product development stage.[2,3]In order to overcome the limitations and disadvantages of traditional chromatographic methods we can adopt the AQbD principles as it includes the statistical DoE to know a "method operable design space" of a robust analytical procedure. The "method operable design space" gives the practically possible working areas or region in which changes to the method aspects will not reasonably affects the quality and data of the procedure. It will be technically and practically important to know if a "method operable design space" for change in chromatographic parameters can be generated to assist the optimization of a rugged and robust procedure.[4] Many scientists have implemented the AQbD approaches and guidelines to chromatographic procedures.[5-10]The systematical and rational utilization and implementation of elements of QbD to analytical procedure optimization to obtain good performance of technique is known as AQbD.[11,12] This avenue gives surety of reliability and high quality of the analytical procedures and reduces the failure risk in the phase of standardization as well as routine quality analysis. "It is a scientific and risk-based method for understanding of the critical analytical attributes (CAAs) and affected independent parameters impacting the performance of procedure". AQbD gives the detailed information on the risks and effects between the variables of the method.[13,14]MET is an oral antidiabetic agent chemically categorized to biguanides class and effectively utilized for the management of type 2 diabetes. It mainly shows its mechanism by reducing the glucose secretion. It decreases the level of cholesterol (LDL), also reported achieves the weight loss in few cases.[15] It is also used for the treatment of "polycystic ovary syndrome".[16] MET is marketed in single and also in combined dosage form with other antidiabetic drugs. In the year 1922 MET was prepared by reaction of dimethylamine hydrochloride and 2-cyanoguanine in the presence of heat.[17] The adverse effects of MET includes the lactic acidosis and few related to gastro intestinal tract. MET is contraindicated in the patients with diseases of liver, kidney, lung and heart disorders.[18] Chemically MET is N, N-dimethylimido dicarbonimidic diamide with molecular formula $C_4H_{11}N_5$ and molecular mass 129.164 gm/mol. It is soluble very freely in H_2O. MET is sensitive to light and degrades when heated emitting nitric oxide. It appears as white to off-white crystalline powder with pKa value 12.4. The chemical structure of MET is presented in Figure 1.

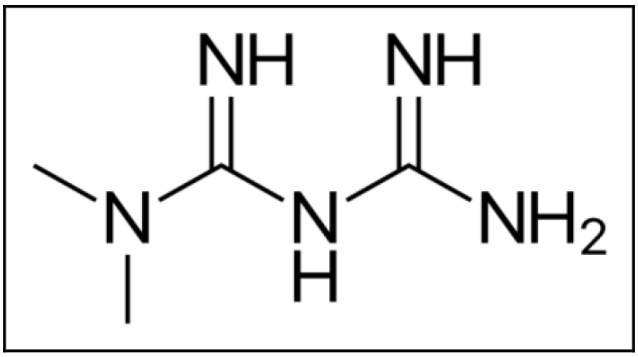

Figure 1: Chemical Structure of MET.

Literature review revealed that various analytical methods have been reported for quality assessment of MET in bulk and pharmaceutical dosage forms. MET is analysed by few chromatographic methods in its single and combined dosage forms. But the reported chromatographic assessment procedures suffer with various limitations and disadvantages like critical steps, complicated methods, extraction steps are multiple which leads to more time consuming procedure. Most of the published chromatographic procedures for MET have limitations as they have complex solvent system composition, longer retention time and use of costly solvents and also methods are developed with traditionally approach.19The scientific, BBD assisted and AQbD driven chromatographic optimization of MET has not discussed in detail till date. Hence there is strong and solid requirement for the optimization of simple, robust, accurate and sensitive procedure for assessment of MET employing BBD to overcome the limitations and issues as mentioned above and also to assure the quality of the proposed method.The main objectives of proposed research work is to develop and optimize an simple, sensitive, rapid, rugged, robust, accurate and reliable RP-HPLC methodology by employing BBD and AQbD approaches for quality analysis of MET in its pharmaceutical tablet dosage forms as well as in bulk powder.

MATERIALS AND METHODS

Reagents and Chemicals

MET (99.8%) was obtained as a gift sample from FDC Ltd. Verna-Goa. The HPLC grade methanol was purchased from Merck India Limited. The solvent system was filtered employing 0.45 µm nylon filters obtained from Pall India Private Limited, Mumbai, India. The solvent system was degassed and sonicated using sonicator. The marketed tablets of MET was obtained from local Pharmacy shops of Belagavi, Karnataka, India and analyzed for its quality using BBD assisted RP-HPLC methodology.

Apparatus, Equipments and Chromatographic Conditions

In the proposed research work HPLC system of Agilent 1100 (Autosampler), Chemstation possessing software was used. The chromatographic separation of the MET was achieved on Agilent C_{18} column (250 × 4.6 mm i.d., 5µm). The solvent system composed of methanol: 0.1% ortho phosphoric acid (OPA) in water with pH 2.8 in the proportions of 4:96 v/v. Elution of MET was done at 0.7 mL/min with UV identification at 231 nm. The temperature of column was maintained at ambient, and the injection volume of 20 µL. The Chromatographic grade water was used and it was again filtered using a 0.45 µm membrane filters (Nylon) before taking it for the preparation of solvent system composition. The micro weighing balance of Sartorius was used for weighing the standard drug and powdered sample of MET. In order to degas the solvent system as well to solubilize the drug and powdered drug sample into solvent, Sonicator of MJL lab was used. All the glassware's used such as pipettes, measuring cylinders and volumetric flasks were calibrated before use.

Preparation of solvent system

1 mL of OPA was measured and transferred into 1000 mL milli-Q water and pH was maintained at 2.8 and filtered through 0.45-µm nylon membrane filters. The mobile phase used was methanol to 0.1% OPA in water with pH 2.8 in a ratio of 4:96 (v/v).

Preparation of standard stock solution

The MET stock was made by dissolving 10 mg of MET in 10 ml of methanol. The resulting stock was sonicated to solubilize the MET, and then 1 ml of this solution was transferred into 10 ml of volumetric flask (VF), and it was diluted 10 ml with solvent system as diluent. This stock was utilized for optimization and validation of experimental parameters. The further working solutions of MET were obtained by serial dilutions. The series of concentrations were obtained by using solvent system as diluent. The final solutions were filtered using a 0.45 µm syringe filter. The final dilutions were then transferred to vials before chromatographic runs.

Preparation of sample solution

Weighed and powdered 20 MET tablets. The powder amount equal to 10 mg of MET was taken and transferred into a 10 ml of the VF. Then, 2 ml of solvent system was added and 15 min sonicated to solubilize it and volume was made up to the mark with solvent system. The resulting solution, filtered using Whatman filter paper and then 1 ml of this solution diluted to 10 ml with solvent system. Further 2.5 ml of the above solution transferred in 10 ml of VF and made up to the mark with solvent system (25 µg/ml).

Preliminary Method Development

In order to select the suitable stationary phase, initial experimental runs were performed for identification of possible combinations and selection of column and solvent system. Initial trials were carried out by using literature review. In order to develop cost effective methodology, methanol was chosen for trial study. Aqueous solvent composed of different concentration of OPA were chosen for effective separation with different pH. By the analysis of the above trials, the C_{18} stationary phase was used for further study.

Implementation of AQbD Avenue

QbD is defined as "a systematic approach to development that begins with predefined objectives and emphasizes product and process understanding based on sound science and quality risk management." "The approach of QbD covers a better scientific understanding of critical parameters and qualities of product, designing tests and controls based on the scientific limits of understanding during the development stage and using the knowledge and data generated during the life cycle of the product to work on a constant improvement". In the present investigation we have worked on various elements of AQbD and implemented in the practical methodology. It has started with identification of Analytical Target Profile (ATP), followed by study of Critical Quality Attributes (CQAs) and Risk Assessment (RA) by employing fish-bone approach. Further approach is mainly deals with application of DOE which helps in the factor screening and optimization of RP-HPLC methodology.

Analytical Target Profile (ATP)

Identification and setting up the ATP is first and most important step in the QbD based analytical procedure

development and it can be defined as collection of information required for desired quality profile needed for the development of method. This helps for assuring the safety, quality, and practical usefulness of the analytical procedure at the site of commercial application of it in the pharmaceutical industry. Various components of ATP include the "target drug, target samples and method application, analytical method, instrument characteristics and requirements, sample preparation steps, and analytical procedure quality attributes".

Critical Quality Attributes (CQAs)

In order to meet the requirements of target profile of analytical procedure, various CQAs need to be selected and it is very important and essential step in the AQbD based method development. The CQAs can be defined as "the measurable parameters of the chromatogram of analyte that should be within an appropriate and acceptance limit or range or criterions to warrant the desired quality and ability of the analytical procedure". In case of chromatographic development usually the critical quality attributes includes the tailing factor (TF), retention time (RT), peak area (PA), peak spectral purity, theoretical plates (TP), and assay limit etc. Out of these attributes, RT, TP, and TF have real impact on the performance of analytical procedure.

Risk Assessment (RA)

The assessment of risk in the very important and essential step in the QbD based procedure development. In this study RA was conducted for determining the probability of failures in the procedure. In order to perform this, a cause to effect model for relationship of "critical material attributes" and "critical method parameters" with the Critical Analytical Attributes (CAAs) was done and executed by construction of an "Ishikawa Fish-Bone" (IFB) diagram.

Factor Screening by Design of Experiment Avenue using Box-Behnken Design (BBD)

A new chromatographic procedure was optimized for MET using software of Design Expert. In this software, design of Box-Behnken was employed to optimize the "Critical Process Parameters" (CPPs) or "Critical Method Parameters" (CMPs), and to assess the interaction of these parameters on the CQAs. BBD is a three factor two level design and it requires fewer experimental runs.

Box-Behnken Optimization Study Design

The chromatographic optimization of parameters was done by utilizing 3 factors, 2 levels BBD to estimate the main, effects and quadratic effects of critical factors on the variables of identified responses.[20-22] The BBD composed of 14 experiment runs and screening phase includes the following steps. In the first phase selection of CMPs was done which includes the flow rate, organic phase (% methanol), and wavelength of detection. The second phase of study includes selection of CQAs. In the present study CQAs selected are RT, PA, TP, and TF. These responses were monitored during the practical trials.

Method Development According to Experimental Design

As per factor screening method, the selection of the CMPs which are affecting the performance of procedure was optimized employing 3 factor at two equidistant levels (low (-1) and high (+1) levels). A 10µg/mL of MET was used for all chromatographic runs, and analyzed for CAAs (RT, PA, TP, and TF).

Data Analysis and Model Validation

The responses generated after running suggested trial were added to software and various plots like "3D response surface plots" and "graph plots" were obtained. These plots suggest the effect of CMPs on the selected CQAs. The assessment of these plots was utilized to analyse as to which parameter give the reasonable responses. Based on these data, the final CMPs of the procedure were identified and the optimized conditions were selected. The statistical tool, like "analysis of variance" (ANOVA) for every response was used to study the significance of each parameter used for the study using the p value.

Method Validation

In order to standardize the suitability of the proposed method for its intended use, method validation was carried out as per ICH guidelines by assessing the system suitability, linearity and range, detection and quantification limit, precision, ruggedness, robustness and accuracy. The proposed RP-HPLC procedure was then applied successfully for the quality assessment of MET in its marketed table forms.

System Suitability

The suitability of system and testing is an essential part of any analytical method. It was performed by injecting three replicates of 10 µg/mL standard MET solution and then evaluating the each chromatogram with respect to its RT, TP and TF.

Specificity and Selectivity

The specificity and selectivity of the procedure was done by obtaining the HPLC peak of solution of MET (10 µg/mL), sample and blank chromatogram of solvent system. It helps to identify the analyte, interference from other peaks, and also the peak purity.

Linearity and Range

The linearity of procedure was assessed between the ranges of 5 to 25 µg/mL of MET. The linearity response was determined by preparing different concentrations and injecting three times into HPLC, and recording chromatograms and noting down the peak area for all the graphs. The standard calibration curve was graphed as peak area *vs* the concentration of the MET in µg/mL.

Detection and Quantification Limit

"Limit of detection (LOD) is the lowest amount of drug in a sample that can be detected but not necessarily quantified as an exact value". Detection limit was obtained using the following equation:

$$LOD = 3 \times \sigma/S$$

Limit of quantification (LOQ) of the developed method is "the lowest amount of analyte or drug in a sample which can be quantified with suitable precision and accuracy". Quantification limit was obtained using the following equation:

$$LOQ = 10 \times \sigma/S$$

"Where σ is the standard deviation of y-intercepts of regression lines, and S is the slope of the calibration curve" of MET.

Precision

The precision is estimated in terms of relative standard deviation **(**RSD). The precision was tested by injecting three replicate injections of concentration 10, 15 and 20µg/mL. The intraday and interday precision study was done by injecting 3 replicates of different concentration of MET and calculating %RSD for PA. (It was assessed on the same day and on three different days).

Robustness

The robustness of the method was evaluated by injecting the standard solution of MET at deliberately changing the chromatographic conditions which includes a change in composition of organic phase in solvent system, flow rate, and wavelength.

Ruggedness

The ruggedness of the proposed method has been done by analyzing the standard solution of MET by two different analysts. The overall mean, and % RSD was calculated for peak area obtained in the chromatogram of each peak.

Accuracy

Accuracy of the method was assessed from the recovery study of MET through 5µg/ml solution of MET spiked with 80, 100, and 120%. The mean recovery and % RSD was calculated at each level.

Quality Assessment of MET using Box-Behnken Design Assisted RP-HPLC Method

Weighed and powdered 20 MET tablets. The powder amount equal to 10 mg of MET was taken and transferred into a 10 ml of the VF. Then, 2 ml of solvent system was added and 15 min sonicated to solubilize it and volume was made up to the mark with solvent system. The resulting solution, filtered using Whatman filter paper and then 1 ml of this solution diluted to 10 ml with solvent system. Further 2.5 ml of the above solution transferred in 10 ml of VF and made up to the mark with solvent system (25 µg/ml) and injected into stabilized HPLC for further elution and analysis.

RESULTS AND DISCUSSION

Initial Method Development

In order to optimize a simple, cost-effective, precise and accurate method for assessment and quality control of the MET in its bulk as well as pharmaceutical tablet dosage forms, preliminary experiments on the basis of literature search and trial and error basis were carried out. Initially, development was started with selecting mobile phase by utilizing various combinations of solvents such as methanol, and o-phosphoric acid. The buffer phase is tried at different concentrations in order to achieve better separation of the MET. In initial runs, it was ientified that the selection of OPA in 0.1% concentration with pH 2.8 which leads to faster and effective separation of drug with elution of MET at lower RT along with low peak tailing and satisfactory peak symmetry.

Analytical Quality by Design Approach and its Implementation

Various approaches and methodologies of AQbD were applied to proposed RP-HPLC method as discussed in methods section. The ATP, CQAs and process parameters of method were studied, identified and presented in Table 1.

Risk Assessment and Screening Studies

RA study was performed to analyze the CMPs, which are high-risk factors and have a critical effect on the CAAs. In this study, IFB diagram (Figure 2) was prepared to list out serious risk factors that may have an effect on performance of method. The risk factors listed may includes the method of extraction of sample, time of extraction, solvent used for extraction etc. and instrumental related parameters such as ratio of solvent system, chromatographic mode, flow rate, and injection volume. The high-risk procedure variables were listed and exposed to further analysis by applying suitable experimental optimization design. The high-risk method variables selected as flow rate of mobile phase, % composition of organic phase (methanol) in mobile phase and wavelength of detection.

Table 1: QTPP, CQAs and Process Parameters of Proposed RP-HPLC Method.

Sl. No.	AQbD Parameters	Description
1	Quality target product profile	Flow rate, mobile phase composition, wavelength of detection, pH, temperature, and column attributes, injection volume, pressure, buffer attributes.
2	Critical quality attributes	Retention time, Peak area, theoretical plates, peak purity, tailing factor.
3	Process parameters	Specificity, selectivity, linearity, range, precision, robustness, ruggedness, accuracy, LOD, LOQ.

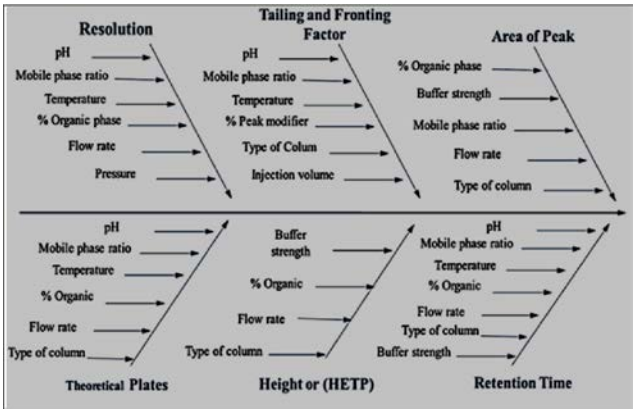

Figure 2: Ishikawa Fishbone Diagram.

Box–Behnken Optimization Design

It is very important component of DOE avenue and in order to implement for the RP-HPLC method, we have selected high-risk factors such as flow rate of mobile phase, % organic phase in mobile phase and wavelength of detection. They were optimized by BBD to detect main, interaction and quadratic effects of these factors on RT, PA, TP and TF. The 14 runs were performed, and obtained results were assessed statistically using software. The independent variables selected were flow rate of mobile phase ($X1$), % organic phase in mobile phase, and wavelength of detection, whereas RT, PA, TP, and TF were selected as the dependent responses. The BBD optimization is shown in Table 2. The software carried analysis of variance (ANOVA), and statistical optimization. The ANOVA results are presented in Table 3. This implies that the model is valid. The "3D-surface" and "2D-contour plots" were also analyzed to define design space and to visualize the effect of independent parameters and their effects on the

Table 2: Box–Behnken Optimization Design.

	Factor 1	Factor 2	Factor 3	Response 1	Response 2	Response 3	Response 4
Run	A:Flow rate	B:Methanol	C:Wavwlength	RT	PA	TP	TF
	ml/min	%	nm				
1	0.7	3	230	3.45	4850.37	11675	0.82
2	0.8	2.3	231	2.95	4019.71	10892	0.84
3	0.7	3	232	3.369	4521.28	11693	0.83
4	0.6	4	231	3.89	5516.24	12789	0.8
5	0.7	5	230	3.33	4896.5	11458	0.83
6	0.9	3	232	2.61	3555.04	9810	0.86
7	0.7	5	232	3.32	4665.06	11373	0.82
8	0.8	4	229.3	2.92	4373.36	10645	0.84
9	0.9	3	230	2.62	3755.37	9777	0.85
10	0.8	4	231	2.62	3707.75	9733	0.86
11	0.9	5	230	2.71	4018.88	9950	0.86
12	0.9	5	232	2.69	3785.56	9793	0.85
13	0.8	5.7	231	3	4326.7	10679	0.84
14	0.8	4	232.7	3	4484.22	10716	0.84

Table 3: Statistical Analysis Data.

ANOVA Parameters	Retention Time	Peak Area	Theoretical Plates	Tailing Factor
R-Squared	0.9960	0.8277	0.9970	0.9987
Adjusted *R*-squared	0.9913	0.8133	0.9936	0.9971
Predicted *R*-Squared	0.9583	0.7698	0.8559	0.8529
Standard Deviation	0.0363	239.06	74.53	0.0009
%C.V	1.19	5.53	0.69	0.1116
F-value	211.69	57.63	288.01	646.40
p-value	<0.0001	<0.0001	<0.0001	<0.0001

R-squared = Coefficient of determination.
F-value = Value on the F distribution.
p-value = Probability of falsely detecting a significant effect.
C.V.% = Percent Coefficients of variance.

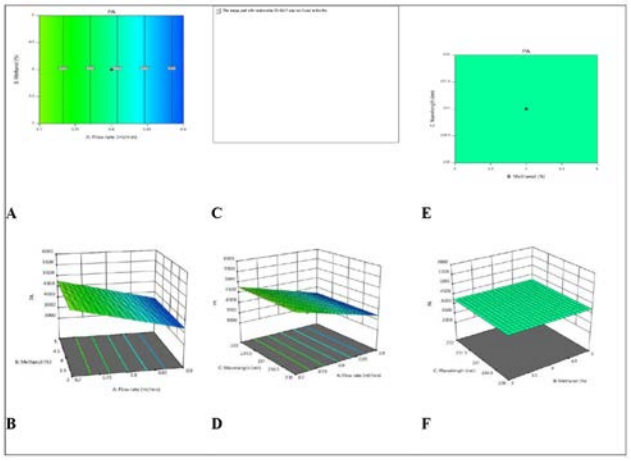

Figure 4: 2D Contour and 3D surface plots depicting the effect of (A and B) flow rate and % methanol on peak area, effect of flow rate and wavelength (C and D) on peak area, %methanol and wavelength (E and F) on peak area.

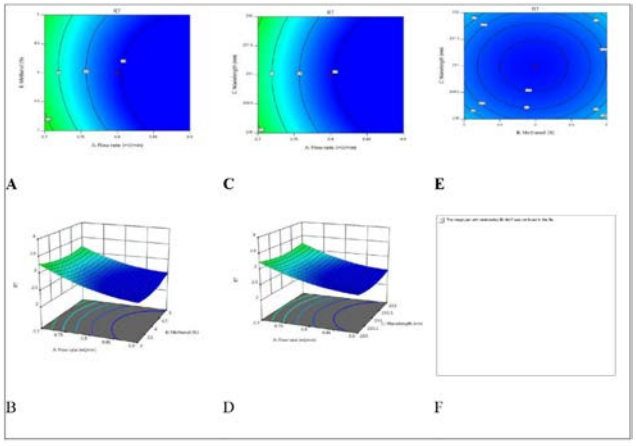

Figure 3: 2D Contour and 3D surface plots depicting the effect of (A and B) flow rate and % methanol on retention time, effect of flow rate and wavelength (C and D) on retention time, %methanol and wavelength (E and F) on retention time.

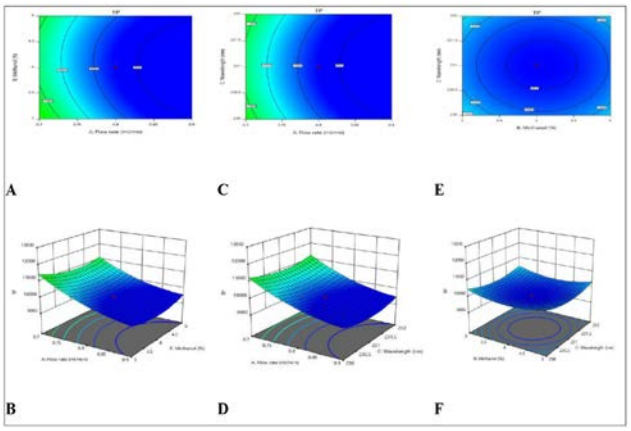

Figure 5: 2D Contour and 3D surface plots depicting the effect of (A and B) flow rate and % methanol on theoretical plates, effect of flow rate and wavelength (C and D) on theoretical plates, %methanol and wavelength (E and F) on theoretical plates.

respective response variables. As the proposed model has more than two independent factors, one factor was kept constant for each plot. Figure 3-6 presents the portrays 3D surface plot, and it's corresponding 2D contour plot showing the effects of flow rate, % composition of organic phase, and wavelength on RT, PA, TP and TF.

Analytical method validation

In the system testing, parameters, like RT, TP, and TF were assessed and %RSD was calculated prior to perform the analysis. Figure 7A presented the HPLC chromatogram of standard MET with RT 2.96 min. Figure 7B shows the chromatogram of MET in sample with R_t 2.96 min. The blank chromatogram also showed no appearance of any peak at R_t of

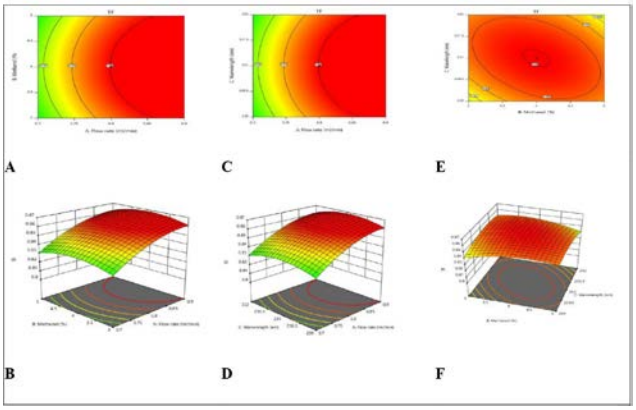

Figure 6: 2D Contour and 3D surface plots depicting the effect of (A and B) flow rate and % methanol on tailing factor, effect of flow rate and wavelength (C and D) on tailing factor, %methanol and wavelength (E and F) on tailing factor.

MET. No interference observed at the retention time of MET peak in standard and sample chromatogram; thus, method was found to be specific and selective for MET. The overlay chromatograms of MET were presented in Figure 7C. The standard calibration curve of MET was plotted in the concentration range of 5 to 25 µg/mL (Figure 8). The procedure was linear in the concentration range of 5-25 µg/mL with R^2 value 0.999. Limit of detection and limit of quantitation were found to be 0.30 and 0.93 µg /ml respectively. Data in Table 4 suggest the precision, robustness, and ruggedness of proposed method. The %RSD of each parameter for standardization falls within the limit (<2%). The robustness of procedure was assessed by changing solvent composition methanol: aqueous phase (3:97 to 5:95 v/v), change in wavelength from 230 to 232 and change in the flow rate from 0.6 to 0.8 ml/min. Acceptable %RSD values were obtained after making small deliberate variations in developed method parameters. This suggests that the procedure is robust for the industrial analysis. The ruggedness of the procedure was done by different analysts and values of results were found to be within the acceptance. An recovery with %RSD of less than 2% was obtained from all 3 level recovery studies. The %RSD of all the 3 levels is presented in Table 5.Quality Assessment of MET by Box–Behnken Optimized RP-HPLC Method

The BBD driven RP-HPLC analysis of marketed formulation of MET presented excellent recovery. The % recovery was found to be 99.89%. The retention time of MET in dosage was not changed with regard

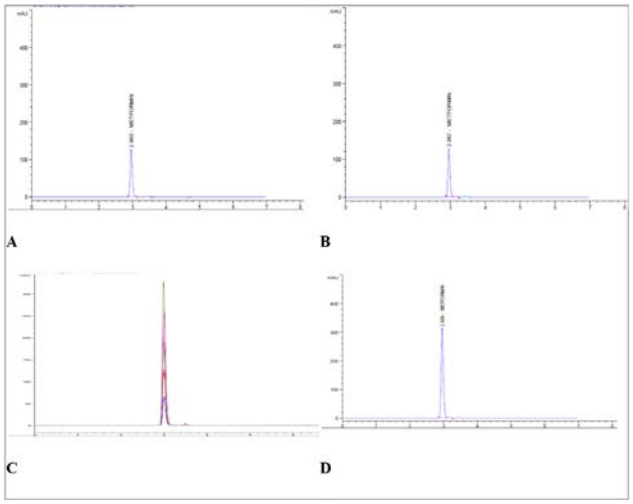

Figure 7: Chromatograms depicting (A) MET Standard (B) MET sample (C) Overlay of MET Standard (D) MET assay sample.

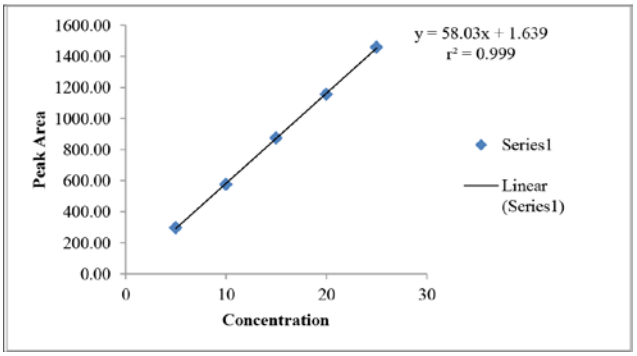

Figure 8: Standard Calibration Curve of Metformin.

Table 5: Accuracy Study Data of MET.

Level	Sample	Recovery (%)	Mean Recovery (%)	% RSD
80%	S1	99.18	98.98	0.69
	S2	99.53		
	S3	98.22		
100%	S1	99.84	99.79	0.48
	S2	99.29		
	S3	100.25		
120%	S1	99.79	99.20	0.70
	S2	99.38		
	S3	98.44		

Table 4: Analytical Method Validation Report.

Precision	Intraday		Interday-1		Interday-2		Interday-3	
Conc.	Peak Area	%RSD	Peak Area	%RSD	Peak Area	%RSD	Peak Area	%RSD
10 µg/mL	579	0.12	585	0.29	577	0.65	581	0.71
15 µg/mL	873	0.37	878	0.31	876	0.11	876	0.13
20 µg/mL	1163	0.15	1175	0.29	1176	0.80	1159	0.24
Ruggedness by Change in the Analyst			Robustness					
			Flow Rate		Mobile Phase		Wavelength	
5 µg/mL	297	0.39	25 µg/mL		25 µg/mL		25 µg/mL	
15 µg/mL	876	0.12	1637		1443		1485	
25 µg/mL	1457	0.21	0.19		0.84		0.11	

to MET standard. The TF and TP factor were found to be within the limits of acceptance. All the solutions of samples showed no additional peaks in chromatograms that showed no inference of dosage form additives with MET. This showed ability of proposed RP-HPLC method for routine quality assessment and analysis of MET in its bulk and tablet forms. The assay chromatogram is presented in Figure 7D.

CONCLUSION

The present investigation successfully implemented the BBD and AQbD avenue to optimize the RP-HPLC procedure for MET assessment with a proper understanding of the critical factor response relationship for augmenting the procedure performance. The BBD and AQbD assisted RP-HPLC method optimization of MET ensured the robustness of the analytical procedure before standardization studies. This novel pathway helps the researcher and analyst to set control strategies to reduce the unwanted effect of these critical method variables on performance of method. The validation reports confirmed the specificity, selectivity, excellent linearity, detection and quantification limit, accuracy, precision robustness, and ruggedness of proposed method. The BBD assisted optimized and validated RP-HPLC method further utilized for the quality assessment of marketed MET tablets to ratify the applicability of the proposed procedure.

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ABBREVIATIONS

AQbD: Analytical Quality by Design; **ANOVA:** Analysis of Variance; **ATP:** Analytical Target Profile; **BBD:** Box-Behnken Design; **CPPs:** Critical Process Parameters; **CMPs:** Critical Method Parameters; **CAAs:** Critical Analytical Attributes; **CQAs:** Critical Quality Attributes; **DOE:** Design of Experiment; **IFD:** Ishikawa Fish-Bone Design; **ICH:** International Council for Harmonization; **LOD:** Limit of Detection; **LOQ:** Limit of Quantification; **MET:** Metformin; **OPA:** Ortho Phosphoric Acid; **PA:** Peak Area; **QbD:** Quality by Design; **RA:** Risk Assessment; **RSD:** Residual Standard Deviation; **RT:** Retention Time; **RP-HPLC:** Reverse Phase-High Performance Liquid Chromatography; **TF:** Tailing Factor; **TP:** Theoretical Plates.

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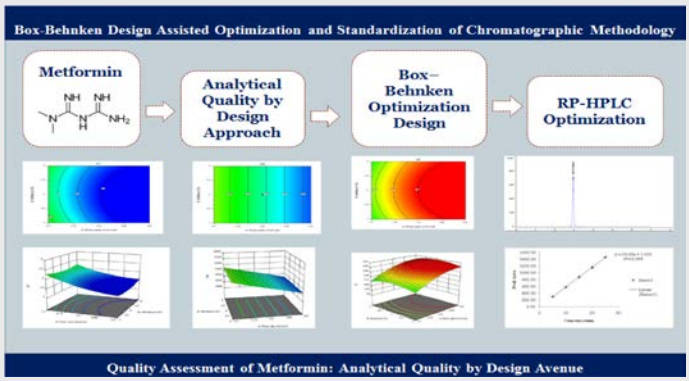
SUMMARY

In methodology BBD was used to identify and optimize the critical process parameters for augmenting the performance of proposed methodology. The optimum RP-HPLC separation was achieved on Agilent C_{18} column (250 x 4.6 mm i.d., 5µm) employing methanol and 0.1% ortho phosphoric acid in water with pH 2.8 in the proportions of 4:96 v/v as solvent system. The elution was carried out at the flow rate of 0.7 mL/min and detection at 231 nm using UV detector. The BBD driven optimized method was standardized as per guidelines of International Council for Harmonisation Q2 (R_1) in terms of validating method parameters such as specificity, linearity, Limit of detection and limit of quantitation, precision, robustness, ruggedness and accuracy. The method was found to be linear in the concentration range of 5-25µg/ml with $r^2 = 0.999$. The detection and quantitation limit were obtained at concentration of 0.30 and 0.93 µg /ml. The values of precision, robustness, and ruggedness parameters were found to be well within the acceptance limits with relative standard deviation < 2%. The accuracy of MET was observed 99.22% to 100.25% at three different recovery experiments. At the end the proposed design of experiment (DOE) oriented methodology successfully applied for quantification of MET. In conclusion, the BBD and AQbD approaches are highly useful for the quality assessment of MET in bulk and its marketed tablet dosage forms.

PICTORIAL ABSTRACT



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Dr. M S Palled, has completed his Ph.D in Pharmaceutical Chemistry in 2011 from RGUHS, Bengaluru and M.Pharm in Pharmaceutical Chemistry from KLE College of Pharmacy, Belgaum, KUD University, Dharwad. Karnataka. His area of interest is development and validation of analytical methods and synthetic chemistry. Currently working as Professor in Department of Pharmaceutical Chemistry, KLE College of Pharmacy, KLE Academy of Higher Education and Research, Belagavi. Dr. Palled has published over 22 scientific papers in national and international journals and presented over 15 posters in various conferences and symposiums. He has guided more than 12 students for PG level research projects. He has attended many national and international conferences, workshops and symposiums and also participated as resource person.

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Mr. Suryawanshi has published over 22 scientific papers in national and international journals and presented over 15 posters in various conferences and symposiums. He have published a book entitled "Quality Control and Standardization of Phytomedicines" in Nirali along with Dr. Sunil S. Jalalpure, Principal, KLE College of Pharmacy, Belagavi and Dr. Bhaskar Kurangi, Assistant Professor, Department of Pharmaceutics, KLE College of Pharmacy, Belagavi.

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A green chemistry approach to establish a conductometric technique for quantifying Metformin HCl in pharmaceutical samples and its greenness assessment using an analytical greenness metric calculator

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Metformin HCl, conductometric method, green chemistry, validation, pharmaceuticals.

ABSTRACT

Green analytical chemistry focuses on making analytical processes safer and more environment friendly for the analyst. The primary goals of the proposed research are to develop and validate a conductometric method with green chemistry assistance for the estimation of Metformin HCl (MET), as well as to assess the method's level of greenness using the AGREE-analytical greenness measure tool. The conductometric method for quantification of MET was developed in two steps. Step 1: Preparation of working standard solutions of MET and Step 2: Estimation of electrical conductivity of working standard solutions of MET. During the development, various dilutions of MET were prepared in Millipore water (MW) ranging from 50 to 250 µg/ml. The prepared solutions were subjected to the measurement of electrical conductivity using the conductometer ELICO CM 183EC-TDS analyzer version 2.3 instrument. The created approach was authenticated to regulate its routine capabilities in accordance with the most recent International Council for Harmonisation guidelines for regulatory recommendations. With an r^2 value of 0.999, MET demonstrated linearity concerning amount ranges from 50 to 250 µg/ml. Less than 2% RSD was observed for each set of validation parameters, which was substantially within acceptable limits. The observed recovery levels ranged from 99% to 100%. According to the AGREE metric tool calculation, the suggested method was deemed to be greener and more suitable for analyzing MET in pharmaceutical samples. The proposed research found to show advantages over reported methods in terms of usage of solvents, cost, and time of analysis. The method suggests the usage of MW as a cheap solvent, only simple dilutions and measuring the conductance of the analyte solution makes the method less time-consuming.

INTRODUCTION

Green analytical chemistry (GAC) encourages analytical chemists to think about environmental, human health, and safety issues at every stage of their work. Green analytical process development is essential in the pharmaceutical industries. Till date, various GAC measures were developed to appraise the greenness of analytical techniques. To determine the method's greenness, the "analytical greenness calculator" combines all 12 GAC principles. It is a wide-ranging, analyst-approachable, instructive, and sensitive GAC metric calculator for evaluating analytical methods. The assessment criteria in this procedure are the 12 principles of GAC which are transformed into a common scale of 0–1 and all GAC rules are used to determine the final score. The outcome is a representation that shows the concluding score, performance of the analytical technique for each criterion, and the user-assigned weights (Armenta *et al*., 2008; Pena-Pereira *et al*., 2020; Tobiszewski *et al*., 2015)

A significant element in the product development stage is the quality valuation and assessment of pharmaceuticals. The pharmaceutical industries place a great deal of importance on the development of drug assays and their formulation. The crucial step in pharmaceutical quality control is analytical valuation. A poor analytical evaluation may produce erroneous values and assessment results, which could be dangerous for the stage

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of medicine and product creation. The quality of drugs and formulations can be controlled by using a variety of modern instruments and investigative techniques, such as optical, physical, thermo-analytical, electrochemical, biological, microbiological, titrimetric, radioactive methods, and other analytical methods. The electrochemical methods evaluate the electrochemical characteristics or properties of pharmacological compounds. The conductometric analysis is an analytical tool for quantitative analysis of various analytes, which includes measuring the conductivity of analyte solution. It is widely known that the concentration of analyte and the number of free charges present in the sample affect an electrolytic solution's conductivity. It is also particularly helpful in the analysis of very weak acids and dilute solutions (Sankar, 2010; Siddiqui *et al*., 2013).

The biguanides moiety of the well-known orally accessible antidiabetic drug Metformin (MET) is excellently used in the control of diabetes mellitus (Type-2). It mostly demonstrates its mode of action by reducing glucose secretion. In a few cases, it was said to lower cholesterol, which led to weight loss (El Messaoudi *et al*., 2011; Radosh, 2009). It is advertised and sold in many different dose forms, both alone and in combination with other anti-diabetic drugs. It was created through the reaction of 2-cyanoguanine with dimethylamine hydrochloride in the presence of temperature (Werner and Bell, 1921). It has been revealed to have a few side effects, including lactic acidosis and a few gastrointestinal problems. The use of this medication is both advised and prohibited in those with kidney, liver, lung, or cardiac illnesses (Sonnett *et al*., 2009). MET is *N*, *N*-dimethylimidodicarbonimidic diamide with a compound mass of approximately 129 g/mol (Fig. 1). It is a crystalline powder that ranges from white to off-white, soluble in water, and has a pKa value of 12.4 (Suryawanshi and Palled, 2022).

It is evident for a literature study that only a few spectroscopic, chromatographic, and electrochemical techniques were used to analyze the drug. The described techniques were found to have certain drawbacks, including the usage of expensive instruments, hazardous reagents, and excessive use of organic solvents, expensive reagents, longer processing times, crucial extraction steps, and difficult-to-handle instrumentation. Multiple extraction steps also result in longer processing times. A complex solvent system composition and a higher retention factor are drawbacks of the majority of chromatographic procedures that have been documented (Alessandro *et al*., 1974; Aly and El Rayes, 1983; Barbieri and Gargiulo, 2004; Gul, 2016; Mubeen and Noor, 2009; Suryawanshi *et al*., 2019; Wang *et al*., 2011).

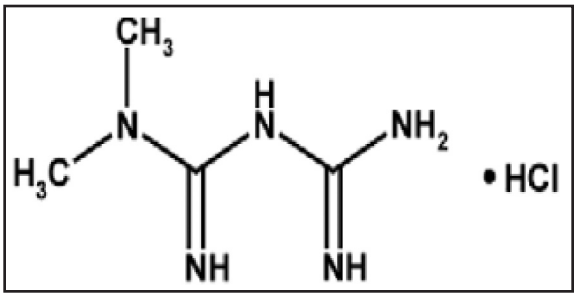

Figure 1. Chemical structure of MET HCl.

The use of conductometric titrations to quantify MET is documented in the literature. In this procedure, titration against silver nitrate was used to quantify the amount of MET. The primary reaction involved in the titration is that between the MET and silver nitrate. Using conductometric, potentiometric, and visual methods, the method's endpoint was examined. The study designed a hypothetical conductance value of the MET when a known amount of titrant is added. In a different approach that has been published, silver-nitrate-based reagents were used to measure the conductivity-metric titration of MET in the marketed formulation. The technique was established based on the chemical interaction among the chloride ions of MET with silver iodide ions giving the precipitate as silver chloride (Sartori *et al*., 2009; Valdivia *et al*., 2019).

Titrimetric procedures are thought to be time-consuming and require the use of additional, expensive substances like silver nitrate. This demonstrates the constraints and drawbacks of methods that have been published. There is a need for the development and validation of a straightforward, financially viable, novel, and a precise electro-analytical method for the valuation of MET in bulk and marketed formulations in order to minimize a few drawbacks of published analytical tools using conductometric titration.

The main goals from the proposed investigation are to develop an inexpensive, quick, robust, sensitive, accurate, reliable, and economical electrochemical technique for estimating MET in marketed preparations and relate its performance to the reported published works of literature.

MATERIAL AND METHODS

Instruments, apparatus, and solvents

The weighing balance of SARTORIUS was used to measure the MET powder, the "Ultrasonic bath Sonicator" was used for the sonication of solutions, the ELICO CM 183EC-TDS analyzer was used to analyze the conductance of the analyte solution and the Direct Q UV aquatic distillation system was used to collect the Millipore Water (MW) for analysis.

Method development

The conductometric method for quantification of MET was developed in two steps. Step 1: Preparation of working standard solutions of MET and Step 2: Estimation of electrical conductivity of working standard solutions of MET. During the development, various dilutions of MET were prepared in MW ranging from 50 to 250 µg/ml. The prepared solutions were subjected to the measurement of electrical conductivity using a conductometer. After the estimation of the electrical conductance of every solution, it was observed that every concentration was found to show a linear response, which is a basic requirement for any analytical method development (i.e., as the concentration increases the electrical conductance increases in linear fashion). The linear response of the method was estimated by constructing a standard calibration curve (Yadav *et al*., 2021).

A standard stock solution of MET

A 50-ml calibrated volumetric flask containing 50 mg of MET was carefully balanced, transported, and filled to the appropriate level using MW as the diluent to create 1,000 µg/ml (Suryawanshi *et al*., 2020).

Working standard solutions of MET

Various volumes were pipetted out from primary stock and transferred separately into 50-ml volumetric flasks, and the volume was marked with MW to yield 50–250 µg/ml of MET (Pancham *et al*., 2020; Uday *et al*., 2021).

Sample preparation and extraction

Weighed and powdered were 20 MET (500 mg label statement) tablets. A fraction measuring 500 mg was weighed and then transferred to a 100-ml volumetric container. MW was utilized to make the mark to 100 ml, then the solution was sonicated for 20 minutes before being strained through Whatman filter paper. To acquire a concentration of 100 µg/ml, dilution was performed using the above-mentioned stock solution. This solution underwent testing to determine its purity level in % (Bossunia *et al*., 2017; Murugesan and Annapurna, 2022).

Method validation

The validation of the analytical method was performed as per the regulatory guidelines for analytical methods (International Council for Harmonisation, 2023).

Selectivity and specificity

The conductance of MW was calculated in the directive to determine the specificity of the technique proposed. The standard amount of MET (100 µg/ml) was produced, and conductance was noted to demonstrate the specificity of the procedure.

Linearity and range

The linearity and range were determined by creating various strengths and evaluating the conductivity. The electrical conductivity of each standard concentration, which contained 50–250 µg/ml of MET, was created. By plotting the accurate quantity of substance against conductance (S), the calibration standard was built, and arithmetic calculations were used to generate data of range and linearity reports.

The limit for detection and estimation

The lowest amount of substance that can be detected but not accurately estimated is known as the detection limit, and it was determined by a statistical method: LOD = 3.3

$$\frac{\sigma}{s}$$

The amount of substance that can be precisely measured and quantified with correctness is known as the quantification limit and it was determined by a statistical method: LOQ = 10

$$\frac{\sigma}{s}$$

where σ = standard deviance and S = slope of the standardization typical curve.

Precision

Measurement in triplicate solutions at higher, middle, and lower concentration levels confirmed the precision in terms of intraday and interday. The conductance of three replicate MET concentrations at three different quantities was measured, as well as the %RSD standards for each obtained conductance. The intraday precision was calculated by running the analysis three times on the same day at three different time intervals. By running the analysis on three different days, the interday precision was determined.

Ruggedness

The ruggedness of the process was done by changing the analyst to test the technique's reproducibility. The electrical conductivity of triplicate solutions at lower, middle, and higher concentration levels was measured and %RSD values for conductance were computed and examined for correctness.

Accuracy

Accuracy was determined using recovery studies, which involved estimating %mean substance recovery at three different levels (150%, 100%, and 50%). Three determinations were performed at each level, and a % mean recovery was calculated. To determine the accuracy of the proposed analytical technique, the standard addition method was referred to.

Conductometric assay

MET tablets (500 mg label statement) were obtained from the market and powdered. A 500 mg powder was weighed accurately and added to a 100-ml volumetric container, and the final capacity was made up to 100 ml with MW and sonicated for 20 minutes before filtering with Whatman sieve paper. The above-mentioned stock solution was diluted to obtain a concentration of 100 µg/ml. This solution was tested to determine its purity percentage. A small amount of methanol was used to extract the exact amount of analyte, and trial studies were conducted.

Table 1. Linearity data of MET HCl by conductometric method.

Concentration µg/ml	Set-1 electrical conductivity (µS)	Set-2 electrical conductivity (µS)	Set-3 electrical conductivity (µS)	Mean electrical conductivity (µS)
50 µg/ml	31.10	30.33	30.20	30.54
100 µg/ml	61.66	61.50	60.90	61.35
150 µg/ml	90.80	91.10	90.70	90.87
200 µg/ml	120.10	120.12	121.12	120.45
250 µg/ml	150.15	149.95	150.10	150.07
r^2	0.9998	0.9998	0.9999	0.9999

Greenness assessment

To systematically assess the greenness of the analytical procedure, we have transformed each of the 12 GAC codes into scores. The following 12 GAC principles are used for assessing the greenness of the method:

1. Direct analytical methods must be used to avoid analyte treatment
2. Use of a limited number of samples and limited sample size
3. *In situ* analysis must be accomplished
4. Incorporation of innovative techniques and methods that saves energy and reduces the use of chemicals
5. Programmed and miniaturized approaches must be designed
6. Avoid the use of derivatization
7. Avoid the generation of large amounts of analytical waste and provide appropriate measures for generated analytical waste
8. Multi-sample analysis approach is preferred
9. Minimum amount of energy should be used
10. Preference should be given to the use of reagents and chemicals manufactured from a renewable source
11. Harmless reagents can be preferred
12. Analyst safety must be considered with more attention

Table 2. Intraday precision data of MET HCl.

Concentration	Time	Conductance (µS)($\bar{x}$)	SD	%RSD
50 µg/ml	Morning	30.62	0.51	1.65
	Afternoon	30.74	0.36	1.18
	Evening	30.86	0.23	0.75
150 µg/ml	Morning	91.03	0.07	0.08
	Afternoon	90.83	0.06	0.07
	Evening	91.02	0.18	0.20
250 µg/ml	Morning	150.78	0.59	0.39
	Afternoon	150.56	0.47	0.31
	Evening	150.77	0.40	0.27

$\bar{x}$ = Mean conductance of three replicates.

Table 3. Interday precision results of MET HCl.

Concentration	Time	Conductance (µS)($\bar{x}$)	SD	%RSD
50 µg/ml	Day-1	31.18	1.12	3.58
	Day-2	30.94	0.16	0.51
	Day-3	30.82	0.17	0.55
150 µg/ml	Day-1	90.76	0.05	0.06
	Day-2	91.02	0.19	0.21
	Day-3	90.97	0.08	0.08
250 µg/ml	Day-1	150.480	0.33	0.22
	Day-2	151.040	0.12	0.08
	Day-3	150.790	0.60	0.40

$\bar{x}$ = Mean of three replicates of conductance.

RESULTS AND DISCUSSION

Method development and validation

In the proposed study, we have chosen MET as an oral anti-diabetic agent that is primarily available in tablet dosage

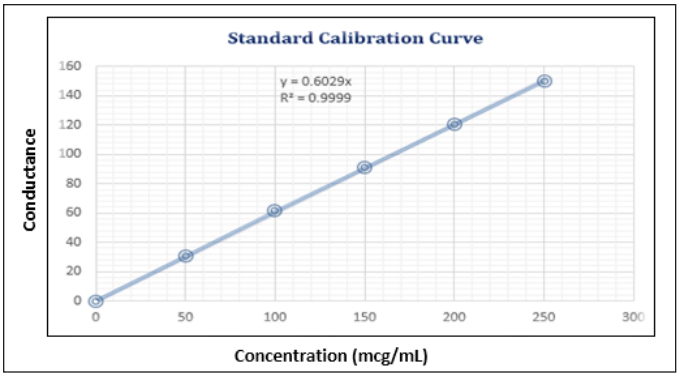

Figure 2. Standard calibration curve of MET HCl.

Table 4. Ruggedness data of MET HCl.

Replicates	Concentration	Change in analyst-1	Concentration	Change in analyst-2
1	50 µg/ml	31.11	150 µg/ml	91.78
2	50 µg/ml	31.80	150 µg/ml	93.80
3	50 µg/ml	30.90	150 µg/ml	92.70
4	50 µg/ml	30.66	150 µg/ml	90.90
5	50 µg/ml	31.11	150 µg/ml	91.05
6	50 µg/ml	30.80	150 µg/ml	90.95
	Mean	31.063	Mean	91.863
	SD	0.401	SD	1.173
	%RSD	1.292	%RSD	1.277

Table 5. Accuracy data of MET HCl.

Sl. No.	Level	Replicate	% Recovery	Mean % Recovery	%SD
1	50%	1	99.90	99.90	0.05
		2	99.95		
		3	99.85		
2	100%	1	100.10	100.04	0.06
		2	100.05		
		3	99.98		
3	150%	1	99.86	99.98	0.15
		2	100.15		
		3	99.94		

Table 6. Assay data of MET HCl in marketed formulation.

Tablet	Drug	Label claim	Sample concentration prepared	% Assay
1	MET	500 mg	100 µg/ml	99.78%

Table 7. Greenness assessment score for conductometric method.

Sl. No.	Principle of green chemistry	Calculated score	Justification
1	Direct analytical techniques should be applied to avoid sample treatment:	0.60	In the proposed analytical method we estimate the study analyte directly without any derivatization and it is a type of at-line analysis. Hence it is given a score of 0.60.
2	Minimal sample size and a minimal number of samples are goals	1.0	In the proposed method in order to prepare the standard solution we require 10 mg of analyte and according to the principle of the researcher uses the sample amount of 100 mg then it is to be assessed as green and the score is given as 1.0.
3	*In situ* measurements should be performed.	1.0	In the proposed method we perform the in-line analysis of MET HCl and hence scoring is given as 1.0.
4	Integration of analytical processes and operations saves energy and reduces the use of reagents	1.0	The proposed method involves a limited number of steps like preparation of standard and sample solutions and the measurement of conductance. It involves simple two steps and hence reduces the energy as well as the additional use of any reagents. According to the GAC principle, the score is given as 1.0 as the proposed procedures involve less than three steps.
5	Automated and miniaturized methods should be selected	0.5	The proposed method is not fully automated and we need to perform manual dilutions as well as measurements. It is considered as the proposed analysis is manual and miniaturized and as per the principle the score is given 0.5.
6	Derivatization should be avoided	1	According to the GAC principle, derivatization should be avoided to reduce the use of extra chemicals. The score is given as 1 as there is derivatization for the proposed method.
7	Generation of a large volume of analytical waste should be avoided and proper management of analytical waste should be provided.	1.0	The generation of a large amount of analytical waste is to be avoided As the waste generated in this procedure is less than 0.1 g/ml.
8	Multianalyte or multiparameter methods are preferred versus methods using one analyte at a time.	0.0	The method analyzes the analysis of multiple samples of the same analyte and hence as per the equation score is calculated as 0.0.
9	The use of energy should be minimized.	1	The score is given 1 as the proposed analytical method consumes <0.1 kWh per sample for the analysis.
10	Reagents obtained from renewable sources should be preferred.	1	The water is used as a solvent and it is obtained from a bio-based source, and hence, the score is 1.
11	Toxic reagents should be eliminated or replaced.	0.059	As the method replaces the use of organic solvents with distilled water it eliminates the use of toxic reagents. Very small amount of methanol has been used in the study for extraction purposes. Based on its requirement per sample analysis of assay and the score has been calculated.
12	The safety of the operator should be increased.	0.8	The safety of the operator is essential while performing the analysis. The proposed research involves the use of water as a solvent and a very less amount of methanol for extraction purposes. Hence one threat is selected as per the guidelines that are toxic to aquatic life. As the methanol and MET HCl, we need to through as waste and which may be not suitable or toxic for aquatic life.
	Final score	0.75	As the final score was found to be more than 0.5 the method is considered greener according to the principles of GAC.

form, with numerous preparations available in the market. A few spectrophotometric, chromatographic assays and electrochemical approaches are reported in the literature to estimate MET formulations. Potentiometric and conductometric titrations are commonly used in reported electrochemical methods. The electrochemical analysis found to exist its limitations and disadvantages. In this research, a novel principle was used to quantify MET, in which we prepared concentrations ranging from 50 to 250 µg/ml and then measured the conductance for every prepared concentration of MET. We observed that as the concentration of analyte increases the conductance value also increases in a linear fashion.

Specificity and selectivity

The technique was found to be selective and specific because the conductance of MW was found to be 1.18 seconds, whereas the conductance of 100 µg/ml MET was found to be 60.66 µS which indicates the selective and specific performance of the method.

Linearity and range

MET demonstrated a linear relationship between electrical conductance (µS) and concentration levels ranging from 50 to 250 µg/ml. Linearity plot calculation yielded an r^2 value of 0.9999. Table 1 displays the statistical linearity and range data. The standard calibration curve, shown in Figure 2, was obtained by plotting the standard amount against the measured conductance of standard solutions.

Detection and quantification limit

MET had detection and quantification limit values of 3.12 and 9.47 µg/ml, respectively. Both outcomes are within the acceptable range.

Table 8. Comparison of the proposed conductometric method with literature methods.

Parameters	Reported/published methods (Sartori *et al*., 2009; Valdivia *et al*., 2019)	Proposed research work method	Conclusion
Development	Conductometric titrimetric methods	Conductometric methods	The newly developed conductometric method is easy to perform with less time and less cost as compared to reported conductometric titrimetric methods.
Solvents and reagents	Diluents and reagents-silver nitrate and Ag(I)	MW	
Performance of method	titrations are involved	Only simple dilutions and measuring the conductance of analyte solution	
Cost with respect to the use of solvents and reagents	Approximately 3,000 to 4,000/- for each 25 g.	0% (As we have used MW)	
Time of analysis	More time is required as it involves the use of titrations	Less time is required as it does not suggest the use of titrations	

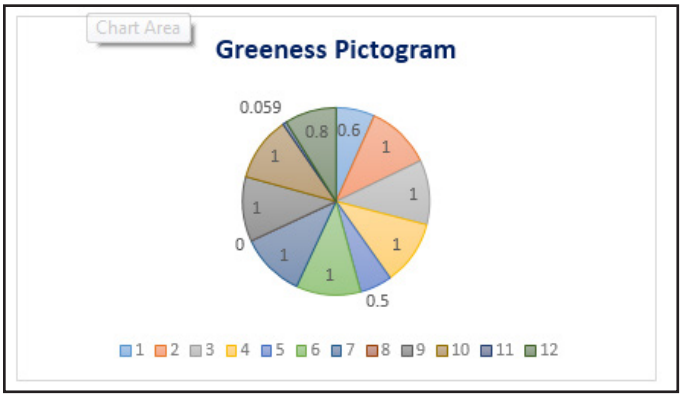

Figure 3. Greenness pictogram of analytical method.

Precision

The analysis reports indicate the preciseness of the suggested technique on similar and altered days. Precision values gained at different concentration levels were less than the 2% RSD limit. Tables 2 and 3 indicate the results of the analysis.

Ruggedness

The ruggedness of the process was tested by different experts and the %RSD values for noted conductance were found to be less than 2%. Table 4 contains the statistics of the ruggedness experiment.

Accuracy

The recovery experiments were carried out at three different levels, namely 100%, 150%, and 50%, and the individual % mean recovery were established between 99% and 100% indicating that the suggested technique is accurate. Table 5 represents the percentage mean recovery values and data of the accuracy experiment.

Conductometric assay

MET tablets (claim 500 mg) were used for the assay, and the comparable weight was measured and diluted to obtain a concentration of 100 µg/ml. Table 6 represents the assay and %purity data of MET quantitative estimation in marketed formulation. The assay value was found to be 99.78%.

Greenness assessment of method using analytical greenness metric tool

The greenness of the method was assessed by giving inputs of the method into the greenness calculator as per the principles of GAC. The principles of GAC, the score calculated for the proposed methodology, and the justification for the inputs have been given in Table 7 and the pictogram is presented in Figure 3.

DISCUSSION

The planned technique was discovered to the usage of MW as the solvent for investigation, and the method does not propose any additional solvents or reagents for the execution of standard and sample preparation. The method involves the measured conductance after preparing various concentrations of analytes in MW. This allows for simple analysis at a low cost and in a short amount of time, which is extremely important and necessary in the pharmaceutical industries that manufacture MET formulations. The suggested process was used to estimate the marketed pharmaceuticals comprising MET in order to assess the applicability and reproducibility during real-time analysis. The suggested research methodology was compared with published literature methods, and the comparison was shown in Table 8.

CONCLUSION

The newly designed and validated method for MET in its powder and tablet dosage form was observed to be novel, cost-effective, easy, and accurate. A simple, accurate, quick, and inexpensive conductometric approach was created and verified using ELICO CM 183EC-TDS analyzer version 2.3. The planned approach was established to employ water as the analysis solvent, and it does not advise the use of any additional solvents or expensive chemicals for executing the dilutions as well as the need for derivatization. The method was found to be green according to green chemistry principles. According to the AGREE metric tool calculation, the suggested method was deemed to be suitable for analyzing MET in pharmaceutical samples.

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AUTHOR CONTRIBUTIONS

All authors made substantial contributions to the conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/ guidelines.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ETHICAL APPROVAL

This study does not involve experiments on animals or human subjects.

DATA AVAILABILITY

All data generated and analyzed are included in this research article.

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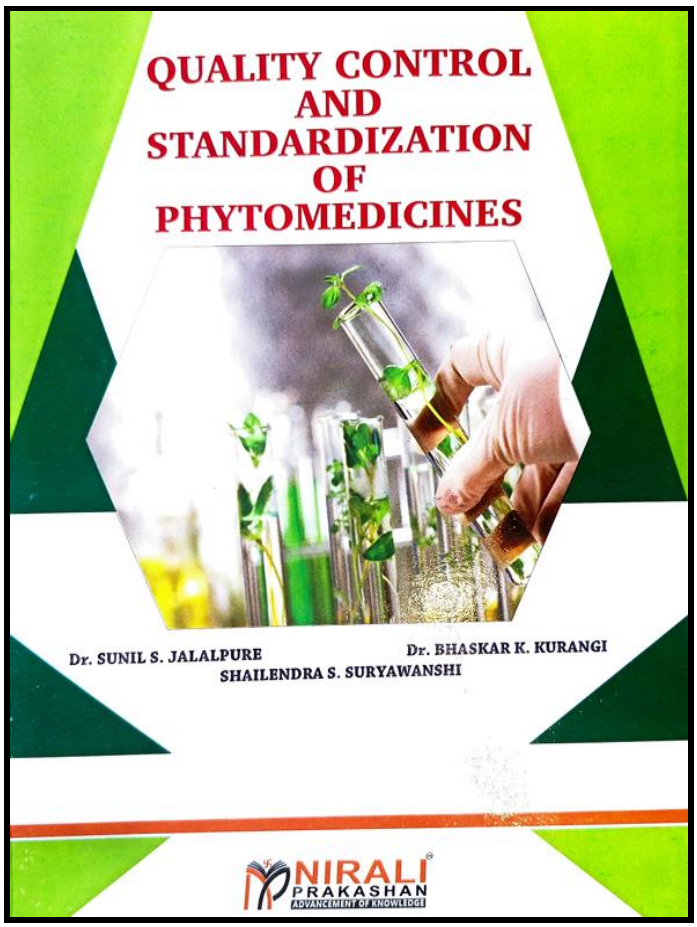
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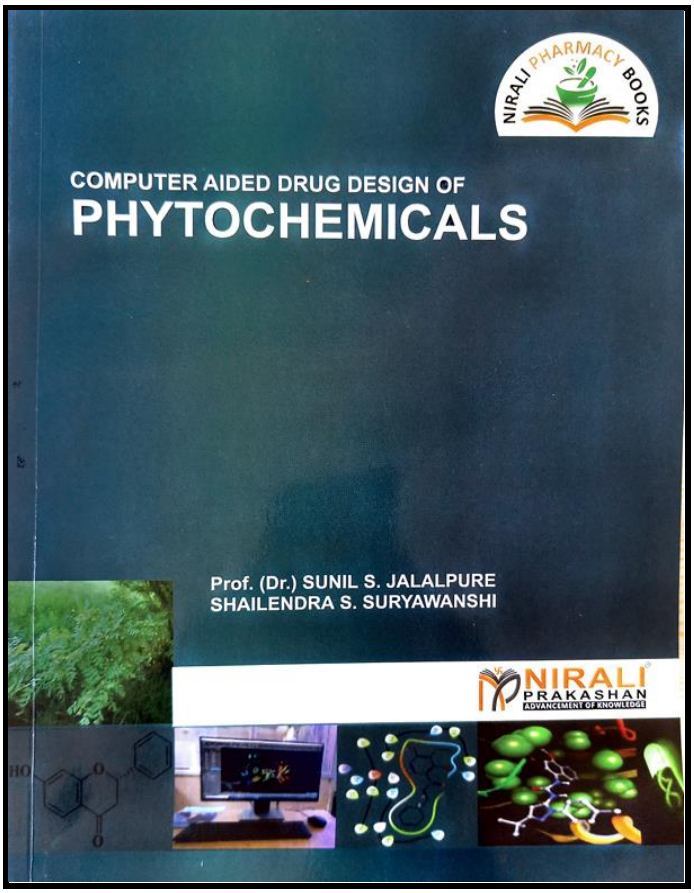
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(57) Abstract :

The present invention relates to a simple, cost-efficient stability-indicating RP-HPLC method for simultaneous estimation of embelin and piperine for routine analysis of marketed polyherbal capsules and tablets. Embelin (EMB) is a benzoquinone-alkaloid, and Piperine (PIP) is an alkaloid; both are reported for antidiabetic, hypolipidemic, antioxidant, anti-cancer activities. According to a literature survey, there is a lack of methods for simultaneous estimation of EMB and PIP. So, we have developed the RP-HPLC method with mobile phase as MeOH:0.1%TEA, pH-adjusted to 4.2 by OPA (92:08) using CHEMSIL-ODS, C18 (4.6×250mm, 5µm) columns, flowrate 1.2 ml/min. PIP and EMB had Rt=3.2, Rt=6.2 minutes at 289nm and validated as per Q2(R1) ICH-guidelines. Linearity ranging from 2-10µg/ml with regression coefficients, LOD and LOQ for PIP were 0.793µg/ml, 2.403µg/ml with r2=0.9979, and for EMB were 0.851µg/ml, 2.578µg/ml with r2=0.9974. Other validation parameters like, precision, robustness, and ruggedness, the %RSD values were <2%, for PIP and EMB, respectively. %purity for PIP was 90.36%, 94.33%, and EMB was 91.65, 92.85%, in both tablets and capsules, respectively. As per forced-degradation studies, acidic degradation was 18.78% and 16.67%, alkaline: 19.27% and 24.36%, oxidative: 5.34%, 7.17%, photolytic :8.93% and 23.59%, and thermolytic: 12.34% and 25.13% degradation for PIP and EMB, respectively. Thus in summary, the developed method is claimed to be specific, precise, linear, accurate, robust, and could be used for detection of EMB and PIP in polyherbal-formulations containing Embelia ribes Brum. and Piper nigrum Linn. extracts on a regular basis in laboratories.

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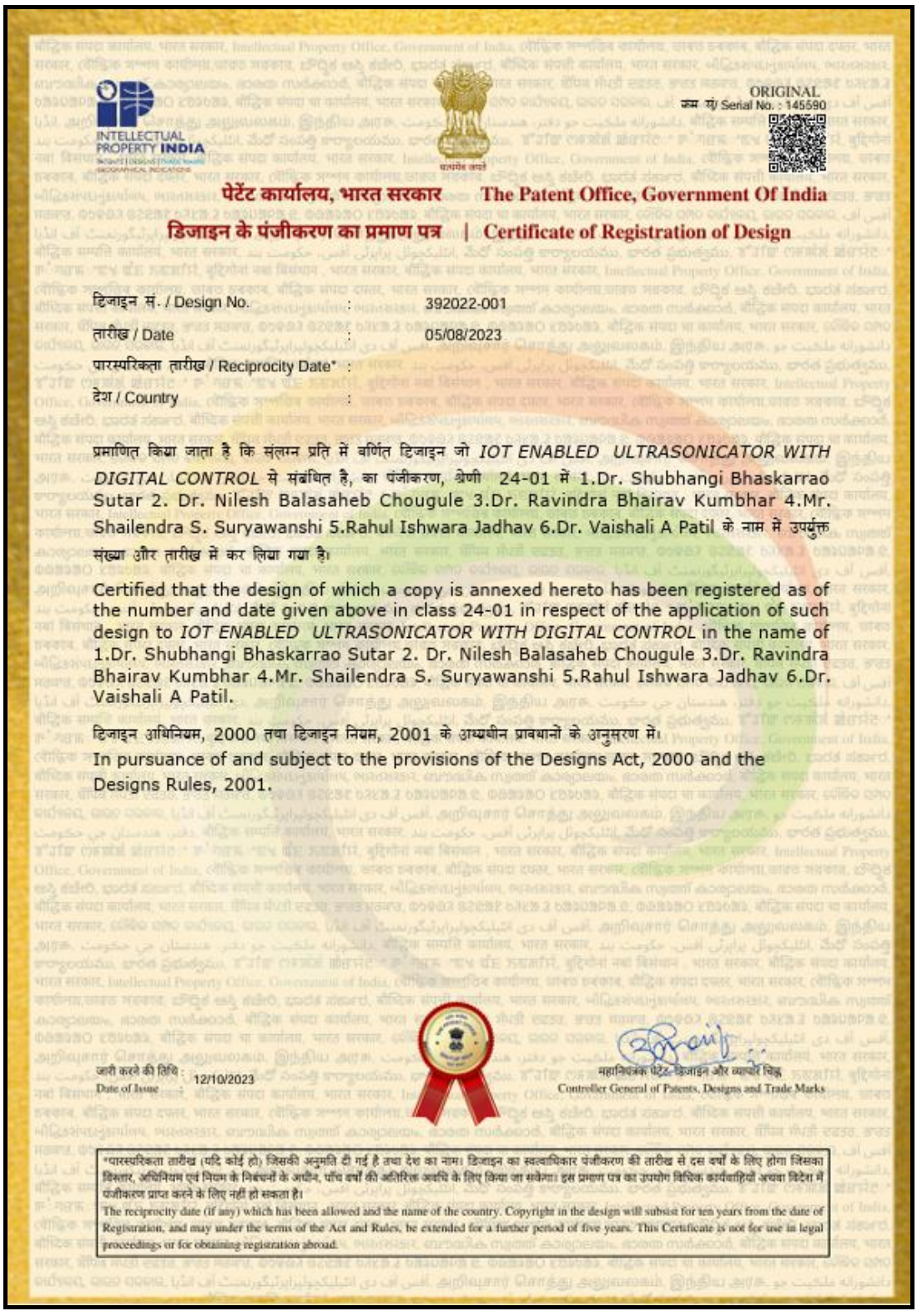

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डिजाइन सं. / Design No.	:	392022-001
तारीख / Date	:	05/08/2023
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